Gas-Phase Covalent And Non-Covalent Ion/ion Chemistry Of Biological Macromolecules

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By John R. Stutzman

Entitled GAS-PHASE COVALENT AND NON-COVALENT ION/ION CHEMISTRY OF BIOLOGICAL MACROMOLECULES

For the degree of Doctor of Philosophy

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GAS-PHASE COVALENT AND NON-COVALENT ION/ION CHEMISTRY OF BIOLOGICAL MACROMOLECULES

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Submitted to the Faculty
of
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by
John R. Stutzman

In Partial Fulfillment of the
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of
Doctor of Philosophy

December 2013
Purdue University
West Lafayette, Indiana
This dissertation is dedicated to my loving family, especially my wife, Wendy, who has been supportive and patient throughout my time at Purdue. I hope that this dissertation will inspire my son, Landon, and my nephews, Beckett and Brooks, to pursue a life of learning.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>ix</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>xiv</td>
</tr>
</tbody>
</table>

## CHAPTER 1. INTRODUCTION TO BIOLOGICAL MASS SPECTROMETRY AND ION/ION CHEMISTRY  

1.1 Tandem Mass Spectrometry ............................... 1  
1.1.1 Collision Induced Dissociation of Biological Macromolecules ....... 2  
1.2 Proteomics ............................................. 3  
1.3 Lipidomics ............................................. 5  
1.4 Gas-Phase Ion/Ion Reactions ............................ 7  
1.4.1 Instrumentation ..................................... 7  
1.4.2 Thermodynamics of Proton Transfer Reactions ............... 8  
1.4.3 Kinetics ............................................. 10  
1.4.4 Proton Transfer Ion/Ion Reactions ...................... 11  
1.4.5 Charge Inversion Ion/Ion Reactions ..................... 13  
1.4.6 Covalent Ion/Ion Chemistry .......................... 14  
1.4.6.1 Schiff Base Covalent Chemistry .................... 15  
1.5 Future Insights of Ion/Ion Chemistry .................... 17  
1.6 References ............................................. 18  

## CHAPTER 2. DISSOCIATION BEHAVIOR OF TRYPTIC AND INTRAMOLECULAR DISULFIDE-LINKED PEPTIDE IONS MODIFIED IN THE GAS-PHASE VIA ION/ION REACTIONS  

2.1 Introduction ............................................. 25  
2.2 Experimental Section .................................... 28  
2.2.1 Materials ............................................ 28  
2.2.2 Tryptic Digest ....................................... 29  
2.2.3 Disulfide Reduction ................................... 29  
2.2.4 Mass Spectrometry ................................... 30  
2.3 Results and Discussion ................................... 30  
2.3.1 Tryptic Peptides ....................................... 30  
2.3.2 Intramolecular Disulfide-Linked Peptides .............. 34
### Chapter 3. Covalent and Non-Covalent Binding in the Ion/Ion Charge Inversion of Peptide Cations with Benzene-Disulfonic Acid Anions

#### Introduction

1. Introduction
2. Experimental Section
   - Materials
   - Mass Spectrometry
3. Results and Discussion
   - Angiotensin II Covalent and Non-Covalent Interactions
   - Leucine Enkephalin-Arginine
   - Leucine Enkephalin-Histidine/Lysine
   - Leucine Enkephalin-phenylalanine and Leucine Enkephalin
4. Conclusions
5. References

### Chapter 4. Ion/Ion Reactions of MALDI-Derived Peptide Ions: Increased Sequence Coverage via Covalent and Electrostatic Modification Upon Charge Inversion

#### Introduction

1. Introduction
2. Experimental Section
   - Materials
   - Methods
   - Tryptic Digest
   - Mass Spectrometry
3. Results and Discussion
   - Lysine Terminated Tryptic Peptides
   - Arginine Terminated Tryptic Peptides
4. Conclusions
5. References

### Chapter 5. Schiff Base Reagent Cluster Anions for Multiple Gas-Phase Covalent Modifications of Peptides and Protein Cations

#### Introduction

1. Introduction
2. Experimental
   - Materials

---

Page

2.4 Conclusions ................................................................. 37
2.5 References ................................................................. 39

CHAPTER 3.  COVALENT AND NON-COVALENT BINDING IN THE ION/ION CHARGE INVERSION OF PEPTIDE CATIONS WITH BENZENE-DISULFONIC ACID ANIONS INTRODUCTION .......................................................... 52

3.1 Introduction ............................................................... 52
3.2 Experimental Section ..................................................... 56
   - Materials
   - Mass Spectrometry
3.3 Results and Discussion .................................................... 57
   - Angiotensin II Covalent and Non-Covalent Interactions
   - Leucine Enkephalin-Arginine
   - Leucine Enkephalin-Histidine/Lysine
   - Leucine Enkephalin-phenylalanine and Leucine Enkephalin
3.4 Conclusions ................................................................. 67
3.5 References ................................................................. 69

CHAPTER 4 ION/ION REACTIONS OF MALDI-DERIVED PEPTIDE IONS: INCREASED SEQUENCE COVERAGE VIA COVALENT AND ELECTROSTATIC MODIFICATION UPON CHARGE INVERSION ......................... 87

4.1 Introduction ............................................................... 87
4.2 Experimental Section ..................................................... 90
   - Materials
   - Methods
   - Tryptic Digest
   - Mass Spectrometry
4.3 Results and Discussion .................................................... 93
   - Lysine Terminated Tryptic Peptides
   - Arginine Terminated Tryptic Peptides
4.4 Conclusions ................................................................. 101
4.5 References ................................................................. 103

CHAPTER 5. SCHIFF BASE REAGENT CLUSTER ANIONS FOR MULTIPLE GAS-PHASE COVALENT MODIFICATIONS OF PEPTIDES AND PROTEIN CATIONS ................................................................. 117

5.1 Introduction ............................................................... 117
5.2 Experimental .............................................................. 119
   - Materials
5.2.2 Mass Spectrometry ................................................................. 120
5.3 Results and Discussion .................................................................................. 120
  5.3.1 FBMSA Reagent Cluster Anions ......................................................... 120
  5.3.2 Multi-Derivatized K10 Cations ............................................................ 121
  5.3.3 Multi-Derivatized Ubiquitin Cations .................................................... 122
5.4 Conclusions ............................................................................................... 125
5.5 References ................................................................................................... 126

CHAPTER 6. CONVERSION OF MALDI-DERIVED PEPTIDE MONOANIONS INTO MULTIPLY CHARGED CATIONS VIA GAS-PHASE ION/ION REACTIONS .......................................................... 134

6.1 Introduction ............................................................................................... 134
6.2 Experimental Section .................................................................................. 137
  6.2.1 Materials .......................................................................................... 137
  6.2.2 Methods .......................................................................................... 137
  6.2.3 Disulfide Reduction ............................................................................. 138
  6.2.4 Mass Spectrometry ............................................................................. 138
6.3 Results and Discussion .................................................................................. 139
  6.3.1 Optimal Protein Reagent Cations ......................................................... 140
  6.3.2 Conversion of Peptide Anions Outside of the Native Mass Range ...... 143
  6.3.3 Increasing Structural Characterization upon Charge Inversion .......... 144
6.4 Conclusions ............................................................................................... 145
6.5 References ................................................................................................... 147

CHAPTER 7. GAS-PHASE TRANSFORMATION OF PHOSPHATIDYLCHOLINE CATIONS TO STRUCTURALLY INFORMATIVE ANIONS VIA ION/IION CHEMISTRY ......................................................... 157

7.1 Introduction ............................................................................................... 157
7.2 Experimental ............................................................................................ 161
  7.2.1 Materials .......................................................................................... 161
  7.2.2 Solution Phase PDPA Spiking ............................................................. 161
  7.2.3 Mass Spectrometry ............................................................................. 162
7.3 Results and Discussion .................................................................................. 163
  7.3.1 Transformation of PC Cations ............................................................. 163
  7.3.2 MS/MS of [PC-CH₃]- Anions ................................................................. 166
7.4 Solution Phase PDPA Mixture .................................................................. 168
7.5 Conclusions ............................................................................................... 169
7.6 References ................................................................................................... 171
LIST OF FIGURES

Figure 1.1: Generic energy diagrams for (a) an proton transfer ion/ion reaction (b) proton transfer ion/molecule reaction .................................................................23

Figure 2.1: Ion trap CID product ion spectra of a) modified product, [M+♦], b) [M-H], c) [M+2H]^2+ derived from M = TLSDYNIQK ................................................................................42

Figure 2.2: Ion trap CID product ion spectra of A) [M + ♦], B) [M - H], C) [M + H]^+ derived from M = MQIFVK ...........................................................................................43

Figure 2.3: Ion trap CID product ion spectra of A) [M + ♦], B) [M - H], C) [M + H]^+ derived from M = LIFAGK .......................................................................................44

Figure 2.4: Ion trap CID product ion spectra of A) [M + ♦], B) [M - H], C) [M + 2H]^2+ derived from M = TITLEVEPSDTIENVK .............................................................45

Figure 2.5: Ion trap CID product ion spectra of A) [M + ♦], B) [M - H], C) [M + H]^+ derived from M = EQIPPDQQR ...................................................................................46

Figure 2.6: Ion trap CID product ion spectra of a) [M+♦], b) [M-H], and c) [M+2H]^2+ derived from M = ESTLHLVLR .........................................................................47

Figure 2.7: Ion trap CID product ion spectra of A) [M+♦], B) [M-H], C) [M+H]^+ ions derived from M = AGCKNFFWKFTFTSC (somatostatin). The b,♦ symbols in the somatostatin sequence at the top of the figure represent modified b-ions that originate from the loop defined by the disulfide linkage. These ions may contain different numbers of sulfur atoms depending upon which of the bonds of the disulfide linkage was cleaved. ........................................................................48

Figure 2.8: Ion trap CID product ion spectra of a) [M+♦], b) [M-H], c) [M+H]^+ derived from M = AGCKNFFWKFTFTSC (reduced somatostatin-14) .........................49

Figure 2.9: Ion trap CID product ion spectra of a) [M+♦], b) [M-H], and c) [M+H]^+ derived from M = CYIQNCPLGL-NH2 (oxytocin) ..................................................50
Figure 2.10: Ion trap CID product ion spectra of a) [M+\bullet]-, b) [M-H]-, and c) [M+H]+ derived from M = CYIQNCPLG-NH₂ (reduced oxytocin) .................................................................51

Figure 3.1: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+\bullet]-, b) BDSA-modified product, [M+BDSA-H]-, M = angiotensin II ..................................................72

Figure 3.2: Ion trap CID product ion spectrum of [M+BDSA-H-H₂O]-, derived from M = angiotensin II (DRVYIHPF) .....................................................................................73

Figure 3.3: Ion population of [M+\bullet], a) Schiff base formation with singly protonated angiotensin II and doubly deprotonated FBDSA, b) One of the possible strong electrostatic interactions that may play a role in the observation of the y\bullet-ion of Figure 1(a). This interaction involves a deprotonated sulfonate group on FBDSA and the charged guanidinium side chain of arginine. ...............................................................74

Figure 3.4: Ion trap CID product ion spectrum of the BDSA adduct of methyl esterified angiotensin II (D(OMe)RVYIHPF(OMe)), i.e., [M+BDSA-H]-. ..................................75

Figure 3.5: Four examples of possible non-covalent interactions between protonated YGGFLR and the dianion of BDSA. (a) Strong acid-base interaction. (b) Negatively charged proton-bound dimer. (c) Combined strong acid-base and proton-bound dimer interactions. (d) Salt-bridge interaction. .................................................................76

Figure 3.6: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+\bullet]-, b) BDSA-modified product, [M+BDSA-H]-, c) methyl esterified YGGFLR-BDSA adduct, [YGGFLR(OMe)+BDSA-H]-. ........................................................................77

Figure 3.7: Ion trap CID product ion spectrum of [M-H]- derived from YGGFLR ..........78

Figure 3.8: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+\bullet]-, b) BDSA-modified product, [M+BDSA-H]- derived from M = YGGFLH ......................79

Figure 3.9: Ion trap CID product ion spectrum of [M-H]- derived from M = YGGFLH .........................................................................................................................80

Figure 3.10: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+\bullet]-, b) BDSA-modified product, [M+BDSA-H]- derived from M = YGGFLK .......................81

Figure 3.11: Ion trap CID product ion spectrum of [M-H]- derived from M = YGGFLK .........................................................................................................................82

Figure 3.12: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+\bullet]-, b) BDSA-modified product, [M+BDSA-H]-, derived from M = YGGFLF ......................83
Figure 3.13: Ion trap CID product ion spectrum of \([M-H]^—\) derived from \(M = YGGFLF\). ..........................................................................................................................84

Figure 3.14: Ion trap CID product ion spectra of a) FBDSA-modified product, \([M+♦]^+\), b) BDSA-modified product, \([M+BDSA-H]^—\), and c) the deprotonated molecule \([M-H]^—\) derived from \(M = YGGFL\). ........................................................................................................85

Figure 3.15: Ion trap CID product ion spectra of a) [ac-YGGFL+BDSA-H]^—, b) [YGGFL-OMe+BDSA-H]^—, and c) [ac-YGGFL-OMe+BDSA-H]^—. .........................................................86

Figure 4.1: Modified AP-MALDI source, attached to a QTRAP 4000, capable of multisource injection...............................................................................................................107

Figure 4.2: a) Ion/ion product spectrum of peptide cation mixture and \([FBDSA-2H]^2\) b) Positive ion spectrum of peptide mixture LIFAGK, EGIPPDQQR, and TLSDYNIQK...................................................................................................................108

Figure 4.3: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= TITLEVEPSDTIENVK\) ........................................................................................................109

Figure 4.4: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= TLSDYNIQK\) ...........................................................................................................110

Figure 4.5: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= GLSDGEWQQVNLNVWGK\) .....................................................................................111

Figure 4.6: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= MQIFVK\) ...................................................................................................................112

Figure 4.7: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= LIFAGK\) ....................................................................................................................113

Figure 4.8: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= EQIPPDQQR\) ............................................................................................................114

Figure 4.9: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= APPGFSPFR\) .............................................................................................................115

Figure 4.10: Ion trap CID product ion spectra of (a) \([M+♦]^-\) (b) \([M+H]^+\) derived from \(M= ESTLHLVLR\) ........................................................................................................116

Figure 5.1: nESI generated clusters of R, where R denotes FBMSA. ............................ 128
Figure 5.2: Ion/ion reactions between $[K_{10}^+3H]^3^+$ and FBMSA reagent clusters, (a) $[2R_{2Na}-Na]$ , (b) $[3R_{3Na}-Na]$ , $[4R_{4Na}-Na]$ , $[5R_{5Na}-Na]$ ........................................................................................................................................ 129

Figure 5.3: (a) Post ion/ion reaction spectrum of $[Ubiquitin + 7H]^2^+$ and $[5R_{5Na}-Na]$ , (b) Rapid sequential collisional activation over the isolated ion population in the insert of (a) .......................................................................................................................130

Figure 5.4: Ion trap CID of (a) $[Ubiquitin + 6H]^6^+$ (b) $[Ubiquitin + 7H + \diamond]^6^+$ , (c) $[Ubiquitin + 7H + 5\diamond]^6^+$ ................................................................................................................................................131

Figure 5.5: Ladder structure of ubiquitin, (a) $[Ubiquitin + 6H]^6^+$ (b) $[Ubiquitin + 7H + \diamond]^6^+$ , (c) $[Ubiquitin + 7H + 5\diamond]^6^+$ ..................................................................................................................132

Figure 5.6 Ion trap CID of (a) $[Ubiquitin + 7H + 2\diamond]^6^+$ (b) $[Ubiquitin + 7H + 3\diamond]^6^+$ , (c) $[Ubiquitin + 7H + 4\diamond]^6^+$ ........................................................................................................................................133

Figure 6.1: Modified AP-MALDI source, attached to a QTRAP 4000, capable of multisource injection ..................................................................................................................................................150

Figure 6.2: Negative ionization mode of melittin via AP-MALDI, M is equivalent to GIGAVLKVLTTGLPALISWIKRKRQQ-NH2. ..................................................................................................................151

Figure 6.3: Positive mode microionspray ionization of a) ubiquitin, b) myoglobin, c) carbonic anhydrase, d) bovine serum albumin. The red color represents the isolated protein cation subjected to ion/ion reactions ........................................................................................................................................ 152

Figure 6.4: Product ion spectrum following the ion/ion reaction between $[Melittin-H]^-$ and $[Ubiquitin+8H]^8^+$. M is representative of melittin, while the R+ is equivalent to the charge state of ubiquitin ........................................................................................................................................ 153

Figure 6.5: Product ion spectrum following the ion/ion reaction between $[melittin-H]^-$ and a) $[myoglobin+16H]^{16^+}$ , b) $[carbonic anhydrase+23H]^{23^+}$ , c) $[BSA+39H]^{39^+}$ , M is representative of melittin, while the red numbers represent the isolated precursor reagent cation ........................................................................................................................................ 154

Figure 6.6: a) Negative ionization mode of chain B via AP-MALDI, b) ion/ion reaction between $[Chain+B-H]^-$ and $[BSA+36H]^{36^+}$ , M is equivalent to FVNQHLCGSHLVEALYLVCG ERGFFYTPKA ........................................................................................................................................ 155

Figure 6.7: a) ion/ion reaction between $[M-H]^-$ and $[cytochrome c+ 12H]^{12^+}$ , b) Ion trap CID of $[M+2H]^{2^+}$ , c) Ion trap CID of $[M+H]^+$ , M is equivalent to HDMNKVLDL ........................................................................................................................................ 156
Figure 7.1: (a) Product ion spectrum following ion/ion reactions between PDPA dianions and 16:0/18:1 PC monocations, (b) Ion trap CID of the long-lived complex, [PC\textsubscript{16:0/18:1}+PDPA-H\textsuperscript{-}]. ..........................................................................................................................175

Figure 7.2: (a) Product ion spectrum following ion/ion reactions between PDPA dianions and 18:1/16:0 PC monocations, (b) Ion trap CID of the long-lived complex, [PC\textsubscript{18:1/16:0}+PDPA-H\textsuperscript{-}]. ..........................................................................................................................176

Figure 7.3: (a) Ion trap CID of [PC\textsubscript{16:0/18:1}-CH\textsubscript{3}]\textsuperscript{-}. (b) Ion trap CID of [PC\textsubscript{16:0/18:1}+H\textsuperscript{+}]. (c) Relating bond cleavage and product ion type for [PC\textsubscript{16:0/18:1}-CH\textsubscript{3}]\textsuperscript{-}. ..................................................176

Figure 7.4: (a) Ion trap CID of [PC\textsubscript{18:1/16:0}-CH\textsubscript{3}]\textsuperscript{-}. (b) Ion trap CID of [PC\textsubscript{18:1/16:0}+H\textsuperscript{+}]. ..................................................177

Figure 7.5: (a) Ionization of PDPA and 16:0/18:1 solution phase mixture via negative mode nESI (b) Ion trap CID of [PC\textsubscript{16:0/18:1}-CH\textsubscript{3}]\textsuperscript{-}. ..................................................178

Figure 7.6: (a) Ionization of PDPA and PC\textsubscript{18:1/16:0} solution phase mixture via negative mode nESI (b) Ion trap CID of [PC\textsubscript{18:1/16:0}-CH\textsubscript{3}]\textsuperscript{-}. ..................................................179
ABSTRACT


Gas-phase ion/ion chemistry involves the interaction of oppositely charged ions inside of the mass spectrometer. During this gas-phase chemistry, particle transfer (i.e., proton and electron) or synthesis can occur at rapid reaction rates. Particle transfer represents a mature area of ion/ion chemistry, while selective covalent modification represents a fairly new area of gas-phase chemistry. Gas-phase covalent chemistry is based on traditional solution phase organic chemistry.

The work demonstrated in this dissertation greatly involves gas-phase covalent and non-covalent Schiff base chemistry on peptide and protein ions. The reagent dianion, 4-formyl 1,3-benzene disulfonic acid, has been used to covalently modify unprotonated primary amines present in peptide and protein ions. In addition, strong non-covalent interactions have also been observed with arginine-containing peptides ions. Studies of their dissociation behavior as well as the nature of their interaction (i.e., covalent versus non-covalent) have been investigated. Application of this Schiff base ion/ion chemistry has been demonstrated on matrix assisted laser desorption/ionization (MALDI)-derived peptide ions. Such Schiff base ion/ion chemistry, whether electrospray or MALDI-derived, can produce complementary or even an increase in structural information.
Multiple covalent modifications within one ion/ion encounter have been demonstrated on peptide and protein cations via Schiff base cluster anions. This dissertation also highlights the gas-phase transformation of phosphatidylcholine cations into demethylated phosphatidylcholine anions, which provides an increase in structural information upon activation. As a whole, gas-phase covalent and non-covalent ion/ion chemistry represents a promising new area for identifying and characterizing biological analytes.
CHAPTER 1. INTRODUCTION TO BIOLOGICAL MASS SPECTROMETRY AND ION/ION CHEMISTRY

1.1 Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) utilizes multiple stages of ion separation (i.e., ion isolation) and identification (i.e., dissociation and detection) in the gas-phase. This methodology has revolutionized mass spectrometry as an analytical tool since its inception in the early 1970’s. MS/MS is based on the detectable difference in the mass/charge (m/z) ratio of a precursor ion prior to the final stage of analysis. A dissociation method or chemical reaction are generally employed to cause a change in the precursor ion. In order to achieve detection following multiple stages of MS, the precursor ion must be stable in the first stage of MS and the product ion must be stable in the second stage of MS. Such a requirement provides high selectivity and a drastic reduction of chemical noise as the other ions do not satisfy the conditions for detection.

The process of tandem MS is analogous to chromatographic separations due to the extra dimension added to mass spectrometry upon separation of one component from an ionization spectrum. Instead of separation based on retention behavior, analytes can be separated on their molecular weight.

Tandem mass spectrometry can be either performed in time or space. MS/MS in space involves multiple stages of MS that are physically separated, i.e. coupling two or more distinct mass analyzers. In space MS/MS has been applied to sectors, time-of-flight
(TOF), and multiple quadrupole devices.\textsuperscript{4,5} For instance, the triple-quadrupole MS (QqQ) has been the standard in tandem MS for the past few decades. The triple quad is comprised of a mass filter (Q1), an rf-only collision cell (q2), and a mass filter for product ion mass analysis (Q3).\textsuperscript{6} Since commercialization of these platforms, many operational modes have been developed for high throughput MS analysis: precursor ion scan, product scan, neutral loss scan, and selected reaction monitoring.\textsuperscript{5} In all of these scan-types, fragmentation occurs via beam-type collisional activation in the collision cell.\textsuperscript{6} Alternatively, MS/MS in time involves multiple stages of MS (i.e., isolation and dissociation) sequentially in the sample ion trap. This type of tandem MS is performed in ion traps (i.e., 2-D and 3-D) and ion cyclotron resonance cells. A significant benefit of tandem MS in time is the ability to perform MS\textsuperscript{n} experiments, where up to seven or eight stages have been demonstrated.\textsuperscript{4,5}

1.1.1 Collision Induced Dissociation of Biological Macromolecules

In tandem mass spectrometry, energetic collisions with a neutral target gas are the most commonly used activation method of biological ions. This process of collisional activation followed by fragmentation is referred to as collision-induced dissociation (CID). CID has proven to be extremely useful and effective for the identification and structural characterization of biological macromolecules. This fact can be attributed to the ease of implementation, universal applicability, and high CID cross sections. As a result, tandem MS with CID has emerged as a powerful tool in proteomics and other “omic” areas as structural information can provide insight on physiological processes. In fact, gas-phase unimolecular dissociation of proteins and peptides is a continuing area of
research in hopes to generate algorithms that predict fragmentation behavior as well as to further fundamental understandings. There are multiple factors that influence the dissociation behavior of biological ions. Some of these factors include the activation time period relative to dissociation or rearrangement, the activation energy deposited, the distribution of deposited energy, the form of deposited energy (i.e., vibrational versus electronic), the time scale of instrument observation, and the efficiency of the CID process.

Ion trap CID is considered a slow heating method (i.e., 10-100 millisecond time frame), where multiple collisions can occur with considerable time periods between each collision. Such a time scale allows for isomerization or dissociation during activation. Typically, 1-100 eV kinetic energy is deposited into the bio-ion in low energy CID processes such as ion trap CID. Alternatively, fast activation methods (i.e., microsecond time frame) deposit kilo-election-volt energies with only a few collisions with neutral bath gas. Generally, high energy/fast activation CID is performed on sector-based and tandem TOF instruments.\(^7\)

1.2 Proteomics

Proteomics can be defined as the study of proteins with a particular interest in their structure and function. Identifying proteins and their covalent structures has become central to the life sciences.\(^8\) The primary sequence (i.e., amino acid composition) of a protein relates to a specific gene, thus providing a link between cell physiology and genetics.\(^9\) Proteomic analysis can be quite complex and difficult due to the relative size and diversity of a proteome. In fact, the number of proteins in a proteome greatly
exceeds the number of genes. This finding can be attributed to alternating splicing of primary transcripts, the present of sequence polymorphism,\textsuperscript{8,9} post-translation modifications (PTMs),\textsuperscript{8,9,10} and other protein processing mechanisms.\textsuperscript{8,9}

The field of proteomics heavily relies on mass spectrometry and tandem MS as an analytical tool to analyze and characterize these analytes.\textsuperscript{11} Mass spectrometry and tandem mass spectrometry provides a fast and robust platform to determine the mass information and relative abundance of the analyte ions and their fragment ions.\textsuperscript{4,5}

Initially, MS was restricted to small and thermostable compounds due to the inability to ionize larger analytes from the solution phase to the gas-phase without extensive fragmentation.\textsuperscript{12,13} The advent of soft ionization techniques, electrospray ionization (ESI)\textsuperscript{14} and matrix assisted laser desorption/ionization (MALDI),\textsuperscript{15,16} afforded the introduction of gaseous biological macromolecules into the MS. These significant contributions have fueled new innovations in MS instrumentation as well as biological applications for proteome characterization.

Two conventional methods of protein identification in MS are bottom-up and top-down proteomics. Bottom-up proteomics is the most common and widely utilized approach to characterize proteins. In bottom-up, peptides generated by an enzymatic digestion of a protein\textsuperscript{17} are subjected to MS/MS,\textsuperscript{18,19} where the precursor masses of the peptide ions and the subsequent dissociation behavior are compared to a protein database for identification.\textsuperscript{20} This process is generally coupled with liquid chromatography and highly automated. Peptide analytes generally ionize easily via ESI or MALDI and their fragmentation behavior is quite straightforward\textsuperscript{18} (i.e., cleavage at the peptide bond forming b- and y-ions). In addition to these advantages, there are several key drawbacks
to this methodology. Foremost, enzymatic digestion generates a fraction of the total theoretical peptides; therefore, only limited primary sequence is observed. Potentially, PTMs like phosphorylation, sulfonation, and glycosylation, can be lost due to the limited production of peptides. In addition, a single peptide or a group of peptides may not be unique to a single protein, but shared among multiple proteins. Such a fact will cause ambiguity with protein assignments. Alternatively, the top-down approach subjects the intact protein to mass analysis and characterization without recourse to extensive separation or digestion. Mass analysis of gaseous protein ions has been demonstrated up to 200kDa. In principle, dissociation of an intact protein ion enables the entire sequence for examination, enabling complete characterization of the protein. Since this approach allows for intact mass information, redundant identifications by peptide sequencing strategies can be eliminated by simple identification of isoforms and PTMs. Additionally, the masses of intact mass of proteins ion generated by ESI are spread over a wider mass range than enzymatically derived peptides, thus alleviating complex mixture analysis. Some drawbacks to this method include complex fragmentation behavior and difficulties generating stable ion signal.

1.3 Lipidomics

The area of lipidomics pertains to the identification and quantification of lipids within a biological system as well as their effect on the function or dysfunction on various physiological pathways. Lipidomics has emerged as an important area of research due to new insights on lipid-lipid and lipid-protein interactions as these biological macromolecules have distinct biochemical roles and biophysical
Traditionally, lipids have been characterized solely on their physical properties, i.e. solubility in nonpolar solvents, instead of their physiological roles. Recent publications and investigators have tried to redefine this traditional definition. Lipids are quite distinct from the other classes of biological macromolecules, i.e. proteins and nucleic acids. Instead of permutations of common monomers, lipids encompass a wide range of molecular structures. Common lipid classes include fatty acids, glycerolipids, glycerolphospholipids, sterol lipids, and sphingolipids. Within each lipid classes, there is high degree of diversity which leads to an extraordinary number of combinations. This diversity does not account for isomeric lipids that differ only by the double bond position, backbone substitution, and stereochemistry. Due to this high degree of diversity, lipidomics progressed and developed much more slowly than proteomics and genomics. As such, MS has become the analytical tool of choice to address the diverse range of molecular structures due to its speed, selectivity, etc. In lipidomics, two strategies have been employed: targeted lipidomics and global lipidomics. Target lipidomics focuses on the identification and quantification of a single lipid within a tissue or cellular extract, while global lipidomics attempts to identify and quantify all of the lipids in a system. Overall, mass spectrometry has advanced the field of lipidomics greatly over the recent decade.
1.4  **Gas-Phase Ion/Ion Reactions**

Gas-phase ion/ion reactions have emerged as an analytically useful reaction within the mass spectrometry.\(^{30}\) These reactions represent a flexible and rapid means to manipulate and characterize biological macromolecules in the gas-phase. Three classes of ion/ion reactions currently exist: acid/base reactions, redox reactions, and covalent bond formation. Acid/base and redox reactions represent areas of detailed investigation, while the selective covalent bond formation at specific functional groups represents a relatively new area of ion/ion chemistry.\(^{31}\) This work in this dissertation extensively utilizes acid/base and covalent bond formation ion/ion chemistry.

1.4.1  **Instrumentation**

Ion/ion reactions have been performed in 3-D quadrupole and linear quadrupole (2-D) ion traps at reduced pressures (millitorre regime).\(^{32,33}\) Linear ion traps have several distinct advantages over 3-D traps in relation to ion/ion chemistry. These advantages include the improved dynamic range and the enhanced efficiency of coupling to external ion sources, detectors, and other mass analyzers.\(^{34}\) Conventional trapping in a 2-D trap utilizes static potentials (DC) on the containment lenses; however, the static potential will only be sufficient to axially trap one ion polarity. In order to perform ion/ion reactions on linear ion traps, an auxiliary alternating current (AC) is superimposed on the containment lenses of the quadrupole ion trap to afford mutual storage of oppositely charged ion populations. The 2-D ion traps provide sufficient spatial overlap of both ion populations, causing these vessels to be well suited for gas-phase ion/ion reactions.\(^{33}\) These ion traps also provide the ability to perform multiple stages of mass spectrometry.
(i.e., ion isolation and activation), which is desirable for characterization, ion manipulation, and mass analysis.

The instrument control of an ion/ion reaction and bipolar ion introduction is fairly straightforward. Ions of opposite polarity are sequentially injected into the reaction quadrupole via alternately pulsed nanoelectrospray (nESI) emitters. During the injection of one polarity, one component of the population may be isolated or activated prior to the ion/ion reaction. Once the desired ion population reaches the reaction quadrupole, the ion population is trapped with static potentials on the containment lenses. Next, the opposite polarity ion population is injected into the reaction cell, whereby the supplemental AC is applied to the containment lenses to trap both polarity of ions. The ions are allowed to react for a predefined period of time, where the product ions are subjected to further tandem MS and mass analysis.\textsuperscript{33,34,35}

1.4.2 Thermodynamics of Proton Transfer Reactions

The generic energy diagram for a proton transfer ion/ion (see process (1)) reaction is displayed in Figure 1 (a).\textsuperscript{36} This energy diagram is analogous to the Brauman diagram. The entrance channel is dominated by the long range, charge dependent attractive potential \(-Z_1Z_2e^2/r\). Here, \(Z_1\) and \(Z_2\) are the nominal charges of the cation and anions, \(e\) is the elementary charger, and \(r\) is the distance between the two ions. Once the orbiting pair is formed, the reactants must overcome ion-dipole and ion-induced dipole (assuming one product is completely neutralized) to proceed over the exit channel barrier. The enthalpy of reaction is established by the difference in the proton affinity (PA) of \([M^{+(n-1)H}]^{0-}\) and \([A-H]^-\), as seen in process (2).
\[ [M+nH]^{nH^+} + [A-H] \rightarrow [M+(n-1)H]^{(n-1)H^+} + A \quad (1) \]

\[ \Delta H_{\text{rxn}} = PA([A-H]) - PA([M+(n-1)H]^{(n-1)H^+}) \quad (2) \]

The significant disparity in energy between the highest proton affinities of neutral bases and the lowest proton affinities of anions generates a highly exothermic process; and therefore, deprotonation of the strongest of gaseous bases will occur in the presence of singly charged anions. Ion/ion reactions are generally exothermic by at least 100 kcal/mol for any charge \( n \).\(^{31}\) As the degree of excess protonation increases (i.e. increase in \( n \)), the reaction exothermicities increase due to the decreasing proton affinity of the cations. In fact, from a thermodynamic standpoint, a reaction should always occur between oppositely charge ions.

In comparison, the energy diagram of gas-phase proton transfer ion/molecule reactions is displayed in Figure 1 (b). The reaction process can be seen in process (3).\(^{36}\) The entrance channel of an ion/molecule reactions is dominated by shorter-range polarization forces, which is analogous to the exit channel of the ion/ion reactions. As for the exit channel of an ion/molecule reaction (same charged product ions), the repulsive \(+Z_1Z_2e^2/r\) potential is dominant and creates a coulomb barrier. Overall, the energy surface can be described by the reaction enthalpy of ion/molecule reactions. The reaction enthalpy is determined by the difference of proton affinities of B and \([M+nH]^{nH^+}\), as seen in process (4).
\[ [M+nH]^{nH^+} \rightarrow [M+(n-1)H]^{(n-1)H^+} + [B+H]^+ \]  

(3)

\[ \Delta H_{mx} = PA([B]) - PA([M+(n-1)H]^{(n-1)H^+}) \]  

(4)

Since the proton affinity of a neutral base (B) is significantly lower than [A-H], proton transfer ion/molecule reactions are always less exothermic than the ion/ion reactions and are likely to be endoergic processes.

1.4.3 Kinetics

When there is a vast excess of reagent ions, pseudo first order kinetics are achieved for forming a bound orbiting pair of oppositely charged ions. Under these conditions, the rate of analyte ion depletion has shown a charge square dependence, which is consistent with the \( r^{-1} \) attractive potential for the formation of a bound orbiting pair (\textit{vide supra}).\(^{36}\) The rate constant for the formation of a stable orbit is shown in equation (1):

\[ k_c = \nu \pi \left[ \frac{Z_1 Z_2 e^2}{4 \pi \varepsilon_0 \mu v^2} \right]^2 \]

where \( \varepsilon_0 \) is the vacuum permittivity, \( \mu \) is the reduced mass of the two reactants, and \( \nu \) is the relative velocity of the bipolar ions. Stable orbits can be achieved at relatively long distances due to the long-range attractive potential. At such long distance, chemistry cannot occur between the two ion populations. Bound orbits can either have high or low
eccentricity. High eccentricities will ultimately reach a distance during the orbit where a reaction will occur. At low eccentricities, a mechanism to bring the orbiting pair sufficiently close must occur in order for a reaction to proceed. Two mechanisms can account for diminishing orbiting distances: a third body interaction and the tidal effect. A third body interaction with the orbiting pair can either destroy or reduce the relative velocities of the orbiting pair. By reducing the relative velocities of the orbiting pair, smaller orbital distances are achieved and reaction will proceed. Bath gas (i.e. helium, nitrogen) in the reaction quadrupole at sufficient number densities will act as a third body. The tidal effect, however, is a more plausible mechanism for macroions (i.e., peptides, proteins). Macroions have significant internal structure that can be affected by the changing electric field associated with the bound orbit. These rapid changes cause translational energy to internalize, thus reducing the relative velocities.

1.4.4 Proton Transfer Ion/Ion Reactions

Single proton transfer reactions have been routinely performed in electrodynamic traps. The charge state of reactant ions can range drastically: multiply charged cations with singly charged anions, multiply charged anions with singly charged cations, and both multiply charged anions and cations. Single proton transfer can occur via a proton hopping mechanism or a long-lived intermediate complex. The proton hopping mechanism occurs when reactant ions fly past one another in their bound orbit and the energy is sufficient for proton transfer (vide supra). Complex formation, or long-lived intermediate complex, involves a sticky collision, where the two reactants come into intimate contact. During the lifetime of the complex, single or even multiple proton
transfers can occur. Collisional activation of long-lived complexes, comprised of a multiply charged cation and monoanion, generates charged reduced product ions (e.g., \( n-I \)), demonstrating the ability to perform proton transfer reactions through a long-lived complex.\(^{40}\)

Since its inception, there have been many applications for proton transfer reactions in the gas-phase. These reactions have been demonstrated on mixtures of biomolecular ions (i.e., nucleic acid and proteins).\(^{41,42,43,44}\) Reducing the mixture of multiply charged ions to predominately singly charged ions, regardless of the initial charge, provides multiple benefits: simplifying the mass spectrum and removal of overlapping charge state distributions, etc.\(^{31}\) In addition to reducing the charge state of ESI-generated mixtures, proton transfer reactions can be applied to collisional activation product ions. Collisional activation of multiply charged ions can lead to complex mixtures of different masses and charges.\(^{45,46,47,48,49,50,51,52,53,54}\) The proton transfer reactions can simplify the MS/MS data by converting all the product ions to mainly singly charged ions. Application of this chemistry to MS/MS product spectra allows for confident assignment, less complexity, and higher database confidence scores. In addition, the extent of consecutive proton transfer reactions can be controlled by a technique deemed ion parking.\(^{55}\) Using selective ion acceleration techniques (i.e., low amplitude resonant excitation on desired \( m/z \)), inhibition of consecutive ion/ion reactions can be selected at a desired analyte charge state.
1.4.5 Charge Inversion Ion/Ion Reactions

The ion/ion chemistry discussed to this point has exclusively dealt with single proton transfer or consecutive ion/ion reactions. A unique ion/ion reaction involves the conversion of analyte ions from one polarity into the opposite polarity.\textsuperscript{56,57,58} Charge inversion can only be achieved by forming a long-lived intermediate complex, where multiple protons can be transferred within the complex prior to dissociation, as seen in process (5).\textsuperscript{40} In order to perform charge inversion, the number of transferred protons to the analyte ion must exceed the number of charges that neutralize the analyte. Achieving consecutive ion/ion reactions that invert the polarity of the analyte are highly unlikely due to neutralization and the resulting low number densities of the neutral and reactant ions.\textsuperscript{56,57} In addition to multiple proton transfers, adduct formation with multi-charged and highly polarizable ions can undergo charge inversion.\textsuperscript{56} Here, the charge of the adduct exceeds that of the analyte ion by at least one and the long-lived complex does not dissociated in the time frame of the experiment, as seen in process (6).

\[
[M+H]^+ + [R-nH]^n- \rightarrow [M-H]^\cdot + [R-(n+2)H]^{(n+2)^\cdot} \quad (5)
\]

\[
[M+H]^+ + [R-2H]^2- \rightarrow [M+R-H]^\cdot \quad (6)
\]

Charge inversion ion/ion chemistry has owned a wide range of applications.\textsuperscript{59,60,61,62} Simply changing the polarity of the analyte ion can provide complementary or even additional sequence information. This chemistry is utilized in situations where the most abundant ionization mode is not necessarily the best polarity for activation. Charge
inversion has been utilized to concentrate multiply cations types (i.e., [M+Na]^+, [M-H+2Na]^+, [M-2H+3Na]^+) into a singly analyte anion type (i.e., [M-H])^60 as well as the reduction of chemical noise in a complex mixture. Charge inversion has also been utilized to increase the charge, or “charge up”, analyte anions into multiply protonated analyte ions.62,63

1.4.6 Covalent Ion/Ion Chemistry

The chemistries discussed above have been restricted to particle transfer and non-covalent interactions. A new area of ion/ion chemistry that has emerged recently involves forming covalent bonds at specific functional groups.64,65,66,67,68 Much of this gas-phase chemistry has been largely based on classic solution phase organic chemistry. Solution phase derivatization in mass spectrometry has been used to facilitate analyte ionization,69 quantification,70,71 and characterization.72,73 Mainly, gas-phase covalent chemistry has been incorporated into typical MS^n workflows to provide characterization in various biological macromolecules (i.e., proteins and peptides). Gas-phase derivatization provides multiple inherent benefits: speed (100s of milliseconds), quick comparison between modified and unmodified ions, high degree of control over reactants via mass selection, ability to control the number of modifications, avoidance of complex reaction mixtures, etc.31

In order to perform a successful covalent modification in the gas-phase, certain characteristics of the reagent must be present. A reagent ion must contain at least one polarizable (“sticky”) group to generate strong electrostatic interactions between the reactant ions as well as a reactive functionality, i.e. a bifunctional reagent ion. The sticky
group serves to anchor the reagent to the analyte ion so as to allow sufficient time for the reactive functionality to undergo a chemical reaction. The long lifetime of the complex generates high reaction efficiencies due to multiple interactions between the reactants and the effectively high concentration of reagent per analyte. Sulfonate and quaternary amines have been demonstrated to be excellent functionalities for promoting strong non-covalent interactions. Sulfonates, which are anionic in nature, generate strong electrostatic interactions with biological cations (i.e., lipids, nucleic acids, proteins, and peptides). Alternatively, quaternary amines, which contain fixed positive charges, generate strong electrostatic interactions with biological anions. Site specific covalent modification of unprotonated amines in the gas-phase has been demonstrated via Schiff base and N-hydroxysuccinimide (NHS) ester chemistry. In addition to primary amine modification, covalent modification has been demonstrated on carboxylic acid functionalities with carbodiimide reagents. Both the carbodiimide and NHS chemistry generate amide linkages, while the Schiff base chemistry generates imine bonds. For the most part, gas-phase covalent chemistry has been used to covalently tag, increase structural characterization, map three dimensional structure, and discover unique or rare chemistry.

1.4.6.1 Schiff Base Covalent Chemistry

Covalent ion/ion chemistry involving schiff base reagents will be discussed in greater detail than the NHS or carbodiimide chemistry due to the relevance with this dissertation. Schiff base formation involving either the N-terminus or the $\epsilon$-NH$_2$ group of
lysine residues has been demonstrated by reacting peptide cations with either singly or doubly deprotonated 4-formyl-1,3-benzenedisulfonic acid (FBDSA). A sulfonic acid group in the reagent anion can interact strongly with a protonated site on the peptide resulting in a long-lived complex (Scheme 1). Upon collisional activation of the complex, the aldehyde group can undergo nucleophilic attack by an available unprotonated primary amine resulting in loss of water and formation of a Schiff base. Water loss from the complex is a signature of Schiff base formation, although water loss is a common process from peptide ions.

Singly deprotonated FBDSA is useful for modifying multiply-protonated peptides, as shown in process (7). Note that the diamond symbol (♦) corresponds to the addition of FBDSA to M with loss of a water molecule. Doubly deprotonated FBDSA can be used to modify singly protonated peptides via charge inversion ion/ion chemistry. In the case of singly protonated peptides, reaction with doubly deprotonated FBDSA results in the conversion of the peptide cation to an anionic product, as indicated in process (8).

\[
[M+nH]^{n+} + [FBDSA-H]^− \rightarrow [M+FBDSA+(n-1)H]^{(n-1)+} \rightarrow [M+♦+(n-1)H]^{(n-1)+} + H_2O (7)
\]

\[
[M+H]^+ + [FBDSA-2H]^2− \rightarrow [M+FBDSA-H]^− \rightarrow [M+♦]^- + H_2O \quad (8)
\]
1.5  **Future Insights of Ion/Ion Chemistry**

Ion/ion reactions represent a flexible methodology to manipulate gaseous biological macromolecules. With the recent discovery of selective covalent chemistry, a promising new area of gas-phase reactivity has emerged. Potential areas of impact include primary sequencing, highly selective cleavages, selective modification of new biologically relevant functionalities, higher order structure analysis, *etc.* While many of the current reagent ions are bifunctional (i.e. reactive and sticky group), there is a need for trifunctional reagents. In addition to a sticky group to facilitate a long-lived complex and a reactive group to covalently bind the reagent to the analyte, a third functionality is needed to perform new chemistries that may not be accessed without covalent attachment (i.e., disruption of the long-lived complex prior to reaction). Other promising areas include laser activation/dissociation in conjunction with ion/ion chemistry as well as metal coordination chemistry.
1.6 References


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Figure 1.1: Generic energy diagrams for (a) an proton transfer ion/ion reaction (b) proton transfer ion/molecule reaction
Scheme 1: Summary of the charge inversion process involving covalent modification of unprotonated primary amines via gas-phase ion/ion reactions with FBDSA dianions.
CHAPTER 2. DISSOCIATION BEHAVIOR OF TRYPIC AND INTRAMOLECULAR DISULFIDE-LINKED PEPTIDE IONS MODIFIED IN THE GAS-PHASE VIA ION/ION REACTIONS

2.1 Introduction

Primary structure characterization by mass spectrometry is generally dependent upon fragmentation of ions derived from the molecule of interest. The two main options for maximizing the structural information of interest are selection of ion-type (e.g., protonated molecule versus radical cation versus deprotonated molecule, etc.) and ion activation conditions.\textsuperscript{1} The ion-type is usually determined by the means for ionization. It is also common to subject analyte species, such as peptides or proteins, to a derivatization reaction in solution to facilitate ionization,\textsuperscript{2} quantification,\textsuperscript{3} or structural characterization\textsuperscript{4} via mass spectrometry or tandem mass spectrometry. Alteration of the ion-type can also take place in the gas phase via ion/electron,\textsuperscript{5} ion/molecule,\textsuperscript{6} or ion/ion reactions.\textsuperscript{7} Most gas phase reactions have involved the gain or loss of protons or electrons by the analyte ions. Examples of selective covalent modification of polypeptide ions in the gas-phase are restricted to ion-molecule\textsuperscript{8} and ion/ion reactions and are still rare. However, several recent findings suggest that the selective modification of primary amines in peptide ions via ion/ion chemistry is straightforward via either Schiff base formation\textsuperscript{9} using aldehyde containing reagents or via amide bond formation\textsuperscript{10} using N-hydroxysuccinimide ester containing reagents. The ability to form covalent bonds selectively in the gas-phase
opens up new possibilities for probing the structures of gaseous polypeptides. For example, the cross-linking of peptide ions in the gas-phase has recently been demonstrated using bi-functional N-hydroxysuccimide ester reagent ions.11

Schiff base formation involving either the N-terminus or the $\epsilon$-NH$_2$ group of lysine residues has been effected by reacting peptide cations with either singly or doubly deprotonated 4-formyl-1,3-benzenedisulfonic acid (FBDSA). A sulfonic acid group in the reagent anion can interact strongly with a protonated site on the peptide resulting in a long-lived complex. Upon collisional activation of the complex, the aldehyde group can undergo nucleophilic attack by an available unprotonated primary amine resulting in loss of water and formation of a Schiff base. Water loss from the complex is therefore consistent with Schiff base formation, although water loss is a common process from peptide ions. Strong electrostatic interactions between the reagent anion and the peptide cation can occur, particularly between the sulfonate group and protonated arginine. Hence, either covalent or non-covalent modification of the peptide may alter the fragmentation behavior of a peptide ion. The identities of the fragmentation products arising from further activation of the water loss product to give cleavages along the peptide backbone can provide clues regarding the nature of the modification. Loss of intact FBDSA, for example, from the water loss product is a clear indication of an electrostatic interaction for those ions that undergo this loss.
Singly deprotonated FBDSA is useful for modifying multiply-protonated peptides\textsuperscript{9}, as shown in process (1):

\[ [M+nH]^n+ + [FBDSA-H]^- \rightarrow [M+FBDSA+(n-1)H]^{(n-1)+} \rightarrow [M+\bullet+(n-1)H]^{(n-1)+} + H_2O \quad (1) \]

Note that the diamond symbol (\(\bullet\)) corresponds to the addition of FBDSA to M minus a water molecule. Doubly deprotonated FBDSA can be used to modify either singly protonated peptides or peptides with three or more excess charges in a single step. In the case of singly protonated peptides, reaction with doubly deprotonated FBDSA results in the conversion of the peptide cation to an anionic product, as indicated in process (2):\textsuperscript{12}

\[ [M+H]^+ + [FBDSA-2H]^{2-} \rightarrow [M+FBDSA-H]^- \rightarrow [M+\bullet^-]^- + H_2O \quad (2) \]

Doubly protonated peptides can also undergo charge inversion in reactions with FBDSA di-anions but this entails two consecutive ion/ion reactions with the first involving single proton transfer (process (3)) to yield the singly protonated peptide, which can then undergo process (2):

\[ [M+2H]^{2+} + [FBDSA-2H]^{2-} \rightarrow [M+H]^+ + [FBDSA-H]^- \quad (3) \]

Single proton transfer, as represented by process (3), competes with long-lived complex formation, which constitutes the first step of processes (1) and (2).\textsuperscript{13} Complex formation
is the dominant process for the species relevant to this work but single proton transfer typically occurs as a competitive process.

All work to date using ion/ion reactions involving FBDSA anions with peptide cations has indicated that the modified products upon collisional activation often yield sequence information that is complementary to that derived from unmodified ions.\textsuperscript{9,12} However, the work has been restricted to a limited number of model peptide ions. In this work, we have extended the range of observations to include peptides with an intramolecular disulfide linkage as well as ions derived from tryptic digestion of a protein. Tryptic peptides are of interest because of their relevance to bottom-up protein identification.\textsuperscript{14} Peptides with intra-molecular disulfide linkages often present a challenge for deriving sequence information, particularly for protonated peptides, due to the fact that the disulfide linkage must be cleaved along with a backbone bond.\textsuperscript{15,16} In this work, somatostatin-14 and oxytocin, two peptide hormones, were chosen as model cyclic peptides.

2.2 Experimental Section

2.2.1 Materials

Methanol, glacial acetic acid, and ammonium hydroxide were purchased from Mallinckrodt (Phillipsburg, NJ). Ubiquitin from bovine erythrocytes, TPCK treated trypsin from bovine pancreas, somatostatin-14, 4-formyl-1,3-benzenedisulfonic acid, tris (2-carboxyethyl) phosphate hydrochloride, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Peptides MQIFVK, TITLIVEPSDTIENVK,
EGIPPDQQR, LIFAGK, TLSDYNIQK, and ESTLHLVLR were generated from the digestion of ubiquitin by trypsin. Oxytocin was obtained from Bachem Bioscience Inc. (Torrance, CA). Peptide analytes were prepared in ~100 µM aqueous solution prior to positive nanoelectrospray ionization (nESI). The anion reagent was prepared at a concentration of 3.5 mM in solution of 49.5/49.5/1 (v/v/v) water/methanol/ammonium hydroxide for negative nESI.

2.2.2 Tryptic Digest

The procedure for the tryptic digestion of ubiquitin has been described previously. Separation of the tryptic peptides was performed using a reverse-phase HPLC (Agilent 1100, Palo Alto, CA) equipped with an Aquapore RP-300 (7µm pore size, 100 x 4.6 mm i.d.) column (Perkin-elmer, Wellesley, MA). The gradient for the HPLC separation has been described previously. Following separation, collected fractions were lyophilized and dissolved in 250 µL of water.

2.2.3 Disulfide Reduction

The procedure for disulfide reduction has been described previously. Reduced cyclic peptides were filtered through a disposable PD-10 desalting column (GE Healthcare) using a 1% acetic acid solution. Collected fractions were lyophilized and dissolved in 250 µL of water.
2.2.4 Mass Spectrometry

Experiments were performed on QqQ tandem mass spectrometers (QTRAP 2000 and QTRAP 4000, AB Sciex, Concord, ON, Canada), which have been modified for ion/ion reactions. The QTRAP 4000 was used in cases in which the Schiff base product ions were of $m/z$ greater than 1700 due to its higher mass range. Alternately pulsed ESI emitters allowed for sequential ion injection into the q2 reaction cell. First, doubly or singly deprotonated FBDSA was accumulated in the q2 reaction cell. Next, the peptide cations were generated and transferred to the q2 cell to undergo mutual storage with the FBDSA anions for 500-1200 milliseconds. The product ions were then transferred to the Q3 ion trap for subsequent mass analysis via mass-selective axial ejection (MSAE) or for further interrogation via MS$^n$.

2.3 Results and Discussion

2.3.1 Tryptic Peptides

Ubiquitin was subjected to digestion with trypsin and cations derived from the following peptides were observed in the positive nESI mass spectrum: MQIFVK, LIFAGK, TITLEVEPSDTIENVK, EGIPPDQQR, TLSDYNIQK, and ESTLHLVLRL. Cations of each of these peptides were subjected to ion/ion reactions with anions derived from FBDSA and the results are provided below and in Supplementary Information. In all cases, comparisons were made of the CID behavior of the unmodified [M-H]$^-$ peptide as well as that of the singly or doubly protonated species.
The ion trap CID product ion spectrum of the modified TLSDYNIQK anion is compared to those of the singly deprotonated and doubly protonated forms of the peptide in Figure 1. In this case, cations of TLSDYNIQK were reacted with [FBDSA-2H]^{2-} to yield the negatively charged [TLSDYNIQK+FBDSA-H]^- complex. Ion trap collisional activation of the complex gave rise to a highly abundant water loss product, which is indicated as [M+♦]- in Figure 1(a). The diamond symbol (♦) associated with some products indicates a fragment that is consistent with the indicated product with the expected mass shift associated with the covalent modification. We note, however, that the labeled fragments can also, in principle, arise from non-covalent FBDSA adduction with water loss arising elsewhere in the peptide ion. In this particular case, no clear evidence for electrostatic binding, such as the appearance of [FBDSA-H]^− or the attachment of FBDSA to a product ion (see below) is apparent. The most abundant products are consistent with modified y-type and b-type ions, using the nomenclature that is adapted^{23} from the protonated peptide literature,^{24} which would correspond to α-ions and β-ions, respectively, in the nomenclature employed by Bowie.^{25} When compared to the unmodified anion (i.e., the [M-H]^− ion), the CID of the modified peptide in this case generates more sequence information (compare Figures 1(a) and 1(b)). Figure 1(c) shows the CID product ion spectrum of doubly protonated TLSDYNIQK, as this is the most abundant cation in the nESI mass spectrum. The modified peptide and the unmodified peptide cation of TLSDYNIQK produce similar sequence information (Figures 1(a) and 1(c)). Both spectra reflect cleavages of the same peptide bonds, although the relative contributions differ markedly. For example, the complementary
The $y_7/b_2$ pair dominates the cation spectrum whereas less selectivity in cleavage among amide bonds is apparent in the CID of the modified anion.

The results of TLDYNIQK are illustrative for tryptic peptides with a C-terminal lysine residue. The results for ions derived from MQIFVK (Figure 2), LIFAGK (Figure 3), and TITLEVEPSDTIENVK (Figure 4) are provided as Supplementary Information. In all three cases, the major fragmentation products from the [M+♦]$^-$ ions correspond to $y_♦$- or $b_♦$-type ions. The CID product ion spectra of the [M-H]$^-$ ions, on the other hand, provide fewer sequence-informative fragment ions. The results reported here are consistent with previous work involving condensed-phase covalent derivatization of the C-terminus of peptides with 4-aminonaphthalenesulphonic acid. The resulting anions showed abundant modified y-type ions, the origin of which was attributed to a charge-remote mechanism. The sulfonate anionic site is highly stable and is expected to inhibit intramolecular proton transfer relative to a carboxylate site, thereby reducing the contributions from at least some charge-mediated reactions.

Fragmentation spectra of the modified tryptic peptides with a C-terminal arginine residue shared commonalities with those of the C-terminal lysine peptides but also showed some unique products. As an example, the CID product ion spectra for ions derived from EGIPPDQQR are compared in Figure 2. In the spectrum of the [M+♦]$^-$ ion, both $b_♦$- and $y_♦$-ions are observed (Figure 5(a)), which suggests that the precursor ion population is comprised of a mixture of species with the attachment at either end of the peptide. However, the N-terminus is the only site with an available primary amine for Schiff base formation. The $y_♦$-ions are interpreted to arise from precursor ions with strong electrostatic attachment of a sulfonate group to the guanidinium side-chain of the
C-terminal arginine residue, such interactions are known to be strong in the condensed phase\textsuperscript{29,30} or via formation of a negatively charged proton-bound dimer with the C-terminus. Ion/ion charge inversion studies have shown that the C-terminus can play an important role in adduct formation.\textsuperscript{13,31} The water loss resulting from activation of the [EGIIPPDQQR+FBDSA-H]\textsuperscript{−} complex presumably can arise from several sites on the peptide (e.g., the Glu and Asp residues). The appearance of abundant [y\textsuperscript{+}FBDSA]\textsuperscript{−} products in Figure 2(a) is consistent with this interpretation, as the site from which water is lost is not expected to be dependent upon the site of electrostatic attachment. On the other hand, the water lost upon Schiff base formation must come from the aldehyde group of FBDSA. If all peptide ions were to undergo Schiff base formation, no products with an FBDSA adduct should appear.

Ion trap CID spectra of the [M-H]\textsuperscript{−} and [M+2H]\textsuperscript{2+} ions of EGIPPDQQR are shown in Figures 5(b) and 5(c), respectively. The [M-H]\textsuperscript{−} ion provides relatively few sequence-related product ions, the most prominent of which is the c\textsubscript{5}-ion, presumably arising from the previously noted cleavage associated with the aspartic acid side-chain.\textsuperscript{25} The doubly protonated species generates a series of complementary b- and y-type ions with cleavage N-terminal to the proline at the fourth residue accounting for a large fraction of the observed fragmentation. Clearly, each precursor ion reflected in Figure 2 gives distinct fragmentation pattern. It is particularly noteworthy that the apparent non-covalent attachment of FBDSA can affect cleavage of the peptide backbone, rather than lose FBDSA, at least for the arginine-containing peptides. A similar set of comparisons for ESTLHLVLRLR is shown in Figure 6.
2.3.2 Intramolecular Disulfide-Linked Peptides

Cations of two peptides with an intramolecular disulfide linkage, somatostatin-14 and oxytocin, were subjected to ion/ion reactions with anions of FBDSA to explore the effect of modification on fragmentation of the cyclic portions of the peptide ions. Polypeptide ions with intramolecular disulfide linkages, particularly protonated forms, often show little evidence for cleavage within the peptide loop defined by the disulfide bridge.\textsuperscript{15} For this reason, the peptides are often reduced prior to tandem mass spectrometry.\textsuperscript{32} However, gas-phase means for the selective cleavage of disulfide linkages have also been explored, such as the CID of deprotonated species,\textsuperscript{33,34} CID of alkali or alkaline earth cationized ions,\textsuperscript{35} CID of coinage metal cationized ions,\textsuperscript{15,36,37} photodissociation,\textsuperscript{38} electron induced dissociation,\textsuperscript{39} electron capture dissociation,\textsuperscript{40} and electron transfer dissociation.\textsuperscript{15} Some approaches rely on altering the ionization conditions while ECD/ETD require the reactant polypeptide to be multiply-charged. The use of [FBDSA-2H]\textsuperscript{2-} allows for the modification of singly-protonated species.

Figure 7 compares the ion trap CID product ion spectra of the modified form of somatostatin-14 (i.e., the [M+\textbullet]\textsuperscript{-} species) (Figure 3(a)) with those of the [M-H]\textsuperscript{-} and [M+H]\textsuperscript{+} ions (Figures 7(b) and 7(c), respectively). Ion trap CID of the [M+H]\textsuperscript{+} species yields essentially no sequence information. As previously reported in the CID of deprotonated somatostatin,\textsuperscript{15} the \(z_{12}\)-ion and an associated loss of \(\text{H}_2\text{S}_2\) dominate the product ion spectrum (Figure 7(b)). The formation of the \(z_{12}\)-ion is a two-step process that begins with cleavage of the disulfide linkage at the CH\_2-S bond of Cys\(_3\). Some internal fragments that contain neither the N-terminus nor the C-terminus are also noted, although much of the low level signal is not readily assigned.\textsuperscript{15} CID of the modified
somatostatin ions produces a major loss of FBDSA, which suggests that a significant fraction of the precursor ions are comprised of a non-covalently bound FBDSA. However, a series of $b_n\dagger+32$ ions, where $n=3-9$, is noted. This series of ions is consistent with Schiff base formation N-terminal to the backbone cleavage (e.g., the $b_3\dagger+32$ and $b_4\dagger+32$ ions suggest Schiff base formation at the N-terminus while the $b_5+32$ can be modified at the N-terminus, at Lys$_4$, or at Lys$_9$) as well as cleavage of the CH$_2$-S bond of Cys$_{14}$. Ion trap CID of disulfide linked peptide anions have shown cleavage at all three bonds along the disulfide linkage$^{30}$ (i.e., at the S-S bond or at either of the CH$_2$-S bonds of R-CH$_2$-S-S-CH$_2$-R). Evidence for a few $b_n\dagger$-ions that follow cleavage either at the S-S bond or at the CH$_2$-S bond of Cys$_3$ was noted but the abundances were very low relative to the $b_n\dagger+32$ ions.

The presence of the disulfide linkage in somatostatin has a profound effect on the ion trap CID of all three ion types, as reflected in the respective CID spectra shown in Figure 8. Modification of reduced somatostatin via reaction with FBDSA dianions leads to a spectrum rich in sequence-related ions (Figure 4(a)) that is comprised primarily of $b\dagger$- and $y\dagger$-type ions. As there is no need to break a disulfide linkage in order to observe cleavage products between the cysteine residues, no product ions with either missing or additional sulfur atoms are observed. As expected, the ion trap CID spectrum of the reduced [M-H]$^-\$ ion shows no evidence for a $z_{12}$-ion, as this ion is generated via a sequential fragmentation that begins with cleavage of the Cys$_3$ CH$_2$-S bond (see Figure 8(b)). Rather, the spectrum is dominated by losses of small neutral molecules, such as H$_2$S, and one major product from an apparent backbone cleavage that also involved small molecules losses (i.e., the $y_{12}$-H$_2$S-NH$_3$ ion). Ion trap CID of the protonated peptide
(Figure 8(c)) provides a similar range of backbone cleavages to that observed from the modified anion, although the relative abundances are markedly different. There is a small degree of complementarity associated with the data of Figures 8(a) and 4(c), however, as the modified anion shows unique evidence for cleavage of the Cys$_3$-Lys$_4$ amide bond, while the protonated molecule shows unique evidence for cleavage of the Thr$_{10}$-Phe$_{11}$ amide bond.

Oxytocin represents another peptide hormone with a cyclic portion defined by a disulfide linkage, in this case between Cys$_1$ and Cys$_6$. Figure 5 provides the ion trap CID product ion spectra from the modified anion (i.e., the [M+♦]- species, Figure 9(a)), the deprotonated molecule (Figure 9(b)), and the protonated molecule (Figure 9(c)). Similar to the case for somatostatin, CID of the [M+H]$^+$ and [M-H]$^-$ ions of oxytocin produces limited sequence information. The unmodified peptide anion and cation produce products from cleavages between two and three of eight interresidue linkages, respectively. Activation of the [M-H]$^-$ ion produces a few prominent c-ions, which are commonly observed in CID of deprotonated peptides with an asparagine residue. Dissociation of the protonated molecule shows a dominant N-terminal proline cleavage that falls outside of the loop defined by the disulfide linkage. A cleavage at the same amide bond is also highly abundant for the modified anion. Both the protonated molecule and the modified anion show evidence for cleavage of the disulfide bond in oxytocin by virtue of the appearance of b- or y-type ions from within the loop defined by the disulfide bond. Ions that contain both sulfur atoms or no sulfur atoms are clear indicators of cleavages of the disulfide linkage at CH$_2$-S bonds. Unlike the somatostatin case, no clear preference for cleavage of either of the two CH$_2$-S bonds is noted for the
oxytocin ions. The \([M+\bullet]^+\) ions show evidence for cleavages of five of the eight amide linkages, which yields primary structure information that largely overlaps with that derived from the protonated molecule. Taken together, the \([M+H]^+\) and \([M+\bullet]^+\) ions show cleavages at six of eight interresidue linkages. For comparison, the ion trap CID product ion spectra of the reduced forms of the three ion types are shown in Figure 6. Fragmentation of the \([M-H]^-\) ion (Figure 10(b)) proceeds largely through loss of one or two molecules of \(\text{H}_2\text{S}\). The \([M+H]^+\) (Figure 10(a)) and \([M+\bullet]^+\) (Figure 10(c)) ions of the reduced form of oxytocin show evidence for cleavages at six and five interresidue linkages, respectively. However, the spectrum of the \([M+H]^+\) ion tends to show more extensive losses of small molecules, such as water and ammonia, than does the \([M+\bullet]^-\) ion.

### 2.4 Conclusions

Doubly deprotonated 4-formyl-1,3-benzenedisulfonic acid reacts with protonated peptides largely via complex formation. Schiff base formation involving a primary amine of the peptide and the aldehyde group of the reagent anion can occur in conjunction with dehydration of the complex. However, water loss that is unrelated to the reagent can also occur. An electrostatic (or dipole/dipole) interaction between a sulfonate of the reagent anion and a protonated site on the peptide can be sufficiently strong that covalent bond cleavages can compete with simple detachment of the reagent. This work provides evidence for Schiff base formation associated with many of the ion/ion reactions studied, while, in some cases, the formation of complexes with a strong electrostatic interaction without Schiff base formation is indicated as well. This work
suggests that Schiff base formation can generally be expected for tryptic peptides and is also demonstrated for the peptide hormones somatostatin-14 and oxytocin, which both contain an intramolecular disulfide linkage. In all cases, the modified anions (i.e., those that undergo Schiff base formation as well as those that undergo non-covalent anion attachment) show fragmentation behavior that is distinct from either protonated or deprotonated forms of the peptide. Significantly more structural information, in particular, was derived from the modified anions than from the deprotonated species. The highly stable sulfonate group is expected to inhibit intramolecular proton transfer, which likely accounts for the markedly different fragmentation behavior of the modified anions relative to the deprotonated species. The structural information available from the dissociation of protonated forms of the various peptides overlapped with that from the modified anions but some complementarity was noted in most cases. Modification of peptide ions in the gas-phase via ion/ion reactions is an attractive means for increasing structural information from dissociation because it does not require chemical modification in solution. Schiff base formation and strong electrostatic binding are two possible means for making modifications. Future work will be devoted to understanding when these phenomena compete, which may point to novel reagents that react exclusively by one means or the other.

2.5 References


Figure 2.1: Ion trap CID product ion spectra of a) modified product, \([\text{M+}++]\), b) \([\text{M-H}]^-\), c) \([\text{M+2H}]^{2+}\) derived from \(\text{M = TLSDYNIQK}\)
Figure 2.2: Ion trap CID product ion spectra of A) [M + ♦], B) [M - H], C) [M + H]^+ derived from M = MQIFVK
Figure 2.3 Ion trap CID product ion spectra of A) \([M + \bullet]\), B) \([M - H]\), C) \([M + H]^+\) derived from \(M = \text{LIFAGK}\)
Figure 2.4: Ion trap CID product ion spectra of A) [M + ♦], B) [M - H], C) [M + 2H]$^2+$ derived from M = TITLEVEPSDTIENVK
Figure 2.5 Ion trap CID product ion spectra of A) [M + •], B) [M - H], C) [M + H]^+ derived from M = EQIPPDQQR
Figure 2.6: Ion trap CID product ion spectra of a) \([\text{M}+\bullet]\), b) \([\text{M-H}]^\cdot\), and c) \([\text{M+2H}]^{2+}\) derived from \(\text{M} = \text{ESTLHLVLR}\)
Figure 2.7: Ion trap CID product ion spectra of A) [M+\textbullet\textbullet\textbullet]^{-}, B) [M-H]^{-}, C) [M+H]^+ ions derived from \( M = AGCKNFFWKFTSC \) (somatostatin). The \( b_{n\textbullet\textbullet\textbullet} \) symbols in the somatostatin sequence at the top of the figure represent modified b-ions that originate from the loop defined by the disulfide linkage. These ions may contain different numbers of sulfur atoms depending upon which of the bonds of the disulfide linkage was cleaved.
Figure 2.8: Ion trap CID product ion spectra of a) [M+•], b) [M-H], c) [M+H]^+ derived from M = AGCKNFFWKFTSC (reduced somatostatin-14)
Figure 2.9: Ion trap CID product ion spectra of a) [M+\(\bullet\)], b) [M-H]-, and c) [M+H]+ derived from M = CYIQNCPLG-NH\(_2\) (oxytocin)
Figure 2.10: Ion trap CID product ion spectra of a) [M+\(\text{\textbullet}\)], b) [M-H\(-\)], and c) [M+H\(^+\)] derived from M = CYIQNCPLG-NH\(_2\) (reduced oxytocin)
CHAPTER 3. COVALENT AND NON-COVALENT BINDING IN THE ION/ION CHARGE INVERSION OF PEPTIDE CATIONS WITH BENZENE-DISULFONIC ACID ANIONS INTRODUCTION

3.1 Introduction

Tandem mass spectrometry has become the method of choice in generating sequence information from peptides and proteins.\textsuperscript{1,2,3} The major considerations for structural characterization via tandem mass spectrometry are ion-type and dissociation method.\textsuperscript{[4]} Typically, the ion-type is determined by the ionization method;\textsuperscript{5} however, manipulation of the ion-type in the gas-phase can be achieved via ion/electron,\textsuperscript{6} ion/molecule,\textsuperscript{7,8} and ion/ion reactions.\textsuperscript{9,10} Gas-phase ion/ion reactions allow for the decoupling of the ion-type from the ionization method by enabling the facile transformation of one ion-type into another within the mass spectrometer. One class of ion/ion reactions is charge inversion, where the ion of interest is produced in one polarity and then transformed to the opposite polarity.\textsuperscript{11,12,13} There have been several approaches to charge inversion, which include multiple ion transfer,\textsuperscript{14,15} electrostatic adduct formation,\textsuperscript{13,16} and covalent adduct formation.\textsuperscript{17,18} Charge inversion of a peptide cation by selective modification of a primary amine has been demonstrated via Schiff base formation using an aldehyde containing reagent.\textsuperscript{17,18} Charge inversion via electrostatic adduct formation has also been demonstrated on peptides using sulfonate-containing dianions, which display a propensity to form relatively stable long-lived complexes.\textsuperscript{13}
The nature of the analyte ion transformation that takes place in an ion/ion reaction is highly dependent upon the chemical characteristics of both the analyte and reagent ions. It has recently been demonstrated that selective covalent bond formation can take place in a gas-phase ion/ion reaction via Schiff base formation and via amide bond formation using N-hydroxysuccinimide ester-based reagents. Thus far, all ion/ion reactions that have resulted in covalent bond formation have involved multi-functional reagents. That is, the reagents have one or more groups that give rise to a strong electrostatic interaction to facilitate the formation of a relatively long-lived complex as well as one or more reactive groups. For example, Schiff base formation involving primary amines in peptides has been demonstrated using anions derived from 4-formyl-1,3-benzenedisulfonic acid (FBDSA) as reagents. A charged sulfonate group in the reagent anion strongly interacts with a protonated site on the peptide, thereby facilitating the formation of a long-lived complex. Schiff base formation within the complex involves the reaction of an uncharged primary amine (e.g., the N-terminus or $\epsilon$-NH$_2$ side chain of lysine) with the aldehyde functionality of singly or doubly-deprotonated FBDSA. Upon collisional activation, nucleophilic attack on the carbonyl carbon of the aldehyde by an unprotonated primary amine leads to imine formation and loss of water. In the case of a singly protonated peptide in reaction with doubly deprotonated FBDSA, the process results in charge inversion of the peptide cation via covalent attachment (i.e., Schiff base formation), as indicated in process (1):
\[ [\text{M+H}]^+ + [\text{FBDSA-2H}]^2- \rightarrow [\text{M+FBDSA-H}]^- \rightarrow [\text{M+♦}]^- + \text{H}_2\text{O} \] (1)

The diamond symbol (♦) represents the addition of FBDSA to the peptide with a loss of a water molecule. When the diamond symbol is present with fragment ions, this represents the mass shift associated with a covalent modification.

The original paper describing Schiff base formation upon charge inversion involving a protonated peptide and doubly-deprotonated FBDSA showed fragmentation of the [M+♦]- ion that gave a preponderance of evidence for Schiff base formation, with one exception. A y\textsubscript{7}♦-ion, along with many abundant b♦-ions, was noted for the [M+♦]- species generated by charge inversion of protonated angiotensin II (DRVYIHPF).\textsuperscript{17} The y\textsubscript{7}-ion is, by far, the dominant product ion in the ion trap CID of the [M-H]- ion, while no b-type ions are observed, but this fragment contains no primary amine groups that might be expected to undergo covalent modification. This exceptional case was speculated to result from a sub-population of ions that undergo strong electrostatic binding with the reagent without undergoing covalent reaction and that the loss of water for these ions is unrelated to Schiff base formation. Subsequent work with tryptic peptides also showed formation of modified ions with no obvious sites for Schiff base formation in some, but not all, cases.\textsuperscript{18} Furthermore, the observation of y\textsubscript{n}+FBDSA-ions in those peptides that showed unexpected y♦-ions gave additional evidence for strong electrostatic interactions that survive collisional activation, even when amide bonds are cleaved.

In this work, we examine the origins of the product ions generated from singly protonated peptides that undergo charge inversion with doubly deprotonated FBDSA to shed light on the origins of those products that are inconsistent with Schiff base formation.
For example, we clarify the nature of the electrostatic interaction involved in the negatively charged charge inversion product (i.e., interaction between sulfonate and protonated basic sites, such as arginine,\textsuperscript{21,22,23} and/or negatively charged proton-bound dimer between the sulfonate and the C-terminus or side-chains of Asp or Glu),\textsuperscript{13,16} as well as the nature of amino acid side chains in determining the likelihood for the observation of non-Schiff base dissociation products. To examine the behavior of species with strong electrostatic binding without the possibility for covalent reactions, we compare data collected for FBDSA reagent dianions with data collected using doubly deprotonated benzene disulfonic acid (BDSA). The latter species have been demonstrated to charge invert peptides and proteins via adduct formation (see reaction (2)).\textsuperscript{13}

\[
[M+H]^+ + [BDSA-2H]^2- \rightarrow [M+BDSA-H]^- \quad (2)
\]

These reagent ions have been studied in reactions with model peptides angiotensin II and YGGFLX, where X is F, K, R, or H, and variations thereof. These studies have provided a deeper understanding of the nature of the non-covalent reactions that contribute to stabilizing ion/ion reaction complexes.
3.2 Experimental Section

3.2.1 Materials

Methanol, glacial acetic acid, acetic anhydride, and ammonium hydroxide were purchased from Mallinckrodt (Phillipsburg, NJ). Ammonium bicarbonate, acetyl chloride, 1,3-benzenedisulfonic acid, 4-formyl-1,3-benzenedisulfonic acid, angiotensin II, and YGGFL were purchased from Sigma-Aldrich (St. Louis, MO). The peptides YGGFLK, YGGFLF, and YGGFLH were purchased from CPC Scientific (San Jose, CA). The peptide YGGFLR was purchased from Pepnome Ltd. (Zhuhai City, China). All peptides were used without further purification. The procedures for N-terminal acetylation and C-terminal methyl esterification have been previously described.24 Peptide analytes were prepared at a concentration of ~150 µM in a solution of 50/50 (v/v) water/methanol prior to positive nanoelectrospray ionization (nESI). The anion reagent, FBDSA or BDSA, was prepared at a concentration of ~3.5 mM in a solution of 49.5/49.5/1 (v/v/v) water/methanol/ammonium hydroxide for negative nESI.

3.2.2 Mass Spectrometry

All experiments were performed on a 4000 QTRAP QqQ mass spectrometer (AB Sciex, Concord, ON, Canada), which has been modified for ion/ion reactions.25 Alternately pulsed nESI emitters allowed for sequential ion injection into the q2 reaction cell.26,27 Doubly deprotonated reagent anions were ionized first and accumulated in the q2 reaction cell. Next, peptide cations were generated and transferred to the q2 cell to undergo a mutual storage reaction for 500-1000 milliseconds. The product ions were
transferred to the Q3 ion trap, where subsequent MS^n analyses and mass analysis via mass-selective axial ejection (MSAE) were performed.28

3.3 Results and Discussion

3.3.1 Angiotensin II Covalent and Non-Covalent Interactions

The species that was first noted to give rise to an adducted CID product ion that was inconsistent with Schiff base formation, angiotensin II, is described here to illustrate the phenomenology and to compare its behavior when reacted with dianions of BDSA. The ion trap CID of FBDSA- and BDSA-modified angiotensin II (i.e., [M+♦]- and [M+BDSA-H]-) are shown in Figure 1. In the ion/ion reaction of angiotensin II [M+H]^+ with doubly deprotonated FBDSA, a negatively charged complex comprised of the two reactants (i.e., [M+FBDSA-H]^−) is generated. When this complex is isolated and subjected to CID, an abundant water loss product is formed. The water loss product is represented as [M+♦]−, which is consistent with the dehydration reaction that leads to Schiff base formation. Water loss, however, is a very common process with peptide ions and does not, in itself, signal the covalent reaction. Subsequent collisional activation of the dehydration product generates several product ions with mass shifts consistent with the covalent Schiff base formation or electrostatic modification. These ions are labeled with the diamond symbol (♦). Ion trap CID of the [M+♦]^+ ion produces several b♦-ions (the negative fragment ion nomenclature used here is adapted from protonated peptide literature[29]) and two y_7-related ions (viz., y_7♦ and y_7+FBDSA−) (Figure 1 (a)). The presence of b♦-ions in the product spectrum is consistent with the covalent modification
of the N-terminus, which is the only primary amine site in angiotensin II. The greater contributions of the b\textsubscript{1}\textsuperscript{♦} and b\textsubscript{6}\textsuperscript{♦}-ions compared to the other b\textsuperscript{♦}-ions can be attributed to the well-established C-terminal cleavage at aspartate and N-terminal cleavage at the proline residue.\textsuperscript{[30,31]} The b\textsubscript{1}\textsuperscript{♦}-ion is particularly noteworthy in that b\textsubscript{1}-ions are rarely observed in the CID of unmodified peptide cations or anions.\textsuperscript{32,33} The presence of the y\textsubscript{7}\textsuperscript{♦}-ion is inconsistent with the interpretation that Schiff base formation takes place at the N-terminus, at least for the fraction of precursor ions that fragment to give this product ion. This observation led to the hypothesis that some of the [M+\textsuperscript{♦}]- ions were comprised of attached FBDSA with water loss arising from elsewhere in the peptide. This hypothesis requires that the initial [M+FBDSA]\textsuperscript{-} complex can lose water while retaining an electrostatically bound FBDSA reagent and that subsequent activation of the electrostatically bound [M+\textsuperscript{♦}]- species can fragment along the peptide backbone while retaining the adduct. Evidence that both can occur is provided by the product ion that is consistent with the y\textsubscript{7}+FBDSA assignment (see Figure 1(a)).

While the above observations are supportive of the hypothesis described above, it is possible that, for example, arginine might also undergo Schiff base formation, which could explain the presence of the y\textsubscript{7}\textsuperscript{♦}-ion, and the signal assigned as y\textsubscript{7}+FBDSA may have a different origin. The control experiment with [BDSA-2H]\textsuperscript{2-} as the reagent, which cannot engage in Schiff base formation, provides further support for the hypothesis. The reaction of [BDSA-2H]\textsuperscript{2-} with singly protonated angiotensin II produces a negatively charged, long-lived complex, [M+BDSA-H]\textsuperscript{-}. Ion trap CID of [M+BDSA-H]\textsuperscript{-} is shown in Figure 1(b). Fragments with an associated double dagger symbol (†) are consistent with the mass addition of [BDSA-2H]\textsuperscript{−}. The major contribution to the fragmentation
spectrum is the generation of the \(y_7\)-ion, which supports the \(y_7^{+}\) FBDSA assignment made in Figure 1(a). The dehydration product in Figure 1(b) (i.e., the \([M+BDSA-H-H_2O]^-\) ion) was isolated and subjected to ion trap CID resulting in formation of both \(y_7\) and \(y_7-H_2O\) products (Figure 2). The appearance of the \(y_7-H_2O\)-ion confirms that dehydration can occur within the \(y_7\)-ion and fully supports the hypothesis for the origin of the \(y_7\)-ion in Figure 1(a). The presence of weak signals that correspond to \(b_i\)-ions can be attributed to an interaction of the BDSA with the protonated arginine, which is the second residue of angiotensin II, with cleavage C-terminal to the attachment site. It is noteworthy that no evidence for \(b_1\)-ion formation is apparent in Figure 1(b) while the \(b_1\)-ion is prominent in Figure 1(a). Covalent modification of the N-terminus in solution is a strategy that has been used to enhance \(b_1\)-ion formation in peptide tandem mass spectra.\(^{34,35,36}\) It is also noteworthy that the \([BDSA-H]^-\) signal following CID, which arises from detachment of the reagent from the complex as a singly charged species due to proton transfer, is quite small and essentially no evidence for loss of neutral BDSA is observed. This confirms that cleavage of covalent bonds can be highly competitive with loss of the non-covalently bound BDSA.\(^{37,38,39}\)

The angiotensin II data support the hypothesis that the \([M+\bullet^-]^-\) species formed from the dehydration of the \([angiotensin II+FBDSA]^-\) adduct is comprised of a mixture of structures. While the nominal structure of the Schiff base product is clear (i.e. Schiff base formation at the N-terminus) (see Figure 3(a)), several possibilities exist for the nature of the electrostatic interaction. One possibility involves the interaction of the charged guanidinium group of arginine and the anionic sulfonate group on FBDSA (i.e., a strong acid-base interaction), which is illustrated in Figure 3(b). Another possible non-
covalent interaction could be the negatively charged proton bound dimer between a carboxylate group of the peptide and a sulfonate group of FBDSA (i.e., O$_3$S-C$_7$H$_5$O-SO$_3^-$--H$^+$--OOC-peptide). A third possibility that is open to disulfonic acid reagents is a combination of a strong acid-base interaction involving one of the sulfonate groups with the other sulfonate group being engaged in proton sharing with a carboxylate group (i.e., a combination of the two interactions mentioned above). A fourth possibility is a salt-bridge structure in which sulfonate and carboxylate groups interact with a protonation site, depicted here as HO$_3$S-C$_7$H$_5$O-SO$_3^-$--RH$^+$--OOC-peptide, where R represents the arginine side-chain. Neither the abundant y$_7^-$ and y$_4^-$-ions nor the b$_1$-ions in the spectra of Figure 1 can be used to distinguish between the four possibilities because of the aspartic acid residue at the N-terminus and the carboxylate group of the C-terminus. Therefore, experiments were conducted with protonated methyl esterified angiotensin II (i.e., both the aspartic acid side-chain and the C-terminus were methyl esterified) in reaction with [BDSA-2H]$^2^-$. This experiment serves to eliminate the possibilities for the three electrostatic interactions involving carboxylate groups. Ion trap CID of the [D(OMe)RVYIHPF(OMe)+BDSA-H]$^-$ species generated a spectrum (Figure 4) with methanol loss being the major process while the generation of [BDSA-H]$^-$ and water loss were observed at lower levels. Very little backbone cleavage was noted, which is in stark contrast to the data for the unmodified [DRVYIHPF+BDSA-H]$^-$ ion (Figure 1(b)). This result clearly indicates that at least one of the carboxyl groups of angiotensin II plays an important role in stabilizing the BDSA adduct so that backbone bond cleavage can compete with loss of BDSA.
3.3.2 Leucine Enkepalin-Arginine

A series of model peptides based on leucine enkephalin were examined to provide further insights regarding the nature of the electrostatic binding of FBDSA and BDSA reagent anions to peptide cations. Figure 5 illustrates the four examples for electrostatic binding mentioned above for the YGGFLR case. The model peptide YGGFLR was subjected to charge inversion ion/ion reactions with doubly deprotonated anions derived either from FBDSA or BDSA and then activated via ion trap CID (Figures 6(a) and 6(b), respectively). The fragmentation behavior of the modified peptides was also compared to that of the unmodified deprotonated peptide, [M–H]⁻ (Figure S-7). This peptide was chosen so that the N-terminus and the arginine residues are at opposite ends of the sequence. In the cases of the modified versions of the peptide (i.e., [M+♦⁻] and [M+BDSA]⁻) conditions were used to essentially deplete the precursor ion population completely (i.e., modifying the activation amplitude to effectively activate and fragment all of the precursor ion population) to avoid differential sampling of mixtures of structures, if present, that may have different kinetic stabilities. The [M+♦⁻] ion was completely depleted and its location on the mass scale is indicated with an asterisk symbol (*) in Figure 6(a). Upon collisional activation of [M+♦⁻], only N-terminally modified fragments are observed, which is consistent with covalent modification at the N-terminus. Many a♦- and b♦-ions along with neutral losses are observed in the product spectrum, which have been demonstrated previously to be common ion types generated via collisional activation of the [M+♦]⁺ species of other peptides. In contrast with the angiotensin II data, no evidence for electrostatic binding in the [M+♦]⁺ ion is present in the sequence related product ions, such as y♦-ions. However, a relatively small signal
consistent with [FBDSA-H] indicates that a small fraction of the precursor population may be electrostatically bound and fragments by loss of the neutral peptide, rather than cleavage of backbone bonds. Evidence for electrostatic binding and cleavage at backbone bonds has been observed, however, with arginine-containing tryptic peptides longer than approximately 8 or 9 residues.18

Charge inversion of YGGFLR via BDSA attachment (Figure 6(b)) illustrates a marked difference in fragmentation compared to the FBDSA modified peptide. Ion trap collisional activation of [M+BDSA-H] produces mainly y1-ions, which results from the interaction of the negatively charged reagent with the C-terminal arginine residue and/or the C-terminus. This observation differs drastically from the product spectrum of [M+♦], where the product ions are comprised solely of N-terminally modified fragment ions. The [M+BDSA-H] data provides further evidence that Schiff base modification is the dominant process via ion/ion reactions with the reagent FBDSA for YGGFLR. Charge inversion of YGGFLR via BDSA adduction also produced the most extensive C-terminal fragmentation in comparison to the other BDSA-modified peptides (Figure 6(b) and below). Ion trap CID of [M+BDSA-H] produces mainly y1-ions with a high relative abundance. Most notable is the high contribution of y4, where the relative abundance is higher than that of the loss of [BDSA-H]. In all other model peptides used (i.e., peptides not containing arginine), the most dominant dissociation pathway occurred via loss of negatively charged [BDSA-H] and the generation of the neutral peptide produced by proton transfer within the long lived complex, as shown in process (3):
This observation indicates that the electrostatic interaction between YGGFLR and the reagent is relatively strong and can compete with the dissociation of covalent bonds upon collisional activation. Figure 6(c) shows the ion trap CID product ion spectrum of the methyl esterified version of YGGFLR subjected to charge inversion with BDSA (i.e. CID of \([\text{YGGFLR(OMe)+BDSA-H}^-]\). In this case, process (3) is dominant. Hence, consistent with the methyl esterification experiment with angiotensin II described above, it is apparent that the carboxyl group of the C-terminus is involved in the strong electrostatic binding that allows backbone cleavage to compete with formation of \([\text{BDSA-H}]^-\) in the YGGFLR experiment.

The dissociation behaviors apparent in Figures 6(a) and 6(b) are clearly distinct from one another and illustrate the different ways in which covalent and electrostatic adduction can alter dissociation patterns. The ion trap CID of the unmodified peptide anion fragments quite distinctly from either of the modified forms of the peptide (see Figure 7), as seen previously with other peptide sequences.\textsuperscript{17,18} Ion trap CID of deprotonated YGGFLR produces significantly different relative contributions of the \(c_3\)- and \(b_5\)-ions, as well as the peaks associated with losses from the arginine side chain, such as loss of \(\text{HN=C=NH}\). Ion trap CID of the anions derived from YGGFLH, YGGFLK, YGGFLF, and YGGFL exhibit the similar marked differences compared to the spectra of the respective modified species and are also included as Supplemental Information (Figures 9, 11, 13, 14(c), respectively).
3.3.3 Leucine Enkepalin-Histidine/Lysine

Other model peptides, such as YGGFLH and YGGFLK, were subjected to ion/ion reactions to examine the CID behavior of protonated peptides without arginine but with other basic residues (see Figures 8 and 10, respectively). Ion trap CID of \([YGGFLH+\bullet]^−\) (Figure 8(a)) produces similar dissociation behavior as YGGFLR (Figure 6(a)). The major ion-types observed are N-terminally modified fragments, such as \(a\bullet\)- and \(b\bullet\)-ions, which is consistent with covalent modification of the N-terminus. The relatively small signal consistent with \([FBDSA-H]^−\) is the only indication of electrostatic binding in the \([YGGFLH+\bullet]^−\) ion population. Ion trap CID of \([YGGFLK+\bullet]^−\), on the other hand, exhibits distinct dissociation behavior from all other model peptides due to the major contribution of the \(y\bullet\)-ions, especially the contribution of the \(y_4\bullet\)-ion (Figure 10(a)). Collisional dissociation of \([YGGFLK+\bullet]^−\) also produces a \(b_5\bullet\)-ion of low abundance. The peptide YGGFLK contains two primary amines, where one is located at the N-terminus and the other is present at the C-terminal lysine, so the presence of \(b\bullet\)- and \(y\bullet\)-ions can both be consistent with Schiff base formation. The preponderance of \(y\bullet\)-ions suggests that most of the modification in this case takes place at the lysine residue. A relatively small signal consistent with \([FBDSA-H]^−\) is also observed in the \([YGGFLK+\bullet]^−\) spectrum, which is the only clear indication that some of the \([YGGFLK+\bullet]^−\) ions show electrostatic binding.

The dissociation behavior of BDSA-modified YGGFLH and YGGFLK (Figures 8(b) and 10(b), respectively) exhibit similar characteristics and are also distinct from their respective \([M+\bullet]^−\) product ion spectra. The major fragmentation channel following collisional activation of BDSA-modified YGGFLH and YGGFLK (i.e., \([M+BDSA-H]^−\))
is the loss of the neutral peptide and generation of [BDSA-H]. Smaller contributions of bi- and yi-ions are noted in both cases. The major sequence-related product ion in both cases is the y4-ion. The total signal associated with yi-ions is much greater than that for bi-ions, which suggests that the electrostatic binding is largely at the C-terminal basic residue. However, the fact that any bi-ions are noted at all indicates electrostatic binding at the N-terminus for at least some of the ions. This dissociation behavior of BDSA-modified YGGFLH and YGGFLK is distinct compared to that of BDSA-modified YGGFLR in that the former show largely loss of the neutral peptide whereas the latter shows largely backbone cleavages. This is likely due to the stronger interaction of protonated arginine with sulfonate than either protonated lysine or histidine, in analogy with solution behavior.

3.3.4 Leucine Enkepalin-phenylalanine and Leucine Enkepalin

The model peptides YGGFLF and YGGFL were subjected to ion/ion reactions to investigate the behavior of peptide ions without a basic amino acid residue. Ion trap CID of FBDSA-modified YGGFLF, (i.e., [M+♦]) exhibits dissociation behavior similar to the corresponding ions of YGGFLR and YGGFLH in that the product ion spectra are dominated by N-terminal fragments (see Figure 12(a)). Similar cleavages producing a♦- and b♦-ions are observed as well as similar relative contributions, where major contributions from the b5♦- and b4♦-ions are observed. The results of YGGFL (Figure 14(a)) are highly analogous to those of YGGFLF. The CID product spectrum of [YGGFL♦] produces all N-terminal fragments, which is consistent with the expectation that the covalent modification takes place at the N-terminus. The only evidence for a
contribution from ions with an electrostatic interaction comes from a minor signal consistent with [FBDSA-H].

Collisional activation of the electrostatic complex (i.e., [M+BDSA-H]) for YGGFLF (Figure 12(b)) leads to similar dissociation behavior as that noted for the analogous species from YGGFLH and YGGFLK. Loss of [BDSA-H] again is the dominant dissociation pathway generated via ion trap CID of BDSA-modified YGGFLF and the major backbone cleavage yields the y₄⁻-ion. The results from the [YGGFLF+H]/[BDSA-2H]²⁻ experiment are also highly illustrative of the [YGGFL+H]/[BDSA-2H]²⁻ experiment (Figure 14(b)). Ion trap CID of BDSA-modified YGGFL predominantly leads to [BDSA-H] and the most abundant backbone fragment arises from cleavage of the analogous peptide linkage, which leads to a y₃⁻-ion for this shorter peptide. Data were also collected for cations of N-terminally acetylated YGGFL, methyl-esterified YGGFL, and YGGFL with both N-terminal acetylation and C-terminal methyl-esterification, in reaction with doubly deprotonated [BDSA-2H]²⁻. In all cases, [M+BDSA-H] ions were formed and, in all cases, ion trap collisional activation led to strongly dominant [BDSA-H] formation (see Figure 15). Collectively, the experiments with the YGGFLX model ions clearly show that strong electrostatic binding involving the BDSA anions takes place with peptide cations and that collisional activation of the electrostatically-bound cations can give rise to competitive backbone cleavage. However, backbone cleavage is most strongly competitive, by far, when arginine is present and at least one carboxyl group are both present in the peptide. In all other cases, loss of the peptide and formation of the BDSA anion is the most favored process. The dissociation behavior of BDSA-modified methyl esterified model peptides (i.e., YGGFLR-OMe and
YGGFL-OMe) demonstrates the increased strength of the electrostatic interaction between the carboxyl groups and the reagent anion. This set of observations points to the importance of the interactions that involve both the protonated site of the peptide and a carboxyl group of the peptide (i.e., structures (c) and (d) of Figure 3).

3.4 Conclusions

These studies have added new insights into the nature of the non-covalent interactions of benzene disulfonic acid anions with peptide cations. The substitution of two sulfonic acid groups on the ring of benzaldehyde (i.e., FBDSA) has proved to be important in the observation of gas-phase Schiff base formation involving primary amines in peptide ions. These studies have confirmed earlier speculation that non-covalent interactions in the peptide-BDSA/FBDSA complex can be sufficiently strong to allow covalent bond cleavage to compete with loss of BDSA/FBDSA as an anion or neutral. Such an interpretation was forthcoming from the observation of adduct-containing fragments that did not contain a primary amine for Schiff base formation. Studies with model protonated YGGFLX peptides clearly show that two criteria greatly facilitate the observation of covalent bond cleavage versus disruption of non-covalent binding. These include the presence of arginine, which gives rise to the strongest acid-base interaction of all of the basic side chains, and the presence of a carboxyl group, which can engage in proton sharing with a sulfonate group or stabilize a salt-bridge interaction. While the data presented here cannot be used to distinguish the latter two possibilities, our experience with benzene sulfonic acid (i.e., a single sulfonate group present on the reagent anion) in reaction with multiply protonated peptides (data not
shown), which should be able to undergo a salt-bridge type interaction, show dominant loss of the reagent from complexes with the peptide. This leads us to favor the combined acid-base/proton-bound dimer interaction involving both sulfonate groups (i.e., the interaction of structure (c) in Figure 5).

The improved understanding derived from this work regarding the nature of non-covalent interactions in ion/ion chemistry is useful in considering the design of reagents for specific purposes. For example, the ‘tuning’ of binding strength between reagent and analyte ion via control over the identities of functional groups in both the analyte and reagent can be used to either enhance or minimize particular reaction channel types. For example, it may be possible to increase the cleavage of covalent bonds in ion/ion adducts by incorporating an additional sulfonate group in the reagent. It might also be possible to minimize covalent bond cleavage in arginine containing peptides by using a reagent dianion with one sulfonate group and one carboxylate group instead of two sulfonate groups. This work has indicated, for example, that contributions from covalent bond cleavage from electrostatically bound adducts can be minimized by esterification of the peptide carboxyl groups. The results reported here, therefore, provide useful new insights in the design and application of reagents for gas phase ion/ion reactions.

3.5 References


35  Keough, T.; Youngquist, R.S.; Lacey, M.P. Anal. Chem. 2003, 75, 156A-165A.

Figure 3.1: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+♦], b) BDSA-modified product, [M+BDSA-H]⁻, M = angiotensin II
Figure 3.2: Ion trap CID product ion spectrum of [M+BDSA-H-H_2O]^-, derived from M = angiotensin II (DRVYIHPF).
Figure 3.3: Ion population of [M+1]. a) Schiff base formation with singly protonated angiotensin II and doubly deprotonated FBDSA, b) One of the possible strong electrostatic interactions that may play a role in the observation of the y1+ ion of Figure 1(a). This interaction involves a deprotonated sulfonate group on FBDSA and the charged guanidinium side chain of arginine.
Figure 3.4: Ion trap CID product ion spectrum of the BDSA adduct of methyl esterified angiotensin II (D(OMe)RVYIHPF(OMe)), i.e., [M+BDSA-H].
Figure 3.5: Four examples of possible non-covalent interactions between protonated YGGFLR and the dianion of BDSA. (a) Strong acid-base interaction. (b) Negatively charged proton-bound dimer. (c) Combined strong acid-base and proton-bound dimer interactions. (d) Salt-bridge interaction.
Figure 3.6: Ion trap CID product ion spectra of a) FBDSA-modified product, \([M+\bullet]^-\), b) BDSA-modified product, \([M+BDSA-H]^-\), c) methyl esterified YGGFLR-BDSA adduct, \([YGGFLR(OMe)+BDSA-H]^-\).
Figure 3.7: Ion trap CID product ion spectrum of [M-H]⁻ derived from YGGFLR.
Figure 3.8: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+]\(^{-}\), b) BDSA-modified product, [M+BDSA-H] derived from M = YGGFLH
Figure 3.9: Ion trap CID product ion spectrum of [M-H]- derived from M = YGGFLH.
Figure 3.10: Ion trap CID product ion spectra of a) FBDSA-modified product, [M+♦], b) BDSA-modified product, [M+BDSA-H] derived from M = YGGFLK
Figure 3.11: Ion trap CID product ion spectrum of [M-H]- derived from M = YGGFLK.
Figure 3.12: Ion trap CID product ion spectra of a) FBDSA-modified product, \([M+]\), b) BDSA-modified product, \([M+BDSA-H]^-\), derived from \(M = YGGFLF\)
Figure 3.13: Ion trap CID product ion spectrum of [M-H]⁻ derived from M = YGGFLF.
Figure 3.14: Ion trap CID product ion spectra of a) FBDSA-modified product, \([M^{+}]\), b) BDSA-modified product, \([M+BDSA-H]^-\), and c) the deprotonated molecule \([M-H]^-\) derived from \(M = YGGFL\).
Figure 3.15: Ion trap CID product ion spectra of a) [ac-YGGFL+BDSA-H]⁻, b) [YGGFL-OMe+BDSA-H]⁻, and c) [ac-YGGFL-OMe+BDSA-H]⁻.
CHAPTER 4. ION/ION REACTIONS OF MALDI-DERIVED PEPTIDE IONS: INCREASED SEQUENCE COVERAGE VIA COVALENT AND ELECTROSTATIC MODIFICATION UPON CHARGE INVERSION

4.1 Introduction

Peptide sequencing for identification and structural characterization of proteins is a longstanding activity in molecular biology research. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), two very successful means for the derivation of gas phase ions from proteins and peptides, coupled with tandem mass spectrometry, have become the dominant tools for generating sequence information from mixtures of peptides. While there is significant overlap, ESI and MALDI have each found applications for which they are best suited. For example, ESI has been widely used to interface on-line liquid separations with mass spectrometry, whereas MALDI is widely used for sampling surfaces, as in imaging applications. MALDI has been adapted to several tandem MS instrument geometries, e.g. tandem time-of-flight (i.e., TOF/TOF), quadrupole/time-of-flight (i.e., Q/TOF), and ion trap analyzers. The appeal of ion trap technology is that it permits MS^n experiments to be conducted on MALDI-derived biomolecule-ions.

From the standpoint of sequencing, a significant difference between MALDI and ESI is their propensities for generating multiply charged ions. Under commonly used conditions, MALDI generates ions of lower charge than does ESI. In fact, for peptides,
singly charged ions usually dominate. Low energy collisional activation of singly protonated tryptic peptides, however, often does not produce extensive sequence information.\textsuperscript{17,18} Dissociation behavior is dominated by low energy fragmentation pathways, e.g. cleavage C-terminal to an aspartic acid residue or N-terminal to a proline residue.\textsuperscript{19} Furthermore, MALDI-derived tryptic peptides may generate limited sequence information due to the basic residue present at the C-terminus.\textsuperscript{20,21,22} Tryptic peptides often generate fragment ions associated with the C-terminus (e.g. y-ions) as a result of the C-terminal lysine or arginine sequestering the excess proton.\textsuperscript{20} A variety of approaches have been attempted to improve peptide sequencing in conjunction with MALDI. For example, condensed-phase peptide modification, such as fixed-charged derivatization of the N-terminus, has been attempted with mixed success.\textsuperscript{23,24} A noteworthy approach, developed by Keough et al., to increase sequence information from the dissociation of MALDI-derived tryptic peptides involved conjugating a sulfonic acid group to the N-terminus via solution phase chemistry.\textsuperscript{25,26} The objective was to alter fragmentation pathways by introducing a highly acidic group into the peptide. Fragmentation was dramatically improved in derivatized tryptic peptides compared to the underivatized tryptic peptides. However, the N-terminal position of this modification precluded the presence of N-terminal fragments due to the anionic sulfonate group.\textsuperscript{26}

We and others have been developing approaches to modify ions in the gas phase and within the context of a tandem mass spectrometry experiment (i.e., modification of mass-selected ions) to expand the capabilities of tandem mass spectrometry for ion structure characterization using ion/ion reactions.\textsuperscript{27} Ion/ion reactions can be used to decouple the ion-type initially generated by the ionization method from the ion-type
subjected to tandem mass spectrometry. Structural information derived from fragmentation is highly dependent upon the nature of the ion (e.g., positive ion vs. negative ion, even-electron ion versus odd-electron, etc.). Therefore, ion/ion reactions can expand the range of ion-types that can be subjected to tandem mass spectrometry relative to the ion-types available from the ionization method. For example, ion/ion reactions have been used to manipulate peptide ion charge state and polarity via the transfer of one or more protons, generate odd-electron ions from even-electron ions via electron transfer, and insert metal ions into polypeptides. Recent studies have demonstrated the alteration of peptide dissociation behavior via the gas phase electrostatic attachment of reagents and via the gas phase covalent modification of peptide ions. All such examples to date have been restricted to peptide ions generated via ESI.

In this work, we demonstrate the gas phase modification of peptide ions generated by atmospheric pressure MALDI (AP-MALDI) using a dual source interface developed by Schneider et al. attached to a hybrid triple quadrupole/linear ion trap tandem mass spectrometer and the structural characterization of the modified and unmodified versions of the ions via ion trap CID. The formation of imine bonds (i.e. Schiff base formation) using an aldehyde containing dianion, 4-formyl-1,3-benzenedisulfonic acid (FBDSA) has been demonstrated with peptide ions generated via ESI. Possible sites of peptide covalent modification are the N-terminus and the ϵ-NH₂ of lysine. Strong non-covalent interactions (i.e. electrostatic modification) have also been observed between the sulfonate groups of FBDSA and peptides containing an arginine residue and one or more carboxyl groups. Covalent bond cleavage is competitive with disruption of this strong
electrostatic interaction under collisional activation conditions. In either case (i.e., covalent modification or strong electrostatic binding), the reaction product is a singly charged anion. The overall approach described here differs from the Keough et al. approach in that singly charged anions are subjected to CID, rather than singly charged cations, but is similar in that sulfonate is introduced into the peptide to alter dissociation behavior. Ion/ion reactions have been performed on tryptic peptides of ubiquitin and two synthetic peptides, APPGFSPFR and GLSDGEWQQVLANVWGK. Collisional activation of modified tryptic peptide anions demonstrates modification of the primary amine at the N-terminus and the C-terminal basic residue. Such modifications produce both modified N- and C-terminal fragment ions compared to the solely C-terminal information observed by Keough. Ion trap CID of the modified anions is shown to generate more sequence information than the unmodified ion, especially in the form of b-type ions. This study represents the first demonstration of gas phase modification of MALDI-derived ions via ion/ion reactions.

4.2 Experimental Section

4.2.1 Materials

Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). Ubiquitin from bovine erythrocytes, TPCK treated trypsin from bovine pancreas, alpha-cyano-4-hydroxycinnamic acid (CHCA), FBDSA, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic peptides APPGFSPFR and GLSDGEWQQVLANVWGK were purchased from SynPep (Dublin, CA). Peptides
MQIFVK, TITLEVEPSDTIENVK, EGIPPDQQR, LIFAGK, TLSDYNIQK, and ESTLHLVLRL were generated from a tryptic digestion of ubiquitin.

4.2.2 Methods

The MALDI matrix, CHCA, was prepared in 39.5/59.5/1 (v/v/v) water/acetonitrile/TFA at a concentration of 10 mg/mL. Peptide analytes were prepared in a ~100 µM aqueous solution prior to a 50/50 (v/v) mixture with the MALDI matrix solution. 2.0 µL of the matrix/analyte solution was spotted onto the MALDI stage plate and allowed to air-dry prior to attachment to the AP-MALDI source. The anion reagent was composed of a 3.5 mM solution of 50/50 (v/v) water/acetonitrile for negative microionspray.

4.2.3 Tryptic Digest

The procedure for the tryptic digest of ubiquitin has been described previously. Separation of the tryptic peptides was performed on a reverse-phase HPLC (Agilent 1100, Palo Alto, CA) using an Aquapore RP-300 (7µm pore size, 100 x 4.6 mm i.d.) column (Perkin-Elmer, Wellesley, MA). The gradient for the HPLC separation has been described previously. Following HPLC separation, the collected fractions were concentrated and reconstituted in 200 µL of water.
4.2.4 Mass Spectrometry

Experiments were performed on a 4000 AB Sciex QTRAP (Concord, ON, Canada), which has been modified to perform ion/ion reactions. The ion source is equipped with an AP-MALDI source (MassTech, Columbia, MD) and with a micro-ion spray emitter (New Objective Inc, Waburn, MA) orthogonal to the sample inlet (Figure 1). The micro-ion spray emitter, remote to the MS inlet and MALDI stage plate, allows for the introduction of opposite polarity ions into the MS. Briefly, the AP-MALDI source has a circular opening, where a conductive transfer tube penetrates into the ion source and attaches to the curtain plate. The micro-ion spray is directed towards the transfer tube and upon ionization/nebulization, ions travel into the transfer tube and the ion source.

The reagent anion, doubly deprotonated FBDSA, was generated via micro-ion spray at a continuous flow rate of 8.0 μL/min from a silica sprayer (364 μm outer dimension, 48 μm inner dimension, Polymicro Technologies, Phoenix, AZ). N₂ acts as the nebulizing gas, which helps guide the reagent anions into the ion source. The nebulizing gas was triggered/pulsed via MS Expo software (AB Sciex, Concord, ON, Canada) during negative ion injection conditions. Once doubly deprotonated FBDSA entered the MS, it was transferred to the q2 collision cell and stored. Tryptic peptides, individually spotted in CHCA, were ablated via a 337-nm nitrogen laser (AP-MALDI Ion Source 110, MassTech, Columbia, MD) and transferred to the q2 collision cell to undergo ion/ion reactions with the reagent anions. In the peptide mixture study, multiple peptides from the reconstituted solutions were spotted on the MALDI stage plate with CHCA. All cations produced via MALDI in the mixture study were transferred to q2.
without mass discrimination and reacted with FBDSA dianions. Product ions generated in q2 were transferred to Q3 for further collisional activation and subsequent mass analysis.51

4.3 Results and Discussion

Ubiquitin was subjected to tryptic digestion and the tryptic peptides MQIFVK, TITLEVEPSDTIENVK, EGIPPDQQR, LIFAGK, TLSDYNIQK, and ESTLHLVLRLR were collected. The MALDI-derived tryptic peptide ions were subjected to ion/ion reactions with doubly deprotonated FBDSA. The main product of the ion/ion reaction is a negatively charged, long-lived complex comprised of the reagent dianion and tryptic peptide cation. The complex is formed when a negatively charged sulfonate interacts strongly with a protonated site on the peptide. The aldehyde group of the reagent undergoes nucleophilic attack by a neutral primary amine of the peptide during collisional activation of the complex leading to the formation of an imine bond (i.e., Schiff base) and loss of a water molecule. The Schiff base product anion is represented in spectra by [M+♦]−. The process of forming a Schiff base via gas phase ion/ion reactions is depicted in process (1):

\[ [\text{M}+\text{H}]^+ + [\text{FBDSA}-2\text{H}]^{2-} \rightarrow [\text{M}+\text{FBDSA}-\text{H}]^- \rightarrow [\text{M}+\text{♦}]^- + \text{H}_2\text{O} \quad \text{(1)} \]

The diamond symbol (♦) represents the mass addition of the reagent anion following a water loss. Fragments that are consistent in mass with those that retain the covalent modification are labeled with the diamond. The negative ion mode post-ion/ion reaction
spectrum (Figure 2(a)) and positive MALDI mass spectrum of a mixture of three tryptic peptides (viz., LIFAGK, EGIPPDQQR, and TLSDYNIQK) are compared in Figure 2. Figure 2(a) was generated by exposing peptide cations of Figure 2(b) to reaction with doubly deprotonated FBDSA. The ion/ion reaction generated a product spectrum containing primarily the intact complex (i.e., [M+FBDSA]−) and signals due to [M+♦]− of much lesser abundance. The [M+♦]− ions were likely generated by CID upon transfer from q2 to Q3. Subsequent isolation and activation of the [M+FBDSA]− ion generates abundant [M+♦]− for subsequent interrogation. Contributions from strong electrostatic binding of the reagent were much more apparent in the data for the arginine terminated peptides than for the lysine terminated peptides (vide infra). For this reason, results from lysine and arginine terminated peptides are presented separately.

4.3.1 Lysine Terminated Tryptic Peptides

Ion trap CID spectra of protonated TITLEVEPSDTIENVK and the modified peptide anion (i.e. [M+H]+ and [M+♦]−, respectively) are compared in Figure 3. Collisional activation of [M+H]+ leads to nine of fifteen amide bond cleavages, producing eleven fragment ions (Figure 3(b)). Fragmentation of the singly protonated peptide produces limited sequence informative ions as well as non-informative neutral losses. The product ion spectrum produces relatively large contributions from the fragment ions y6 and y9. The fragment ion y6 arises from the well-established C-terminal cleavage of aspartic acid,52 while the fragment ion y9 originates from the well-established N-terminal cleavage of the proline residue.53 Dominance of low-energy fragmentation
pathways has been frequently observed in tandem MS experiments with MALDI-derived peptide cations. When compared to b-ions, the y-ions clearly dominate the fragmentation spectrum of the unmodified cation. Collisional activation of the peptide cation produced only four b-ions compared to seven y-ions. Limited N-terminal information (e.g. b-ions) from the collisional activation of [M+H]+ can be explained by the presence of a C-terminal basic lysine residue as reported by Biemann.

Collisional activation of modified TITLEVEPSDITENVK (Figure 3(a)) produces distinct dissociation behavior compared to the unmodified spectrum. In the product ion spectrum, the most abundant peaks are associated with modified b- and y-ions, which are represented as b♦ or y♦. The nomenclature of modified fragment anions is adapted from the peptide literature for protonated peptides. The b♦-ions are interpreted to arise from a covalent modification of the N-terminus, while the y♦-ions are interpreted to arise from the covalent modification of the ε-NH₂ of lysine. Collisional activation of the modified peptide anion produces a higher degree of fragmentation than the unmodified peptide cation, where twelve of fifteen amide bonds are cleaved, generating a total of sixteen fragment ions and a significant increase in N-terminal information. In the spectrum of the modified ion, seven b♦-ions are produced compared to four b-ions in the spectrum of the unmodified peptide. We note that previous studies have clearly shown that [M+♦]− ions yield significantly more sequence information upon CID than the corresponding [M-H]+ ions. The incorporation of the two sulfonate groups into the peptide is expected to have a significant impact on proton mobility within the anion, which influences the favored dissociation pathways. This may favor charge remote processes or it may require the ions to be elevated to higher energies in order to promote intramolecular
transfer. In any case, the disulfonic acid modification to the peptide has been shown to lead to consistently greater sequence coverage than singly charged versions of the unmodified peptide (i.e., [M+H]$^+$ or [M-H]$^-$).\textsuperscript{38,44,45} Furthermore, modification at the N-terminus gives rise to much greater contributions from anionic b-top-t-ions, which are not readily observed without the modification. Ion trap CID of [M+\textit{t}]+ also appears to have less selective cleavage along the peptide backbone compared to the peptide cation, which has been observed in previous studies.\textsuperscript{45}

Another ubiquitin tryptic peptide with a C-terminal lysine, TLSDYNIQK, produced results similar to those noted for the peptide TITLEVEPSDTIENVK. Ion trap CID of modified TLSDYNIQK (i.e. [M+\textit{t}]+) produced a high degree of sequence information, where a cleavage at every amide bond is observed (Figure 4(a)). The presence of many b-top-t- and y-top-t-ions is consistent with the covalent modification of the N-terminus and $\epsilon$-NH$_2$ of lysine. The high relative contribution of b$_4$- in the product ion spectrum is attributed to the labile C-terminal cleavage of aspartic acid.\textsuperscript{52} The presence of b$_1$- is particularly noteworthy because the b$_1$-ion is not typically observed in the collisional activation of unmodified protonated and unprotonated peptides.\textsuperscript{57,58} Ion trap CID of the singly protonated peptide generates a product ion spectrum with seven of eight amide bond cleavages (Figure 4(b)); however, many less informative fragment ions are also observed in the spectrum (e.g., internal fragments and neutral losses). The product spectrum of the unmodified peptide cation is dominated by the C-terminal cleavage of aspartic acid, generating the highly abundant y$_5$-ion. Conversely, the modified anion appears to fragment more uniformly. Dissociation of the peptide cation is dominated by the generation of y-ions, while the presence of b-ions is limited. The
modified peptide anion produces a significant number of b♦-ions and an even higher number of y-type ions due to the presence of y1♦ and y2♦. With a near doubled increase of y- and b-type ions (i.e. thirteen versus seven fragment ions), the modified peptide ion clearly produced more sequence information compared to the unmodified peptide.

The peptides TITLEVEPSDTIENVK and TLSDYNIQK are illustrative of the synthetic peptide GLSDGEWQQVLNVWQK. Collisional activation of the modified and unmodified GLSDGEWQQVLNVWQK is provided below (Figure 5). Ion trap CID of [M+♦]- produces a high order of b♦- and y♦-ions (Figure 5(a)), while the protonated peptide produces limited b- and y-ions (Figure 5(b)). The modified peptide anion again produced a higher order of sequence information than the unmodified peptide cation. However, the aforementioned lysine containing peptides are not necessarily illustrative of low m/z peptides, such as MQIFVK (Figure 6) and LIFAGK (Figure 7).

Ion trap CID of modified MQIFVK (i.e. [M+♦]-) produces mainly y♦-ions at four of the five amide bond cleavages (Figure 6 (a)) and only one ion in the b♦-ion series (i.e., the b♦3-ion), which is distinct from the observation of many more b♦-ions from the large modified peptide ions (i.e. TITLEVEPSDTIENVK, TLSDYNIQK, and GLSDGEWQQVLNVWQK). Ion trap CID of the unmodified peptide cation produces many b- and y-ions, which account for four of the five amide bond cleavages (Figure 6(b)). Smaller peptide ions tend to yield more extensive sequence information in general.17 While similar amide bonds are cleaved in the modified and unmodified peptide ions, there is a degree of complementarity from the two peptide ion-types. The modified anion produces lower m/z y♦-ions, while the [M+H]+ produces mainly b-ions. Also, the relative contributions of the fragment ions in the product ion spectra are
markedly different. The modified peptide ion produces a highly abundant \( y_3\)-ion, and the peptide cation generates a highly abundant internal fragment ion, showing the distinct dissociation behavior. The results from the peptide MQIFVK are illustrative of the peptide LIFAGK (Figure 7). Ion trap CID of \([\text{LIFAGK}+\dot{\text{I}}]^-\) produces mainly modified \( y\)-ions, which is consistent with the covalent modification of the \( \epsilon\)-NH\(_2\) of lysine. Collisional activation of the unmodified peptide cation produces a-, b-, and y-ions. Ion trap CID of both the modified and unmodified versions of LIFAGK displays a degree of complementarity, much like MQIFVK.

### 4.3.2 Arginine Terminated Tryptic Peptides

Previous studies of the ion trap CID of modified arginine containing peptides (i.e. angiotensin II (DRVYIHPF) and ubiquitin tryptic peptides) demonstrated product ion spectra consistent with two product populations: Schiff base formation and non-covalent binding of the reagent to the guanidinium side chain of arginine\(^{39,45}\). Product ion spectra showed the appearance of \( y\)-ions; however, the only primary amine in the peptide capable of engaging in Schiff base formation was the N-terminus. The \( y\)-ions were determined to arise from \([M+\dot{\text{I}}]^-\) ions composed of an electrostatic interaction with the arginine guanidinium side chain. The water loss leading to the nominal \([M+\dot{\text{I}}]^-\) originated from elsewhere in the peptide (i.e., it was unrelated to the reagent). The presence of \( y+\text{FBDSA}^-\)-ions also provided evidence of an electrostatic rather than a covalent interaction. Model systems (i.e. YGGFLX peptides) further demonstrated strong interactions between the sulfonate groups and peptides containing an arginine residue and one or more carboxyl groups (e.g. C-terminus) in the gas phase\(^{38}\). These non-
covalent interactions with FBDSA dianions are sufficiently strong that covalent bond cleavage can compete with reagent detachment upon collisional activation.\textsuperscript{38,45}

Ion trap CID of modified EGIPPDQQR is displayed in Figure 8(a). Collisional activation of the modified anion generated fragment ions consistent with covalent and electrostatic modifications. The presence of $b$-ions is consistent with the covalent modification of the N-terminus, while $y$ and $y$-FBDSA-ions are consistent with an electrostatic attachment to the peptide (e.g. reagent interaction with arginine and C-terminus). The presence of $y$-ions can be attributed to the electrostatic attachment of the FBDSA dianion to the C-terminal arginine and a water loss within the fragment ion (e.g. aspartic acid residue). The loss of $[\text{FBDSA-H}]^-$ also indicates an electrostatic interaction between the analyte and reagent. The loss of $[\text{FBDSA-H}]^-$ generates a neutral dehydrated peptide due to proton transfer within the complex, as seen in process (2).

$$[\text{M+FBDSA-H-H}_2\text{O}]^- \rightarrow \text{M-H}_2\text{O} + [\text{FBDSA-H}]^- \quad (2)$$

The high relative abundances from $[\text{FBDSA-H}]^-$ and $y_3$+FBDSA are particularly noteworthy. The significantly higher relative abundance of $y_3$+FBDSA than $[\text{FBDSA-H}]^-$ suggests that the electrostatic interaction can compete with cleavage of covalent bonds upon collisional activation. Such a competition gives rise to the $y$ and $y$-FBDSA-ions.

Collisional activation of the modified anion produces a higher order of sequence information than the peptide cation. The modified anion produces a mixture of ten $y$+FBDSA and $y$-ions, and when combined, the mixture produces seven of the eight
possible C-terminal amide bond cleavages. The modified peptide also produces more N-terminal fragments, i.e., b₃♦, b₆♦, and b₈♦, relative to the unmodified peptide cation. Collisional activation of the singly protonated EGIPPDQQR produces limited sequence information in comparison to the modified version, as seen previously. The unmodified cation produces mainly y-ions, while only a single b-ion was produced. Dissociation of the peptide cation is dominated by cleavage C-terminal to the acidic residues, i.e., the y₃- and y₈-ions, as seen in many MALDI-derived MS/MS experiments. Activation of the unmodified peptide cation produces similar y-type ions to the modified version, while the modified anion produces lower m/z y-type ions that the unmodified version lacks.

Ion trap CID of the modified and unmodified synthetic tryptic peptide, APPGFSPFR, is illustrated in Figure 9. The modified anion produces cleavages at all eight amide bonds, and the most notable observation in the product ion spectrum is the generation of a b♦-ion at each peptide bond (Figure 9(a)), including the b₁♦-ion. Collisional activation of [M+♦]- produces a combination of y♦- and y+FBDSA-ions. The product ion spectrum shows a high contribution from the well-established N-terminal cleavage of the proline residue (i.e. b₆♦-H₂O); however, more uniformity of cleavage among amide bonds is observed compared to the unmodified cation. While the ion trap CID of [M+H]+ (Figure 9(b)) and [M+♦]+ produces similar higher m/z y-type product ions, the modified anion also produces lower m/z modified y-ions (i.e. y₁+FBDSA). The most noteworthy observation of the product ion spectra is the drastic difference between the N-terminal fragment ion information. Collisional acitivation of the peptide cation produces only three b-ions, while the modified anion produces eight b♦-ions. The presence of the b♦-ions significantly increases the observed sequence information. The results of
EGIPPDQQR and APPGFSPFR are illustrative of ubiquitin tryptic peptide ESTHLVLR (Figure 10). Ion trap CID of [ESTHLVLR+♦] produces a mixture of y♦- and y+FBDSA-ions along with higher m/z b♦-ions. The unmodified peptide cation produces similar b- and y-type ions; however, the modified anion additionally produces lower m/z modified y-ions, i.e. y₁+FBDSA.

We note that many of the singly protonated peptides derived by MALDI and subjected to ion/ion reactions in this work have also been derived via electrospray and subjected to reactions with the same reagent. Very similar product ion spectra were derived from the same peptide ions derived by these two ionization methods. Not surprisingly, the main difference in the two approaches was that singly protonated peptides dominated in all cases in the MALDI experiment, whereas multiply protonated peptides were the major ions noted for the larger peptides in the electrospray experiment.

4.4 Conclusions

Covalent and electrostatic modification of MALDI-derived tryptic peptide cations via gas phase ion/ion reactions with doubly deprotonated FBDSA has been demonstrated. Covalent modification is observed at the N-terminus and ϵ-NH₂ of lysine for lysine terminated tryptic peptides. Covalent and electrostatic modification is observed for arginine terminated tryptic peptides, where the covalent modification occurs at the N-terminal primary amine and electrostatic modification occurs at the C-terminal arginine residue. Both modified lysine and arginine terminated tryptic peptides have shown to result in increased sequence information upon collisional activation compared to the unmodified version in high m/z peptides (>1000 Th). Ion trap CID of the modified
anions generally shows an increase in the relative contribution of b-type ions, where unmodified peptide cations have shown limited b-ions. In many cases, the modified anion produces sequence-informative y-ions of lower m/z that are often not observed from the unmodified peptide cation. In general, modified anions have shown more uniform fragmentation compared to the unmodified cations, which often show dominant cleavages due to low energy CID pathways. This study demonstrates the first example of gas phase ion/ion reactions involving MALDI-derived ions (other than those that may occur inherently in the MALDI process). In this case, we demonstrate the gas phase modification of the ions to improve the MS/MS performance of MALDI-derived tryptic peptides without recourse to solution phase chemistry.

4.5 References


Figure 4.1: Modified AP-MALDI source, attached to a QTRAP 4000, capable of multisource injection.
Figure 4.2: a) Ion/ion product spectrum of peptide cation mixture and [FBDSA-2H]^-2 b) Positive ion spectrum of peptide mixture LIFAGK, EGIPPDQQR, and TLSDYNIQK
Figure 4.3: Ion trap CID product ion spectra of (a) [M+♦]− (b) [M+H]+ derived from M= TITLEVEPSDTIENVK
Figure 4.4: Ion trap CID product ion spectra of (a) [M+♦]− (b) [M+H]^+ derived from M= TLSDYNIQK
Figure 4.5: Ion trap CID product ion spectra of (a) [M+♣]⁻ (b) [M+H]^+ derived from M=GLSDGEWQQVLNWVGK
Figure 4.6: Ion trap CID product ion spectra of (a) $[\text{M+}]^-$ (b) $[\text{M+H}]^+$ derived from $\text{M=MQIFVK}$
Figure 4.7: Ion trap CID product ion spectra of (a) [M+♦]⁻ (b) [M+H]^+ derived from M=LIFAGK
Figure 4.8: Ion trap CID product ion spectra of (a) [M+\textbullet]^- (b) [M+H]^+ derived from M= EQIPPDQQR
Figure 4.9: Ion trap CID product ion spectra of (a) \([M+\bullet]^-\) (b) \([M+H]^+\) derived from M= APPGFSPFR
Figure 4.10: Ion trap CID product ion spectra of (a) [M+\(\bullet\)]\(^+\) (b) [M+H]\(^+\) derived from M= ESTLHLVLR
CHAPTER 5. SCHIFF BASE REAGENT CLUSTER ANIONS FOR MULTIPLE GAS-PHASE COVALENT MODIFICATIONS OF PEPTIDES AND PROTEIN CATIONS

5.1 Introduction

Mass spectrometry and tandem mass spectrometry have pivotal roles in the identification and structural characterization of peptides and proteins as such information provides insight on their physiological roles.\(^1,2\) Chemical derivatization of biological macromolecules has been performed so as to facilitate ionization,\(^3\) quantification,\(^4,5\) and characterization.\(^6,7\) Recently, bioconjugation techniques have exploded in popularity, especially in the area of characterization, due to the insight it provides in proteomics. For instance, N-hydroxysuccinimide (NHS) esters have been utilized to determine the relative reactivities of primary amines in denatured and native-state ubiquitin.\(^8\) In addition to determining the relative reactivities, NHS crosslinking reagents have been utilized to elucidate the higher order structure of proteins.\(^9,10,11\) Chemical derivatization has also been used to bioconjugate chromophores to enable photodissociation processes.\(^12\)

While these approaches have been restricted to the solution phase, ion/ion chemistry has afforded bioconjugation techniques to be performed in the gas-phase.\(^13,14,15,16\) Covalent ion/ion chemistry has many inherent benefits over the solution phase reactions: speed (100s of milliseconds), quick comparison between modified and unmodified ions, high degree of control over reactants via mass selection, ability to
control the number of modifications, avoidance of complex reaction mixtures, etc.\textsuperscript{17} Recent demonstrations of bioconjugation chemistries include NHS,\textsuperscript{14,15} Schiff base,\textsuperscript{13,18} and carbodiimide covalent chemistry.\textsuperscript{16} NHS esters have been utilized to tag primary amines as well as unprotonated arginine residues in the gas-phase.\textsuperscript{14,15} NHS crosslinking reagents have been used to crosslink gaseous peptide chains\textsuperscript{19} as well as ubiquitin to determine the relative reactivity of each primary amine and the overall three dimensional structure.\textsuperscript{20} Gas-phase crosslinked ubiquitin has displayed similar dissociation behavior as the solution phase method, providing a faster and more efficient method to perform this bioconjugation chemistry. In addition, the Schiff base reagent 4-formyl, 1, 3-benzene disulfonic acid (FBDSA) has been extensively investigated as a derivatization agent of unprotonated primary amines.\textsuperscript{21,22,23} Gas-phase Schiff base chemistry has shown the ability to increase primary sequence coverage in various biological macromolecules in the context of a ion/ion charge inversion experiment.\textsuperscript{18,21,23} All of the gas-phase covalent chemistry mentioned thus far has been restricted to primary amines. N-cyclohexyl-N’-(2-morpholinoethyl)carbodiimide (CMC) has demonstrated the ability to covalently modify carboxylic acid functionalities present in peptides and dendrimers.\textsuperscript{16}

Covalent chemistry in the gas-phase proceeds through a long lived complex, comprised of the reagent and analyte ions (at least one must be multiply charged). The sufficiently long lifetime allows for multiple interactions to occur so as to generate a chemical reaction. For example, FBDSA modification proceeds via a nucleophilic attack of a primary amine on the aldehyde carbonyl carbon of FBDSA, where a rearrangement occurs causing the loss of water and formation of an imine bond.\textsuperscript{13,18} The extent to which gas-phase modification can be performed on a system is limited by the number of
available reaction sites in the analyte and the initial charge state of the analyte. In some situations, it may be desirable or advantageous to modify multiple reactive sites; however, the initial charge of the analyte ion may be too low to allow consecutive ion/ion reactions prior to neutralization. Ultimately, the number of covalent modifications that can be performed on [M+nH]^{n+} is limited to one less than the total charge, i.e. n-1. Therefore, it is of interest to develop a method to perform multiple covalent modifications within a single ion/ion reaction.

In this study, we demonstrate the ability to perform multiple covalent modifications within one ion/ion encounter via reagent ion clusters. The reagent clusters are comprised of n reactive reagent anions and n-1 counter cations; generating an overall singly charged anion. Specifically, 2-formyl-bezenesulfonic acid reagent anion clusters are reacted with protein and peptide cations. Post ion/ion reaction spectra are consistent with multiple modifications, where the number of modifications never exceeds the number of reagents present in the initial cluster.

5.2 Experimental

5.2.1 Materials

Methanol was purchased from Mallinckrodt (Phillipsburg, NJ). FBMSA and bovine ubiquitin were purchased from Sigma-Aldrich (St. Louis, MO). KKKKKKKKKK (K10) was purchased from Pepnome Ltd. (Jida Zhuhai City, Guangdong Province, China). Ubiquitin and K10 were prepared at a concentration of ~10 μM in a solution of 50/50 (v/v) water/methanol prior to positive nanoelectrospray
ionization (nESI). The reagent anion, FBMSA, was prepared at a concentration of 1 mg/ml in a solution of water prior to negative nESI. The high concentration of the reagent solutions aided in the formation of the cluster ions.

5.2.2 Mass Spectrometry

All experiments were performed on a QTRAP 4000 triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Concord, ON, Canada), which has been modified to perform ion/ion reactions. Alternately pulsed nESI emitters sequentially inject mass-selected anions and cations into the q2 reaction cell. Singly deprotonated FBMSA cluster anions were first ionized and accumulated in the q2 reaction cell. Next, the multiply charged cations were ionized and transferred to the q2 cell to undergo a mutual storage reaction for 500-1000 milliseconds. Product ions resulting from the ion/ion reaction were transferred to Q3 to perform MS^n analyses and subsequent mass analysis via mass-selective axial ejection (MSAE).

5.3 Results and Discussion

5.3.1 FBMSA Reagent Cluster Anions

All previous ion/ion chemistry with FBDSA/FBMSA has been restricted to a single derivatization per reaction. While multiple derivatizations have been achieved via consecutive ion/ion reactions, each gas-phase reaction comes at the cost of one charge. Therefore, the extent of modification is highly dependent on the initial charge of the analyte ion. To circumvent such a restriction, a singly charged anion containing multiple
reactive groups can be utilized to perform multiple covalent modifications within one ion/ion encounter. FBMSA cluster anions form as \([nR_{nNa-Na}]^-\), where \(n\) represent a number \(\geq 2\) and \(R\) represents the reagent FBMSA. The reagent FBMSA is utilized instead of FBDSA due to the inability to form clusters with FBDSA. The sodium counter ions in the cluster serve to stabilize the sulfonate moieties of FBMSA. Negative mode nanoelectrospray of the concentrated FBMSA solution generates singly deprotonated reagent cluster anions containing various number of reagent molecules, i.e. the dimer through the hexamer cluster (Figure 1).

5.3.2 Multi-Derivatized \(K_{10}\) Cations

Various reagent (i.e. di-, tri-, tetra, and penta) FBMSA cluster anions were subjected to ion/ion reactions with triply protonated \(K_{10}\). As with FBDSA, the aldehyde functionality of FBMSA undergoes nucleophilic attack by an unprotonated primary amine upon collisional activation. The result of the rearrangement generates the loss of \(H_2O\) which is the signature of covalent modification. Figure 2 (a) displays the post ion/ion reaction between \([K_{10}+3H]^3+\) and \([2R_{2Na-Na}]^-\). The dominant product ion is \([M+3H+2R_{2Na-Na}-2H_2O]^2+\), which is consistent with two covalent modifications. Here, the degree of water loss never exceeds the number of reagent present in the cluster. Even the neutral loss of an intact, sodiated reagent (-\(R_{Na}\)) from the dimer adducted peptide cation is consistent with the number of reactive reagents and water losses (i.e., one reagent and one water loss). It is noteworthy to mention that water loss is observed without ion trap collisional activation. Collision-induced dissociation is necessary to observed water loss following reactions that involve FBDSA anions. Water loss appears
to occur spontaneously; however, energetic transfer conditions cannot be precluded as a source of ion activation.

Ion/ion reactions involving the trimer, tetramer, and pentamer clusters exhibit similar reaction behavior (Figure 2 (b), (c), (d) respectively). All demonstrate covalent modification consistent with the highest number of reagents present within the cluster. For instance, the pentamer FBMSA cluster generates \([M+3H+5R_{5Na}-Na-5H_2O]^{2+}\), which is consistent with five covalent modifications (Figure 2 (d)). The neutral losses from \([M+3H+5R_{5Na}-Na-5H_2O]^{2+}\) never exceed the possible number of covalent modification, e.g. \(-(2H_2O+2R_{2Na})\). These experiments demonstrate the ability to perform multiple gas-phase covalent modifications in one ion/ion encounter via FBMSA-cluster reagent anions.

5.3.3 Multi-Derivatized Ubiquitin Cations

In order to view the applicability of this chemistry to larger systems, we investigated reagent cluster chemistry with protein cations. Ubiquitin is a ~8.5 kDa protein, which contains thirteen basic sites: the N-terminus, seven lysines, four arginines, and one histidine residue. Based on this primary sequence information, there are eight possible sites for covalent modification via the FBMSA-Schiff base chemistry. \([\text{Ubiquitin} + 7H]^{7+}\) was subjected to separate ion/ion reactions with singly deprotonated FBMSA (i.e., \([R-H]\)) and \([nR_{nNa}-Na]\) clusters, where \(n=2-5\). In each case, the maximum number of water loss from the ion/ion reaction complex was consistent with the number of reagents present in the cluster. The product ion spectrum of the ion/ion reaction between \([\text{Ubiquitin} + 7H]^{7+}\) and \([5R_{5Na}-Na]\) is displayed in Figure 3(a). The ion/ion reaction generates an intact complex (i.e., \([\text{Ubiquitin} + 7H + 5R_{5Na} - Na]^{6+}\)) as well as
nH$_2$O losses, where n=1-4. Next, the ions present in the inset were subjected to isolation and rapid sequential resonance excitation designed to sequentially activate each water loss (i.e., higher m/z to lower m/z). The product ion spectrum following activation generates a dominant -5H$_2$O loss and various reagent neutral losses (Figure 3(b)). The five water loss is consistent with the highest number of covalent modifications with the pentamer reagent anion. Similarly, for all of the other reagent clusters, the loss of n water molecules was observed as the dominant number of water loss following sequential activation. However, loss of water associated with the protein cannot be precluded as water loss is a common process in peptide and protein activation. Sequential losses of neutral FBMSA from the parent ion are also observed. Neutral loss of R$_{4}$ is observed with four water losses as well as 2R$_{4}$ loss with three water losses. Here, the water loss never exceeds the highest possible number of covalent modifications (e.g., -(4H$_2$O+R$_{4}$), four potential modifications). Such an observation suggests that a fraction of the reagents in the cluster engage in an electrostatic interaction, while the other fraction undergoes covalent modification.

Collisional activation of [Ubiquitin + 6H]$^{6+}$, [Ubiquitin + 7H + ◊]$^{6+}$, and [Ubiquitin +7H +5◊$_{5Na}$ - Na]$^{6+}$ are compared in Figure 4 and Figure 5, where ◊ represents covalent modification of the FBMSA reagent and the resulting dehydration. Ion trap CID of [Ubiquitin + 7H + 5◊$_{5Na}$ - Na]$^{6+}$ generates a product ion spectrum containing fragment ions that are derivatized multiple times, where two, three, and four modifications are observed (Figure 4 (c)). For instance, b$_{58}$ has up to four modifications (4◊) with six lysine residues within the fragment ion. Also, y$_{18}$◊ is observed in the product ion spectrum, where only one lysine residue is present in the fragment ion. We
note the neutral loss of $R_{Na}$, which indicates that at least some of the water losses are not related to covalent modification. However, the covalent modification of five sites cannot be precluded.

In addition to reactions with the pentamer cluster, ubiquitin cations were subjected to ion/ion reactions with the dimer, trimer, and tetramer cluster monoanions. Ion trap CID was performed on $[\text{Ubiquitin} + 7H + 2\dot{\Diamond}_{2Na} - Na]^{6+}$, $[\text{Ubiquitin} + 7H + 3\dot{\Diamond}_{3Na} - Na]^{6+}$, $[\text{Ubiquitin} + 7H + 4\dot{\Diamond}_{4Na} - Na]^{6+}$ (Figure 6). CID of multi-derivatized (i.e., dimer through tetramer) ubiquitin cations exhibited similar dissociation behavior as the $[\text{Ubiquitin} + 7H + 5\dot{\Diamond}_{5Na} - Na]^{6+}$. In each case, the number of FBMSA-covalent modifications never exceeded the number of primary amines within the fragment ion or the number of reagent molecules present in the cluster.

In comparison to the unmodified, singly and doubly modified ubiquitin cations (i.e., 0-2\dot{\Diamond} ubiquitin), a few of the predominant dissociation channels are minimized in the highly modified analog (i.e., 3-5\dot{\Diamond} ubiquitin). Ion trap CID of $[\text{Ubiquitin} + 6H]^{6+}$, $[\text{Ubiquitin} + 7H + \dot{\Diamond}]^{6+}$, and $[\text{Ubiquitin} + 7H + 2\dot{\Diamond}]^{6+}$ generates large contributions from the $y_{18}$ and $b_{58}$ ions, while their contributions are minimized in the ion trap CID of $[\text{Ubiquitin} + 7H + n\dot{\Diamond}_{nNa} - Na]^{6+}$, where $n=3-5$. In addition, the 3-5\dot{\Diamond} ubiquitin dissociates exclusively through aspartic acid cleavages with a drastic increase in the contribution from $b_{52}/y_{24}$-ions (D$_{52}$ G$_{53}$ residues), while the 0-2\dot{\Diamond} ubiquitin fragments through other pathways (i.e., nonresidue specific and proline cleavages) in addition to the aspartic acid pathways. Previous studies performing lysine guanidination of ubiquitin demonstrated dominant fragmentation through aspartic acid cleavages.$^{28}$ Guanidination lowers proton mobilites, and as a result, inhibits proton catalyzed N-terminal proline and nonresidue-
specific cleavages. At low proton mobilities, charge remote aspartic acid cleavages dominate the fragmentation behavior. Similar dissociation behavior of multiply FBMSA-modified ubiquitin suggests an alteration of proton mobility.

5.4 Conclusions

Multiple covalent modifications within one ion/ion encounter have been demonstrated on peptide and protein cations via FBMSA reagent cluster chemistry. The reagent cluster anions are comprised of n number of FBMSA reagents and n-1 number of counter sodium ions, generating an overall monoanion. Cluster monoanions allows multiple derivatizations at the cost of only one charge. FBMSA-clusters anions have been formed as dimers, trimers, tetramers, and pentamers. Ion/ion reactions between FBMSA-reagent cluster anions and triply protonated K_{10} have demonstrated up to five covalent modifications. The number of covalent modifications never exceeds the total number of reagents present in the cluster. Cluster chemistry was also applied to ubiquitin cations. Upon reaction with FBMSA clusters, varying degree of water loss is observed. Sequential ion trap CID can concentrate the number of water losses to the overall number of reagents present in the cluster. Collisional activation of the multiply modified protein cation generates fragment ions consistent with multiple covalent modifications.

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5.5 References


Figure 5.1: nESI generated clusters of R, where R denotes FBMSA.
Figure 5.2: Ion/ion reactions between $[K_{10}+3H]^{3+}$ and FBMSA reagent clusters, (a) $[2R_{2Na-Na}]$, (b) $[3R_{3Na-Na}]$, $[4R_{4Na-Na}]$, $[5R_{5Na-Na}]$.
Figure 5.3: (a) Post ion/ion reaction spectrum of [Ubiquitin + 7H]$^{7+}$ and [5R$_{SNa}$-Na$^-$], (b) Rapid sequential collisional activation over the isolated ion population in the insert of (a)
Figure 5.4: Ion trap CID of (a) [Ubiquitin + 6H]^{6+} (b) [Ubiquitin + 7H + \Diamond]^{6+}, (c) [Ubiquitin + 7H + 5\Diamond]^{6+}
Figure 5.5: Ladder structure of ubiquitin, (a) $[\text{Ubiquitin} + 6\text{H}]^{6+}$ (b) $[\text{Ubiquitin} + 7\text{H} + \Diamond]^{6+}$, (c) $[\text{Ubiquitin} + 7\text{H} + 5\Diamond]^{6+}$
Figure 5.6 Ion trap CID of (a) [Ubiquitin + 7H + 2◊]$_6^+$ (b) [Ubiquitin + 7H + 3◊]$_6^+$, (c) [Ubiquitin + 7H + 4◊]$_6^+$
CHAPTER 6. CONVERSION OF MALDI-DERIVED PEPTIDE MONOANIONS INTO MULTIPLY CHARGED CATIONS VIA GAS-PHASE ION/ION REACTIONS

6.1 Introduction

Mass spectrometry (MS) has quickly developed into a powerful analytical tool for the analysis of biological macromolecules (e.g., peptides, proteins, nucleic acids, and lipids). Development of soft ionization techniques such as electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) has afforded the introduction of intact gaseous bio-ions into the mass spectrometer for structural and mass analysis. Both of these ionization methods have developed separate niches in the field of MS. For instance, ESI has been widely utilized in interfacing MS and liquid chromatography. Ions generated via ESI tend to be multiply charged, which facilitates extensive fragmentation and structural characterization of biological ions. The higher massed, multiply charged ions enable (i.e., lower mass to charge ratio (m/z)) analysis within the mass range of the MS (e.g., scannable RF amplitude applied to ion traps). Alternatively, MALDI has been widely used on sampling surfaces such as imaging applications. Ionization via MALDI generally produces lower charged ions than ESI, and for the most part, MALDI generates singly charged ions. Activation of singly charged biological ions often does not generate extensive fragmentation. In fact, the dissociation behavior is generally dominated by low energy fragmentation pathways, e.g. C-terminal cleavage of
an aspartic acid residue or N-terminal cleavage to a proline residue.\(^8\) The higher \(m/z\) of ions (e.g., >3,000 Th) can inhibit mass analysis and tandem MS processes, especially in linear ion traps. Inadequacies generally arise from insufficient trapping conditions (i.e., shallow pseudo potential well) as well as the detectable mass range of the instrument platform.

Ion/ion chemistry represents a unique area in the field of mass spectrometry that can manipulate gas-phase ions with a high degree of control.\(^9,10\) Ion/ion chemistry has demonstrated the ability to decouple the initial ion-type generated by the ionization source from the ion-type ultimately subjected to tandem MS.\(^11\) This chemistry has been utilized to manipulate analyte ion charge state,\(^12,13\) polarity,\(^14,15,16,17\) oxidation state (i.e., electron transfer),\(^18,19\) and covalently\(^20,21,22,23\) and/or electrostatically modify ions.\(^17,24,25\) These gas-phase reactions have many benefits: short reaction time (10-100s milliseconds), high control of reactants, avoidance of complex reactions mixtures, ready comparison to modified and unmodified analyte ion, etc.\(^26\) For instance, the process of charge inversion affords ion formation in one polarity and analysis in the opposite polarity via multiple proton transfer within a long-lived complex.\(^27\) Previous studies have investigated such a process for various analytical reasons and applications. These studies include the fundamental studies of charge inversion reagents,\(^16,17\) charge inversion efficiency,\(^14\) and increasing tandem MS performance on phosphopeptides.\(^28\) However, all of these examples have been restricted to analytes ions generated via ESI.

In this study, we demonstrate the gas-phase charge inversion of MALDI-derived peptide monoanions into multiply charged cations so as to enhance tandem MS performance and to afford analysis within the instrument detection range. Experiments
are implemented via a dual-source interface attached to a hybrid triple-quadrupole/linear ion trap tandem mass spectrometer. Here, the peptide monoanions are subjected to ion/ion reactions with various protein reagent cations. Proteins ionized via ESI have a propensity to generate highly charged ions; therefore, these ions are excellent candidates for the charge inversion process. The charge inversion process proceeds via a long-lived intermediate complex, comprised of the peptide anion and protein cation. The lifetime of the long lived complex is sufficient to allow for multiple proton transfers, where multiple factors contribute to the extent of proton transfer (i.e., gas-phase basicities, charge state, and cross section). The products of the ion/ion reaction are charge reduced protein cations and charge inverted peptide cations, as can be seen in process (1).

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[M-H]^- + [R+nH]^n+ \rightarrow [M-H]^- - [R+nH]^n+ \rightarrow [M+yH]^{y+} + [R+(n-y)H]^{(n-y)^+}
\] (1)

Ion/ion reactions have been performed on melittin (i.e., 26 amino acid peptide), HDMNKLVL, and Chain B of insulin monanions. Multiple protein reagent cations have also been utilized to optimize charge transfer: ubiquitin, myoglobin, carbonic anhydrase (CA), and bovine serum albumin (BSA). In this study, we demonstrate two potential applications for charge inverted MALDI monanions: enhance tandem MS performance and analysis in the detection range.
6.2 Experimental Section

6.2.1 Materials

Methanol, glacial acetic acid, and ammonium hydroxide were purchased from Mallinckrodt (Phillipsburg, NJ). Ubiquitin from bovine erythrocytes, myoglobin, BSA, carbonic anhydrase, insulin, melittin, cytochrome c and 1,5-diaminonaphthalene (DAN), tris (2-carboxyethyl) phosphine hydrochloride, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic peptides HDMNKLVL was purchased from SynPep (Dublin, CA). Peptides MQIFVK, TITLEVEPSDTIENVK, EGIPPDQQR, LIFAGK, TLSDYNIQK, and ESTLHLVLRR were generated from a tryptic digestion of ubiquitin.

6.2.2 Methods

The MALDI matrix, DAN, was prepared in 69.5/29.5/1 (v/v) water/acetonitrile/ammonium hydroxide at a concentration of 10 mg/mL. Peptide analytes were prepared in a ~100 µM aqueous solution prior to a 50/50 (v/v) mixture with the MALDI matrix solution. 2.0 µL of the matrix/analyte solution was spotted onto the MALDI stage plate and allowed to air-dry prior to attachment to the AP-MALDI source. The protein reagents were composed of a ~100 µM solution of 49.5/49.5/1 (v/v/v) water/methanol/acetic acid for positive microionspray.
6.2.3 Disulfide Reduction

The procedure for disulfide reduction has been described previously. Reduced insulin was filtered through a disposable PD-10 desalting column (GE Healthcare) using a 1% acetic acid buffer solution. Collected fractions were lyophilized and dissolved in 250 µL of water.30

6.2.4 Mass Spectrometry

Experiments were performed on a 4000 AB Sciex QTRAP (Concord, ON, Canada), which has been modified to perform ion/ion reactions.31 The ion source is equipped with an AP-MALDI source (MassTech, Columbia, MD) and with a microionspray emitter (New Objective Inc, Waburn, MA) orthogonal to the sample inlet (Figure 1).25,29,32 The microionspray emitter, remote to the MS inlet and MALDI stage plate, allows for the introduction of opposite polarity ions into the MS. Briefly, the AP-MALDI source has a circular opening, where a conductive transfer tube penetrates into the ion source and attaches to the curtain plate. The microionspray is directed towards the transfer tube and upon ionization/nebulization, ions travel into the transfer tube and the ion source.

The protein reagent cations were generated via microionspray at a continuous flow rate of 8.0 µL/min from a silica sprayer (364 µm outer dimension, 48 µm inner dimension, Polymicro Technologies, Phoenix, AZ). Nitrogen acts as the nebulizing gas, which helps guide the reagent anions into the ion source. The nebulizing gas was triggered/pulsed via MS Expo software (AB Sciex, Concord, ON, Canada) during positive ion injection conditions. Once protein cations entered the MS, the ions were
transferred to the q0 cell and cooled to help remove neutral adducts. Following trapping, protein cations were then transferred to q2 and stored prior to a mutual storage reaction.\textsuperscript{33,34} Peptides, individually spotted in DAN, were ablated via a 337-nm, 10Hz nitrogen laser (AP-MALDI Ion Source 110, MassTech, Columbia, MD) and transferred to the q2 collision cell to undergo ion/ion reactions with the reagent anions. Product ions generated in q2 were transferred to Q3 for further collisional activation and subsequent mass analysis.\textsuperscript{35}

6.3 Results and Discussion

Singly deprotonated melittin anions were subjected to charge inversion ion/ion reactions with various protein reagent cations (i.e., ubiquitin, cytochrome c, carbonic anhydrase, and bovine serum albumin (BSA)) to ultimately generate multiply protonated analyte ions. Ion/ion reactions of singly deprotonated anions and multiply protonated cations proceed via two distinct proton transfer channels: charge transfer within a long-lived complex or a proton hopping mechanism. The first process generates the charge inverted peptide ions via transfer of two or more protons prior to dissociation of the complex. The second process occurs at sufficiently long distances, where the relatively high electric fields associated with the oppositely charged ions facilitate a single proton transfer. This process ultimately neutralizes the monoanion analyte. Charge inverted products resulting from two or more successive proton transfer reactions are highly unfavorable due to the low absolute number of the neutral analyte and low ion number densities in the ion trap.\textsuperscript{14,16,27}
6.3.1 Optimal Protein Reagent Cations

Initial experiments were devoted to determining an optimal protein reagent cation to maximize the number of protons transferred to the analyte anions; therefore, singly deprotonated melittin (Figure 2(a)) was subjected to charge inversion ion/ion reactions with various multiply protonated reagent cations. Melittin is a 2.9 kDa peptide, containing six basic sites (e.g., N-terminus, lysine), where the presence of multiple basic sites will facilitate multiple proton transfer (Figure 2). Melittin was also selected as an initial analyte anion since the singly deprotonated form falls within 50 m/z of the upper limit of the instrument mass range. Mass analysis of melittin anions allows for the optimization of instrument conditions prior to the ion/ion chemistry with analyte ions outside of the mass range. Ubiquitin cations were used initially to establish a baseline with a small and low-charged protein (Figure 3(a)). The charge state utilized for reaction was selected on the highest charge state that produced abundant signal upon a RF/DC isolation in Q1. Figure 4(a) illustrates the post ion/ion product ion spectrum of [Melittin-H]⁻ and [Ubiquitin +8H]⁸⁺. The reaction results in the conversion of singly deprotonated melittin into doubly and triply charged melittin (i.e., [M+2H]²⁺ and [M+3H]³⁺), while the singly protonated form is not observed. In addition to proton transfer, attachment of melittin to the reagent cation, [M+R+5H]⁵⁺, is observed, where M and R are representative of the analyte and reagent, respectively.

Previous studies involving ion/ion charge inversion have demonstrated multiple factors that influence charge partitioning upon dissociation of the long lived complex: the overall number of basic sites, their gas-phase basicities, reagent charge state, and the electrostatic repulsion present within the long-lived complex. In the case of gas-phase
basicity, charge transfer continues to occur as long as the gas-phase basicity (GB) of the analyte exceeds that of the reagent as it loses charge. However, the GB of the analyte and reagent can be altered by coulomb repulsion within the complex. In addition, smaller reagent cross sections (with the same amount of charge) increase the electrostatic repulsion within the long-lived complex, which tends to favor increased charge transfer so as to minimize repulsion. Larger protein cations tend to minimize repulsion due to the possibility of many intermolecular interactions (e.g., hydrogen bonding, dipole-dipole). Higher charge densities are also expected to lead greater charger transfer (i.e., lower GB and greater repulsion); however, significantly high charge densities have diminishing effects. At very high charge states, the proton hopping mechanism is preferred due to the potential energy of the opposite polarity ions being equal to the reaction energy at longer distances. Ultimately, a reagent cation with a low GB, small cross section, high degree of repulsion, and high charge state would be optimal.

Following ubiquitin, a larger and higher charged myoglobin protein was utilized as a protein reagent cation (Figure 3(b)). In order to achieve higher charge with our ionization platform, a larger cross sectioned reagent must be utilized, assuming that higher massed, higher charged proteins own a larger cross section. Myoglobin has a molecular mass near 14 kDa with a charge state distribution ranging from 8+ to 16+ upon ionization, while ubiquitin has a mass near 8.5kDa with a charge state distribution ranging from 4+ to 9+. Ion/ion reactions were performed between singly deprotonated melittin and [myoglobin+16H]^{16+} (Figure 5(a)). Melittin monoanions are converted to doubly and triply protonated melittin in a 1:1 ratio as similarly observed with the ubiquitin reagent cations. The similar charge inversion behavior can be attributed to
similar GBs of \([\text{myoglobin}+16H]^{16+}\) and \([\text{ubiquitin}+8H]^{8+}\), i.e. 210 kcal/mol (crude linear extrapolations from Williams et al.).\(^{36}\) The charge states of all the protein reagent cations utilized in the ion/ion reaction are considerably lower in overall charge than cations generated by ESI. This observation can be attributed to the dual injection interface, which ionizes the proteins remote to the aperture and likely introduces charge reduction processes prior to introduction in the MS. Utilizing these lower charges, and in turn higher GBs, diminishes the degree of charge transfer since the analyte exceeds the reagents GBs more rapidly upon each charge transfer. In addition, the product ion spectrum also displays evidence of single proton transfer (i.e., \([\text{myoglobin}+15H]^{15+}\) as well as consecutive ion/ion reactions (i.e., \([\text{myoglobin}+12H]^{12+}\) though \([\text{myoglobin}+7H]^{7+}\)) as more than four transferred protons are not observed.

In an attempt to utilized a protein cation with more charge and a lower GB, melittin monoanions were subjected to ion/ion reactions with \([\text{CA}+23H]^{23+}\) (Figure 5(b)). Carbonic anhydrase is a 29 kDa protein with a charge state distribution from 13+ to 24+ (Figure 3(c)). A crude linear extraction of the GB of CA from Williams et al.\(^{36}\) generates a value of 200 kcal/mol at +23. The product ion spectrum between melittin monoanions and CA polycations generates doubly and triply charged melittin in a 3:2 ratio, respectively. The slight decrease in GB does not generate a higher degree of charge transfer, i.e., \([\text{M}+4H]^{4+}\); however, there is a decrease in the relative abundance of triply protonated melittin. This observation may be attributed to the larger cross section of CA than ubiquitin and myoglobin cations, which favors less charge transfer due to the lower coulomb repulsion between melittin and CA ions.\(^{16}\) To extend the range of observation, BSA reagent cations (i.e., \([\text{BSA}+40H]^{40+}\)) were subjected to ion/ion reactions with
melittin monoanions. BSA is a 66.5 kDa protein which generates a charge state
distribution over $26^+$ to $42^+$ (Figure 3(d)). BSA was utilized due the ability to generate
higher charge than the other protein reagent cations; however, upon further investigation,
the GB of $[\text{BSA+40H}]^{40+}$ is approximately 205 kcal/mol. Therefore, it is expected that
similar charge states of melittin will be generated following the ion/ion reaction. Figure
5(c) illustrates the post ion/ion reaction, where the triply and doubly protonated melittin
are observed in a ratio of 3:1, respectively. Reaction with BSA cations generates the
highest degree of $[\text{melittin+3H}]^{3+}$ compared to the other protein reagent cations. A likely
explanation is that the extrapolated GB of BSA is incorrect and the true GB is lower.
With a possible lower GB and higher degree of charge compared to the other protein
reagent cations, a higher degree of charge separation would be expected.

6.3.2 Conversion of Peptide Anions Outside of the Native Mass Range

After optimizing the instrument conditions and ion/ion chemistry, this chemistry
was applied to MALDI-derived analyte monoanions that fall outside of the mass
spectrometer’s mass range (in our case 2900 m/z). Insulin chain B is 3.4 kDa peptide
(FVNQHLCGSHLVEALYLVCG ERGFFYTPKA) that is generated via solution phase
disulfide reduction of insulin. Ionization of chain B via MALDI generates a mass
spectrum void of any analyte signal due to fact that $[\text{Chain B-H}]^-$ falls outside of the
detection range (~500 m/z) (Figure 6(a)). Singly deprotonated chain B is likely reaching
the Q3 linear ion trap but is not detected due to the instrument constraints (i.e., necessary
RF amplitude to perform mass selective axial ejection is not achieved). Hence, insulin
chain B was subjected to ion/ion reactions with BSA reagent protein cations to
demonstrate the ability to lower the \( m/z \) quotient of the gas-phase ions so as to facilitate detection of the analyte ions. Chain B contains five basic sites (e.g., histidine, arginine), and since this nears the number of melittin basic sites, it is expected that chain B will undergo a similar extent of charge transfer (unless their respective GBs are vastly different). The product ion spectrum of the ion/ion reaction between \([\text{Chain B-H}]^-\) and \([\text{BSA}+36\text{H}]^{36+}\) is illustrated in Figure 6(b). Singly deprotonated chain B is converted to doubly and triply protonated Chain B. The transfer of multiple protons to chain B modifies the \( m/z \) ratio (i.e., lowering the quotient), thus facilitating analysis within the detection range. Such an observation suggests that chain B monoanions are at least reaching the q2 cell to undergo ion/ion reactions. This conversion chemistry is practical in situations where a MALDI ionization source is coupled to a trapping analyzer that is not equipped with a sufficient mass detection range.

6.3.3 Increasing Structural Characterization upon Charge Inversion

Singly charged ions, whether cations or anions, often will not fragment extensively.\(^6\)\(^,\)\(^7\) As mentioned previously, ionization via MALDI has a propensity to generate singly charged ions in both the negative and positive ion mode. The conversion chemistry demonstrated in this study can be utilized to increase structural information as multiply charged ions tend to generate a higher degree of primary information. Singly deprotonated HDMNKVLDDL was subjected to ion/ion with \([\text{cytochrome c} +12\text{H}]^{12+}\) so as to highlight the dissociation behavior of each charge state (Figure 7). Following the ion/ion reaction, \([\text{HDMNKVLDDL-H}]^-\) is converted to the singly and doubly protonated forms (Figure 7(a)). Ion trap collision induced dissociation (CID) of \([\text{M}+2\text{H}]^{2+}\) generates
a product spectrum with extensive fragmentation (Figure 7(b)), where seven y-ions and seven b-ions are generated. The fragmentation behavior also exhibits uniformity even in the presence of two aspartic acid residues. Collisional activation of the singly protonated peptide generates a product ion spectrum with limited fragment ions (i.e., four y-ions and five b-ions). Here, the singly protonated peptide can be envisioned as a product of positive ionization mode MALDI. Fragmentation is also dominated by the aspartic acid low energy CID pathway, a selective cleavage well known to be a more dominant and facile cleavage in peptide monocation fragmentation than most other fragmentation processes. This simple comparison displays the utility of this conversion chemistry, i.e. the ability to generate and dissociate multiply charge cations compared to singly charged ions that MALDI generates upon ionization. While there is not a comparison to the monoanion, it is well documented that peptide cations yield significantly more information than peptide anions (i.e., dominated by neutral losses).

6.4 Conclusions

This study demonstrates the gas-phase charge inversion of peptide monoanions into multiply charged cations via ion/ion reactions. The charge inversion process proceeds via a long-lived intermediate complex, comprised of the reagent and analyte ions, where multiple protons are transferred within the complex. Different protein reagent cations (i.e. varying cross section, charge, and gas-phase basicity) have been investigated to optimize the degree of charge transfer from the reagent to the analyte. All of the protein reagent cations are suitable reagents as each transferred up to four protons; however, BSA cations generated the highest relative abundance of four proton transfer products. Conversion
chemistry displays a distribution of charge transfer, which can be attributed to the
competition between various dissociation channels of the long-lived complexes. This
study also demonstrates two potential applications for MALDI-derived ions: (1) lowering
the m/z ratio of the analyte ion to facilitate detection and (2) increasing sequence
coverage upon dissociation. The first application has been demonstrated on insulin chain
B, where conversion from a singly charged ion into a multiply charged ion afforded
detection. Collisional activation of charge inversion product ions also exhibited an
increase in fragmentation of peptide dications compared to singly protonated peptide
cations.

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6.5 References


Figure 6.1: Modified AP-MALDI source, attached to a QTRAP 4000, capable of multisource injection.
Figure 6.2: Negative ionization mode of melittin via AP-MALDI, M is equivalent to GIGAVLKVTTLGPLASWIKRKRQQ-NH$_2$. 
Figure 6.3: Positive mode microionspray ionization of a) ubiquitin, b) myoglobin, c) carbonic anhydrase, d) bovine serum albumin. The red color represents the isolated protein cation subjected to ion/ion reactions.
Figure 6.4: Product ion spectrum following the ion/ion reaction between [Melittin-H]− and [Ubiquitin+8H]8+. M is representative of melittin, while the R+ is equivalent to the charge state of ubiquitin.
Figure 6.5: Product ion spectrum following the ion/ion reaction between [melittin-H] and a) [myoglobin+16H]^{16+}, b) [carbonic anhydrase+23H]^{23+}, c) [BSA+39H]^{39+}, M is representative of melittin, while the red numbers represent the isolated precursor reagent cation.
Figure 6.6: a) Negative ionization mode of chain B via AP-MALDI, b) ion/ion reaction between [Chain+B-H]⁻ and [BSA+36H]^{36+}, M is equivalent to FVNQHLCGSHLVEALYLVCG ERGFFYTPKA.
Figure 6.7: a) ion/ion reaction between [M-H]⁻ and cytochrome c+ 12H⁺²⁺, b) Ion trap CID of [M+2H]²⁺, c) Ion trap CID of [M+H]⁺, M is equivalent to HDMNKVLDDL.
CHAPTER 7. GAS-PHASE TRANSFORMATION OF PHOSPHATIDYLCHOLINE CATIONS TO STRUCTURALLY INFORMATIVE ANIONS VIA ION/IION CHEMISTRY

7.1 Introduction

Mass spectrometry (MS) has become an essential analytical tool for the identification and structural characterization of biological macromolecules.\textsuperscript{1,2,3} In tandem MS (MS/MS or MS\textsuperscript{n}), the polarity in which the analyte ion is most efficiently formed does not always provide sufficient structural information upon collision-induced dissociation (CID). For instance, phosphatidylcholines (PC), a subclass of glycerophospholipids, are efficiently ionized in the positive polarity, but their cationic versions generate limited structural information upon CID. PCs compose thirty percent of cell membranes in mammalian cells\textsuperscript{4} and can act as secondary messengers in metabolic signaling/pathways.\textsuperscript{5} PCs are composed of a glycerol backbone with two fatty acid chains attached at the \textit{sn}-1 and \textit{sn}-2 positions while a phosphocholine moiety is attached at the \textit{sn}-3 position. Due to the presence of a fixed positive charge quaternary amine, PCs readily generate gas-phase cations in positive ion mode electrospray ionization (ESI).\textsuperscript{6} However, collisional activation of PC cations generally yields the phosphocholine moiety (184 \textit{m/z}) as the dominant and often exclusive product. The phosphocholine fragment ion provides insight on only the phospholipid class and does not provide structural information about the individual acyl chains.\textsuperscript{7,8} Elucidating the
composition of the individual fatty acid chains is important due to the diverse physical properties of PCs (i.e., the number of carbons and extent of unsaturation) as these properties relate to their lipid biochemistry. With the presence of the choline moiety, PCs cannot be ionized in the negative polarity by deprotonation and therefore structural information of the individual fatty acid chains cannot be easily accessed without solution phase additives (see below). Traditionally this might have been addressed by gas chromatographic analysis of the hydrolyzed and derivatized fatty acids arising from the PC fraction of the lipid extract, but this process results in a pooling of all fatty acids and a loss of molecular information.

Efforts to better characterize PCs by tandem MS have included CID studies of adducted PCs in both the positive and negative ion modes. Gas-phase lithium adducts of PCs generated by positive ESI of solution phase PC mixtures have shown neutral losses of lithiated fatty acid chains (i.e., RCO2Li, where R is the alkyl chain) upon CID. The difference in m/z between the product and precursor ion corresponds to the degree of unsaturation and chain length. However, the structurally informative peaks were of low relative abundance and the spectra were complicated by additional neutral losses. In the negative ion mode approach, millimolar concentrations of salts were added to a PC solution to produce gas-phase adducted PC anions (e.g., [PC+Cl]− and [PC+CH3CO2]−). Collisional activation of the adducted PC anions produced a neutral loss comprised of the counter anion and a methyl cation from the quaternary ammonium (i.e., CH3Cl and CH3CO2CH3), yielding [PC-CH3]−. Subsequent CID of [PC-CH3]− generated fatty acid carboxylate anions (RCO2−) and neutral losses associated with free acids (RCO2H) or ketene derivatives (RCHCO), which are all indicative of the
composition of the individual fatty acid chains. In both of the solution phase methods, the high salt concentrations can lead to poor ionization efficiency, spectral complexity upon ionization, and incompatibilities with liquid chromatography (LC) solvent systems.

The type of ion subjected to tandem MS is usually determined by the ionization method and conditions. An alternative approach is to transform in the gas-phase an ion-type that is readily generated by an ionization method to another ion-type that more readily yields structural information of interest, preferably mass-selection. Gas-phase ion/ion reactions have been shown to be particularly useful for many ion transformations, particularly when conducted within the context of MS^n, whereby the analyte and reagent ions are each mass-selected prior to reaction. The use of ion/ion reactions within the context of an MS^n experiment allows the definition of the ion-type to be de-coupled from the initial ionization conditions. As a result, the ion-type subjected to activation can be independently optimized to generate the most informative fragmentation. Ion/ion reactions have been utilized in acid/base reactions (i.e., proton transfer), reduction/oxidation reactions (i.e., electron transfer), and covalent and/or electrostatic modification. Previous studies have demonstrated the significance of ion-type transformation and its relation to increased sequence coverage. For instance, MALDI-derived tryptic peptide monocations covalently and electrostatically modified with benzene disulfonic acid dianions via ion/ion reactions demonstrated increased sequence coverage upon collisional activation, especially in the form of b-type ions.
In the case of PC ions, an example of gas-phase ion-type transformation via ion/ion chemistry has been demonstrated in the electron transfer dissociation (ETD) of doubly sodiated PC cations, \([\text{PC}+2\text{Na}]^{2+}\) using azobenzene radical anions as the ETD reagent. ETD induced cleavages at each ester linkage (\(i.e.,\) neutral loss of a fatty acid chain) with additional neutral loss of the quaternary nitrogen moiety. CID of the charged reduced electron transfer product that did not undergo dissociation (\(i.e.,\) the ETnoD ion), \([\text{M}+2\text{Na}]^{+}\), generated results similar to the electron transfer reaction. Similar to the lithiated PC cation study, this method provided information on the number of carbons and the degree of unsaturation for each fatty acid chain via neutral losses.\(^3\)\(^4\)

In this study, we demonstrate the gas phase transformation of PC monocations into demethylated PC anions (\(i.e.,\) \([\text{PC}-\text{CH}_3]^-\)) via ion/ion reactions, where pre- and post-transformation structural characterization is compared. PC monocations are subjected to ion/ion reactions with doubly deprotonated 1,4-phenylenedipropionic acid (PDPA). The product of the ion/ion reaction is a negatively charged complex, comprised of PDPA and PC, \(i.e.\) \([\text{PC}+\text{PDPA-H}]^-\). Collisional activation of the complex generates \([\text{PC-CH}_3]^-\) as seen in process (1):

\[
[\text{PC}+\text{H}]^+ + [\text{PDPA-2H}]^{2-} \rightarrow [\text{PC}+\text{PDPA-H}]^- \rightarrow [\text{PC-CH}_3]^- + [\text{PDPA+CH}_3+\text{H}]^- \quad (1)
\]

Gas-phase ion/ion reactions have been performed on synthetic PC isomers, 1-palmitoyl-2-oleoyl-\(sn\)-glycero-3-phosphocholine (16:0/18:1 PC) and 1-oleoyl-2-palmitoyl-\(sn\)-glycero-3-phosphocholine (18:1/16:0 PC). Collisional activation of \([\text{PC-CH}_3]^-\) generates large relative contributions from fatty acid carboxylate anions (\(\text{RCO}_2^-\)) and less abundant
neutral losses of fatty acids (RCO₂H) and ketene derivatives (RCHCO), whereas collisional activation of [PC+H]+ produces exclusively the phosphocholine cation. Transformation of PC monocations via ion/ion reactions facilitates the production of structurally informative anions and the identification of fatty acid composition without recourse to solution phase salt additives.

7.2 Experimental

7.2.1 Materials

Methanol, chloroform, and ammonium hydroxide were purchased from Mallinckrodt (Phillipsburg, NJ). Acetonitrile and 1,4-phenylenedipropionic acid were purchased from Sigma-Aldrich (St. Louis, MO). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). PC analytes were prepared at a concentration of ~30 µM in a solution of 50/50 (v/v) chloroform/methanol prior to positive nanoelectrospray ionization (nESI). The reagent anion, PDPA, was prepared at a concentration of ~ 220 µM in a solution of 49.5/49.5/1 (v/v/v) acetonitrile/methanol/ammonium hydroxide prior to negative nESI.

7.2.2 Solution Phase PDPA Spiking

A solution mixture of PC (i.e., PC₁₆:₀/₁₈:₁ or PC₁₈:₁/₁₆:₀) and PDPA was prepared at concentrations of 10 µM and 140 µM, respectively. The solution was subjected to
negative mode nESI, where MS\textsuperscript{n} analyses and subsequent mass analysis were performed in Q3.

7.2.3 Mass Spectrometry

All experiments were performed on a QTRAP 4000 triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Concord, ON, Canada), which has been modified to perform ion/ion reactions.\textsuperscript{35} Alternately pulsed nESI emitters sequentially inject mass-selected anions and cations into the q2 reaction cell.\textsuperscript{36,37} Doubly deprotonated PDPA anions were first ionized and accumulated in the q2 reaction cell. Next, the singly charged PC cations were ionized and transferred to the q2 cell to undergo a mutual storage reaction for 1000 milliseconds. Following accumulation of the doubly deprotonated PDPA anions and throughout the ion/ion reaction period in the transformation efficiency study, any singly charged PDPA anions that were formed from ion/molecule reactions due to adventitious basic vapors or ion/ion reactions were subjected to continuous resonance ejection to minimize the possibility of singly charged anions reacting with the singly charged cations. Product anions resulting from the ion/ion reaction were transferred to Q3 to perform MS\textsuperscript{n} analyses and subsequent mass analysis via mass-selective axial ejection (MSAE).\textsuperscript{38}
7.3 Results and Discussion

7.3.1 Transformation of PC Cations

Two PC isomers, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0/18:1 PC) and 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (18:1/16:0 PC), have been subjected to ion/ion reactions with doubly deprotonated PDPA. The ion/ion reaction produces a negatively charged complex (i.e., [PC+PDPA-H]_), which is stabilized by a strong electrostatic interaction between the fixed charge quaternary ammonium group of PC and a carboxylate of PDPA (Scheme 1). Figure 1(a) illustrates the product ion spectrum following an ion/ion reaction between [PDPA-2H]_2 anions and [PC_{16:0/18:1}+H]^+ cations. The post ion/ion reaction spectrum displays product ions corresponding to [PC_{16:0/18:1}+PDPA-H]_ and a charge reduced reagent anion (i.e., [PDPA-H]). Proton transfer to the reagent dianion can occur via two pathways: a fly-by mechanism or charge transfer upon separation of the long-lived complex.

Subsequent isolation and collisional activation of the long-lived complex, [PC_{16:0/18:1}+PDPA-H], produces a highly abundant [PC_{16:0/18:1-CH_3}] ion along with consecutive fragments consistent with charged and neutral losses of the fatty acid chains (Figure 1(b)). Observation of the demethylated PC anions indicates methyl cation and proton transfers to the anionic reagent within dissociation of the complex, thus producing a neutralized PDPA reagent (see Scheme 1). The observation of [PC-CH_3]_ mirrors the results of solution phase acetate and chloride salt experiments, where collisional activation of [PC+CH_3CO_2H]_ or [PC+Cl]_ generated a demethylated PC anion. Gas-phase methyl cation transfer between carboxylates and quaternary ammonium...
groups has been observed previously within the context of an ion/ion reaction. Alkyl (i.e., ethyl, propyl, butyl, isobutyl) cation transfer has also been observed upon CID of ESI-generated complexes composed of sulfophenyl benzoic acid dianions and tetraalkylammonium monocations. Collisional activation of the gas-phase anionic complex produced either proton or alkyl cation transfer to the sulfophenyl benzoic acid dianions, as similarly seen in this work with collisional activation of PDPA-adducted PCs. The low relative abundance of the proton transfer product (i.e., [PDPA-H]) in the dissociation spectrum is particularly noteworthy (Figure 1(b)). This low contribution of [PDPA-H] compared to [PC_{16:0/18:1-CH_3}] suggests that transfer of a proton and methyl cation upon collisional activation is a dominant pathway, while sole proton transfer without methyl cation transfer is a less favorable pathway. The other PC isomer, 18:1/16:0 PC, yields similar results to the 16:0/18:1 compound for both the ion/ion reaction and subsequent collisional activation of the long-lived complex (Figure 2).

The analytical utility of the gas-phase transformation of PC cations to demethylated PC anions is dependent upon the value of the information gained from changing one ion-type to another, as discussed below, as well as the reaction efficiency. A lower limit to the efficiency of converting PC_{16:0/18:1} cations to the anion complex [PC_{16:0/18:1-CH_3}] can be derived from the spectrum of Figure 1(a) from the ratio [PDPA-H]/([PDPA-H]+[PC_{16:0/18:1-CH_3}]). This yields an efficiency of roughly 25%. However, a significant fraction of the observed [PDPA-H] signal in Figure 1(a) arises from ion/molecule proton transfer from polar vapors present in the vacuum system. Furthermore, the accumulation of [PDPA-H] due to ion/ion and ion/molecule reactions leads to an increase in the neutralization of the PC cations from ion/ion reactions with the
We therefore chose to measure an “operational” efficiency of transforming PC_{16:0/18:1} cations to demethylated PC anions, which is defined as:

\[
\text{Transformation Efficiency } \% = \frac{\sum([PC-CH3-H]) + \sum(\text{ConFragments})}{\sum PC_i - \sum PC_f} \times 100
\]

where \(\Sigma[PC-CH3-H]\) is the abundance of demethylated PC anions produced upon CID of the long-lived complex ([PC+PDPA-H]-), \(\Sigma(\text{ConFragments})\) is the abundance of consecutive fragments from [PC-CH3] following CID of the long-lived complex, \(\Sigma PC_i\) is the abundance of [PC+H]^+ after isolation and before ion/ion transformation, and \(\Sigma PC_f\) is the abundance of residual [PC+H]^+ after transformation. A resonance ejection voltage was applied throughout the reaction period to eject [PDPA-H]^- ions generated by ion/ion and ion/molecule reactions to minimize any neutralization of the PC cations associated with ion/ion reactions with [PDPA-H]^-.

By subtracting \(PC_i\) from \(PC_f\), the transformation efficiency is limited to the fraction of [PC+H]^+ that undergoes an ion/ion reaction with [PDPA-2H]^2-. The transformation efficiency determined in this way represents the maximum possible efficiency for conversion of [PC+H]^+ to [PC-CH3]^- because it represents the value that would be obtained if all reactant cations undergo an ion/ion reaction. Complete depletion of analyte ions is achievable provided there is an excess of reagent ions, there is good overlap in the ion populations, and the reaction time is sufficient to allow for the reaction to go to completion. It is also emphasized here that the measurement yields an “operational” efficiency rather than an absolute efficiency due to possible differences in detection efficiencies for positive ions and negative ions. The
absolute values for voltages applied to the conversion dynode and multiplier were the same in both detection polarities with the only difference being the polarity of the conversion dynode. In this study, the transformation efficiency of PC cations to demethylated PC anions was measured to be $49 \pm 7\%$. The major loss process is expected to be neutralization of the cation by $[\text{PDPA-2H}]^{2-}$ as there are no major competing charge inversion channels and little resonance ejection of the $[\text{PC+PDPA-H}]^-$ complex is expected under the collisional activation conditions used to dissociate it. Overall, the results demonstrate that the ion/ion charge inversion/CID process used to generate the $[\text{PC-CH}_3]^-$ ions is clearly efficient enough for analytical use.

7.3.2 MS/MS of $[\text{PC-CH}_3]^-$ Anions

Ion trap CID of 16:0/18:1 PC prior to and after ion/ion transformation (i.e., $[\text{PC}_{16:0/18:1}+\text{H}]^+$ and $[\text{PC}_{16:0/18:1}-\text{CH}_3]$) are compared in Figure 3. Collisional activation of $[\text{PC}_{16:0/18:1}-\text{CH}_3]$ produces a structurally informative product ion spectrum, where loss of neutral and charged fatty acid chains are observed (Figure 3(a)). These fragment ions allow for the determination of chain length and the degree of unsaturation. The most favorable dissociation pathway appears to be the generation of a charged fatty acid chain from the $sn$-1 or $sn$-2 position, i.e., $[\text{C}_{16}\text{H}_{31}\text{CH}_2\text{COO-H}]^-$ and $[\text{C}_{14}\text{H}_{29}\text{CH}_2\text{COO-H}]^-$. Neutral losses of fatty acids as ketenes (e.g., $[\text{PC}_{16:0/18:1}-\text{CH}_3\cdot\text{C}_{14}\text{H}_{29}\text{CH}=\text{C}=\text{O-H}]^-$) and free fatty acids (e.g., $[\text{PC}_{16:0/18:1}-\text{CH}_3\cdot\text{C}_{14}\text{H}_{29}\text{CH}_2\text{COOH-H}]$) are also observed in low abundance. Similar dissociation behavior of demethylated PC anions$^{8,14-16}$ and other glycerophospholipids$^{43,44,45,46}$ has been noted previously in the literature. In contrast, CID of the $[\text{PC}_{16:0/18:1}+\text{H}]^+$ ion yields the phosphocholine cation as the strongly dominant
product (Figure 3(b)). Figure 3(c) depicts the relationship between bond cleavage and product ion-type (e.g., cleavage of the ester bond leads to charged or neutral loss of a single fatty acid chain). In addition, a comparison of \textit{sn}-cleavage position reveals a larger contribution from ions produced from the cleavage at the \textit{sn}-2 position (i.e., [C$_{16}$H$_{31}$CH$_2$COO-H]$^-$ and [PC$_{16:0/18:1}$-CH$_3$-C$_{16}$H$_{31}$CH=C=O-H]$^-$) compared to those produced from cleavage at the \textit{sn}-1 position (i.e., [C$_{14}$H$_{29}$CH$_2$COO-H]$^-$ and [PC$_{16:0/18:1}$-CH$_3$-C$_{14}$H$_{29}$CH=C=O-H]$^-$). Ekroos \textit{et al.} noted the relative favorability of \textit{sn}-2 cleavage in synthetic PC isomers and biological samples;$^{14}$ however, structural characterization via relative abundance comparisons can be misleading due to the likely isomeric mixtures present in synthetic and natural samples. Additionally, relative abundance ratios of the \textit{sn}-1 and \textit{sn}-2 position were previously demonstrated to be dependent on fatty acid chain length and degree of saturation, thus suggesting caution in assigning exact structures.$^{47}$

Figure 4(a) depicts the product ion spectrum following ion trap CID of [PC$_{18:1/16:0}$-CH$_3$]$^-$. Collisional activation of [PC$_{18:1/16:0}$-CH$_3$] produces similar fragment ions to the 16:0/18:1 PC isomer, (i.e., neutral losses of fatty acid ketenes, free fatty acids, and charged fatty acids), thus providing a diagnostic fragmentation spectrum. As seen with the 16:0/18:1 PC (Figure 3(a)), the dissociation behavior of 18:1/16:0 is dominated by the production of fatty acid anions (Figure 4(a)). However, the most significant difference between the two isomers (i.e., between 16:0/18:1 PC and 18:1/16:0 PC) is the reversal of the relative abundances of the [C$_{14}$H$_{29}$CH$_2$COO-H]/[C$_{16}$H$_{31}$CH$_2$COO-H] and [PC-CH$_3$-C$_{14}$H$_{29}$CH=C=O-H]/[PC-CH$_3$-C$_{16}$H$_{31}$CH=C=O-H] ions. This reversal in intensity may be attributed to the position of the fatty acid chain (i.e., \textit{sn}-1 or \textit{sn}-2), where cleavage of \textit{sn}-2 appears to be more favorable. Ekroos \textit{et al.} reported a similar change in relative
abundances between isomeric demethylated PC anions. Collisional activation of [PC<sub>18:1/16:0</sub>+H]<sup>+</sup> is depicted in Figure 4(b). Ion trap CID of the PC cation produces a dominant phosphocholine moiety, as also seen with 16:0/18:1 PC (Figure 3(b)). While caution should be exercised in the interpretation of these ion abundances, the fact that these low abundant spectral features can be detected following charge inversion presents a significant advantage over analysis in the positive ion mode alone.

7.4 Solution Phase PDPA Mixture

The preceding results demonstrate the formation of a [PC+PDPA-H]<sup>-</sup> complex, which dissociates to the [PC-CH<sub>3</sub>]<sup>-</sup> ion that can yield structurally informative products upon CID in the gas-phase. We also sought to generate such a complex directly from solution for two reasons. First, generation of the complex directly from solution would enable access to the precursor ion of interest without recourse to gas-phase ion/ion reactions, in analogy with the work of Ekroos et al. Of course, such an approach might also require a separate positive ion experiment to confirm the phospholipid class information. Second, we were interested to see if a complex formed in solution would differ in its CID behavior from that of the complex formed via ion/ion reaction. Direct ionization of a solution phase mixture containing PDPA and 16:0/18:1 PC via negative mode nESI is illustrated in Figure 5(a). Ionization of the mixture produces low abundance PDPA-adducted PC anions (i.e., [PC<sub>16:0/18:1</sub>+PDPA-H]<sup>-</sup>) among dimers of PDPA and substantial chemical noise. Collisional activation of the complex produces the demethylated PC anion (data not shown). Ion trap CID of the demethylated PC anion (Figure 5(b)) generates similar dissociation behavior compared to the analogous
[PC_{16:0/18:1-CH_3}]^- produced via ion/ion reactions (Figure 3(a)). The solution phase method clearly exhibits spectral complexity as well as limited ion signal from the adducted PC anion upon ionization, which could lead to complications in complex mixture analysis. The main advantages of the ion/ion chemistry are the high degree of control over the identities of the reactants via mass-selection prior to reaction, the avoidance of spectral complexity that can arise in solution phase reaction mixtures, the avoidance of ion suppression in the ionization step arising from the high salt concentration in the sample, and ready access to the original [PC+H]^+ ions and the product [PC-CH_3]^+ ion from a single solution and set of ionization conditions. Solution phase experiments with 18:0/16:0 PC (Figure 6) produced results similar to those observed with 16:0/18:1 PC. The dissociation behavior of [PC_{18:0/16:0-CH_3}]^- derived from the solution phase is also similar to that observed with the ion/ion chemistry.

7.5 Conclusions

Synthetic phosphatidylcholine monocations have been subjected to ion/ion reactions with doubly deprotonated PDPA. Collisional activation of the long-lived complex, comprised of PDPA and PC, generates a demethylated PC anion (i.e., [PC-CH_3]^-), indicating transfer of a proton from the phosphate group and methyl cation from the quaternary amine group to the carboxylate groups of PDPA. Ion trap CID of [PC-CH_3]^- generates product ions consistent with charged fatty acid chains and neutral losses of the acyl chains from the PC analyte as free acids and ketenes. Fragmentation spectra generate structurally informative product ions, which allows for the elucidation of fatty acid composition (i.e., the number of carbons and degree of unsaturation in each acyl
chain). The dissociation behavior of demethylated PC anions also exhibits preferential cleavages at the \( sn\)-2 over the \( sn\)-1 position as reflected in the relative abundances. The dissociation behaviors in this study are consistent with previous solution phase experiments reported in the literature. Solution phase studies demonstrated here, where a mixture of PC and PDPA are directly ionized via negative mode nESI, show that the complex that leads to demethylated PC anions can also be formed directly in solution, although with relatively low abundance. The ion/ion reaction approach is attractive in that a single set of ionization conditions in one polarity, which can be optimized for \([PC+H]^+\) formation, can be used to generate two ion-types, the \([PC+H]^+\) and \([PC-CH_3]^-\) ions, that provide complementary information upon CID.
7.6 References


Scheme 1: The sequence of generating [PC-CH₃]⁻ via ion/ion reactions between PDPA dianions and PC monocations. Top, mass-selected precursor ions; middle, electrostatic complex generated via a mutual storage ion/ion reaction; bottom, the main products following collisional activation of the negative complex.
Figure 7.1: (a) Product ion spectrum following ion/ion reactions between PDPA dianions and 16:0/18:1 PC monocations, (b) Ion trap CID of the long-lived complex, $[\text{PC}_{16:0/18:1}^+\text{PDPA-H}]^-$. 
Figure 7.2: (a) Product ion spectrum following ion/ion reactions between PDPA dianions and 18:1/16:0 PC monocations, (b) Ion trap CI D of the long-lived complex, [PC\textsubscript{18:1/16:0}+PDPA-H\textsuperscript{-}].

Figure 7.3: (a) Ion trap CID of [PC\textsubscript{16:0/18:1-CH\textsubscript{3}}]. (b) Ion trap CID of [PC\textsubscript{16:0/18:1+H\textsuperscript{+}}]. (c) Relating bond cleavage and product ion type for [PC\textsubscript{16:0/18:1-CH\textsubscript{3}}].
Figure 7.4: (a) Ion trap CID of [PC_{18:1/16:0-CH_3}]^-.
(b) Ion trap CID of [PC_{18:1/16:0+H}]^+.
Figure 7.5: (a) Ionization of PDPA and 16:0/18:1 solution phase mixture via negative mode nESI (b) Ion trap CID of $[\text{PC}_{16:0/18:1}^- \text{CH}_3]$.
Figure 7.6: (a) Ionization of PDPA and PC_{18:1/16:0} solution phase mixture via negative mode nESI (b) Ion trap CID of [PC_{18:1/16:0}-CH₃].
VITA
John R. Stutzman was born on April 10th, 1986 in Williamsport, Pennsylvania. He is the youngest son of Charles and Kay Stutzman, and sister to Lisa Hoffman and brother to Charles Timothy Stutzman. He received his high school education from Loyalsock Township High School in 2005. Following high school graduation, John received his Bachelor of Science degree of Chemistry from Lycoming College in May of 2009 with summa cum laude distinction. He joined Professor Scott A. McLuckey’s research group in November 2009 to study organic and biological mass spectrometry with emphasis on covalent ion/ion chemistry. John defended his Ph. D. dissertation November 15th, 2013.
PUBLICATIONS


Ion/Ion Reactions of MALDI-Derived Peptide Ions: Increased Sequence Coverage via Covalent and Electrostatic Modification upon Charge Inversion
Ion/Ion Reactions of MALDI-Derived Peptide Ions: Increased Sequence Coverage via Covalent and Electrostatic Modification upon Charge Inversion

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ABSTRACT: Atmospheric pressure matrix-assisted laser desorption/ionization (AP-MALDI)-derived tryptic peptide ions have been subjected to ion/ion reactions with doubly deprotonated 4-formyl-1,3-benzenedisulfonic acid (FBDSA) in the gas-phase. The ion/ion reaction produces a negatively charged electrostatic complex composed of the peptide cation and reagent dianion, whereupon dehydration of the complex via collision-induced dissociation (CID) produces a Schiff base product anion. Collisional activation of modified lysine-terminated tryptic peptide anions is consistent with a covalent modification of unprotonated primary amines (i.e., N-terminus and ε-NH₂ of lysine). Modified arginine-terminated tryptic peptides have shown evidence of a covalent modification at the N-terminus and a noncovalent interaction with the arginine residue. The modified anions yield at least as much sequence information upon CID as the unmodified cations for the small tryptic peptides examined here and more sequence information for the large tryptic peptides. This study represents the first demonstration of gas-phase ion/ion reactions involving MALDI-derived ions. In this case, covalent and electrostatic modification charge inversion is shown to enhance MALDI tandem mass spectrometry of tryptic peptides.

Peptide sequencing for identification and structural characterization of proteins is a long-standing activity in molecular biology research. Matrix-assisted laser desorption/ionization (MALDI)²⁻³ and electrospray ionization (ESI),⁴ two very successful means for the derivation of gas-phase ions from proteins and peptides, coupled with tandem mass spectrometry, have become the dominant tools for generating sequence information from mixtures of peptides⁵⁻⁸. While there is significant overlap, ESI and MALDI have each found applications for which they are best suited.⁹ For example, ESI has been widely used to interface online liquid separations with mass spectrometry, whereas MALDI is widely used for sampling surfaces, as in imaging applications. MALDI has been adapted to several tandem MS instrument geometries, e.g., tandem time-of-flight (i.e., TOF/TOF),¹⁰ quadrupole/time-of-flight (i.e., Q/TOF),¹¹ and ion trap¹²⁻¹⁴ analyzers. The appeal of ion trap technology is that it permits MS² experiments to be conducted on MALDI-derived biomolecule ions.¹⁵,¹⁶

From the standpoint of sequencing, a significant difference between MALDI and ESI is their propensities for generating multiply charged ions. Under commonly used conditions, MALDI generates ions of lower charge than does ESI. In fact, for peptides, singly charged ions usually dominate. Low-energy collisional activation of singly protonated tryptic peptides, however, often does not produce extensive sequence information.¹⁷,¹⁸ Dissociation behavior is dominated by low-energy fragmentation pathways, e.g., cleavage C-terminal to an aspartic acid residue or N-terminal to a proline residue.¹⁹

Furthermore, MALDI-derived tryptic peptides may generate limited sequence information due to the basic residue present at the C-terminus.²⁰⁻²² Tryptic peptides often generate fragment ions associated with the C-terminus (e.g., y-ions) as a result of the C-terminal lysine or arginine sequestering the excess proton.⁹ A variety of approaches have been attempted to improve peptide sequencing in conjunction with MALDI. For example, condensed-phase peptide modification, such as fixed-charged derivatization of the N-terminus, has been attempted with mixed success.²³,²⁴ A noteworthy approach, developed by Keough et al., to increase sequence information from the dissociation of MALDI-derived tryptic peptides involved conjugating a sulfonic acid group to the N-terminus via solution phase chemistry.²⁵,²⁶ The objective was to alter fragmentation pathways by introducing a highly acidic group into the peptide. Fragmentation was dramatically improved in derivatized tryptic peptides compared to the under derivatized tryptic peptides. However, the N-terminal position of this modification precluded the presence of N-terminal fragments due to the anionic sulfonate group.²⁶

We and others have been developing approaches to modify ions in the gas-phase and within the context of a tandem mass spectrometry experiment (i.e., modification of mass-selected ions) to expand the capabilities of tandem mass spectrometry

Supporting Information

* Supporting Information

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Ion/ion reactions can be used to decouple the ion type initially generated by the ionization method from the ion type subjected to tandem mass spectrometry. Structural information derived from fragmentation is highly dependent upon the nature of the ion (e.g., positive ion vs negative ion, even-electron ion vs odd-electron, etc.). Therefore, ion/ion reactions can expand the range of ion types that can be subjected to tandem mass spectrometry relative to the ion types available from the ionization method. For example, ion/ion reactions have been used to manipulate peptide ion charge state and polarity via the transfer of one or more protons, generate odd-electron ions from even-electron ions via electron transfer, and insert metal ions into polypeptides. Recent studies have demonstrated the alteration of peptide dissociation behavior via the gas-phase electrostatic attachment of reagents and via the gas-phase covalent modification of peptide ions. All such examples to date have been restricted to peptide ions generated via ESI.

In this work, we demonstrate the gas-phase modification of peptide ions generated by atmospheric pressure MALDI (AP-MALDI) using a dual-source interface developed by Schneider and Covey attached to a hybrid triple-quadrupole/linear ion trap tandem mass spectrometer and the structural characterization of the modified and unmodified versions of the ions via ion trap collision-induced dissociation (CID). The formation of imine bonds (i.e., Schiff base formation) using an aldehyde-containing diamin, 4-formyl-1,3-benzenedisulfonic acid (FBDSA) has been demonstrated to alter peptide dissociation behavior via the gas-phase electrostatic attachment of reagents and via the gas-phase covalent modification of peptide ions. Many of these examples to date have been restricted to peptide ions generated via ESI. Possible sites of peptide covalent modification are the N-terminus and the ε-amino group of lysine. Strong noncovalent interactions (i.e., electrostatic modification) have also been observed between the sulfonate groups of FBDSA and peptides containing an arginine residue and one or more carboxyl groups. Covalent bond cleavage is competitive with disruption of this strong electrostatic interaction under collisional activation conditions. In either case, covalent modification or strong electrostatic binding, the reaction product is a singly charged anion. The overall approach described here differs from the Keough et al. approach in that singly charged anions are subjected to CID, rather than singly charged cations, but is similar in that sulfonate is introduced into the peptide to alter dissociation behavior. Ion/ion reactions have been performed on tryptic peptides of ubiquitin and two synthetic peptides, APPGFSFPFR and GLSDGEWQQQLNVWGGK. Collisional activation of modified tryptic peptide anions demonstrates modification of the primary amine at the N-terminus and the C-terminal basic residue. Such modifications produce both modified N- and C-terminal fragment ions compared to the solely C-terminal information observed by Keough. Ion trap CID of the modified anions is shown to generate more sequence information than the unmodified ion, especially in the form of b-type ions. This study represents the first demonstration of gas-phase modification of MALDI-derived ions via ion/ion reactions.

**EXPERIMENTAL SECTION**

**Materials.** Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). Ubiquitin from bovine erythrocytes, TPCK-treated trypsin from bovine pancreas, α-cyano-4-hydroxycinnamic acid (CHCA), FBDSA, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic peptides APPGFSFPFR and GLSDGEWQQQLNVWGGK were purchased from SynPept (Dublin, CA). Peptides MQIFVK, TVMFEPDIENLYK, EGIPDQQR, LFAGK, TLSNYIQK, and ESTLHLVLR were generated from a tryptic digestion of ubiquitin.

**Methods.** The MALDI matrix, CHCA, was prepared in 39.5/59.5/1 (v/v/v) water/acetonitrile/TFA at a concentration of 10 mg/mL. Peptide analytes were prepared in an ~100 μM aqueous solution prior to a 50/50 (v/v) mixture with the MALDI matrix solution. An amount of 2.0 μL of the matrix/analyte solution was spotted onto the MALDI stage plate and allowed to air-dry prior to attachment to the AP-MALDI source. The anion reagent was composed of a 3.5 mM solution of 50/50 (v/v) water/acetonitrile for negative microionspray.

**Tryptic Digest.** The procedure for the tryptic digest of ubiquitin has been described previously. Separation of the tryptic peptides was performed on a reversed-phase HPLC (Agilent 1100, Palo Alto, CA) using an Aquapore RP-300 (7 μm pore size, 100 mm × 4.6 mm i.d.) column (Perkin-Elmer, Wellesley, MA). The gradient for the HPLC separation has been described previously. Following HPLC separation, the collected fractions were concentrated and reconstituted in 200 μL of water.

**Mass Spectrometry.** Experiments were performed on a 4000 AB Sciex QTRAP (Concord, ON, Canada), which has been modified to perform ion/ion reactions. The ion source is equipped with an AP-MALDI source (MassTech, Columbia, MD) and with a microionspray emitter (New Objective Inc., Woburn, MA) orthogonal to the sample inlet (Figure 1).

The microionspray emitter, remote to the MS inlet and MALDI stage plate, allows for the introduction of opposite polarity ions into the MS. Briefly, the AP-MALDI source has a circular opening, where a conductive transfer tube penetrates into the ion source and attaches to the curtain plate. The microionspray is directed toward the transfer tube, and upon ionization/nebulization, ions travel into the transfer tube and the ion source.

The reagent ion, doubly deprotonated FBDSA, was generated via microionspray at a continuous flow rate of 8.0 μL/min from a silica sprayer (364 μm outer dimension, 48 μm inner dimension, Polymicro Technologies, Phoenix, AZ). N2 acts as the nebulizing gas, which helps guide the reagent anions into the ion source. The nebulizing gas was triggered/pulsed via MS Expo software (AB Sciex, Concord, ON, Canada) during negative ion injection conditions. Once doubly deprotonated FBDSA entered the MS, it was transferred to the q2 collision cell and stored. Tryptic peptides, individually spotted in CHCA, were abladed via a 337 nm nitrogen laser (AP-MALDI Ion Source 110, MassTech, Columbia, MD) and transferred to the q2 collision cell to undergo ion/ion reactions with the reagent.
RESULTS AND DISCUSSION

Ubiquitin was subjected to tryptic digestion and the tryptic peptides MQIFVK, TITLEVEPSDTIENVK, EGIPPDQQR, LIFAGK, TLSDYNIQK, and ESTLHLVLR were collected. The MALDI-derived tryptic peptide ions were subjected to ion/ion reactions with doubly deprotonated FBDSA. The main product of the ion/ion reaction is a negatively charged, long-lived complex composed of the reagent dianion and tryptic peptide cation. The complex is formed when a negatively charged sulfonate interacts strongly with a protonated site on the peptide. The aldehyde group of the reagent undergoes nucleophilic attack by a neutral primary amine of the peptide to form an imine bond (i.e., Schiff base) and loss of a water molecule. The Schiff base product anion is represented in spectra by \([\text{M} + \text{FBDSA}^-]^{-}\). The process of forming a Schiff base via gas-phase ion/ion reactions is depicted in process 1:

\[
\begin{align*}
[M + H]^+ + [\text{FBDSA} - 2H]^2- & \\
\rightarrow [M + \text{FBDSA} - H]^+ & \\
\rightarrow [M + \text{H}]^+ + H_2O
\end{align*}
\]

(1)

The diamond symbol (\(\text{⧫}\)) represents the mass addition of the reagent anion following a water loss. Fragments that are consistent in mass with those that retain the covalent modification are labeled with the diamond. The negative ion mode post-ion/ion reaction spectrum (Figure 2a) and positive ion mode spectra of peptide mixture (Figure 2b) are shown.

MALDI mass spectrum of a mixture of three tryptic peptides (viz., LIFAGK, EGIPPDQQR, and TLSDYNIQK) are compared in Figure 2. Figure 2a was generated by exposing peptide cations of Figure 2b to a reaction with doubly deprotonated FBDSA. The ion/ion reaction generated a product spectrum containing primarily the intact complex (i.e., \([M + \text{FBDSA}]^-\)) and signals due to \([M + \text{H}]^+\) of much lesser abundance. The \([M + \text{H}]^+\) ions were likely generated by CID upon transfer from q2 to Q3. Subsequent isolation and activation of the \([M + \text{FBDSA}]^-\) ion generates abundant \([M + \text{H}]^+\) for subsequent interrogation. Contributions from strong electrostatic binding of the reagent were much more apparent in the data for the arginine-terminated peptides than for the lysine-terminated peptides (vide infra). For this reason, results from lysine- and arginine-terminated peptides are presented separately.

Lysine-Terminated Tryptic Peptides. Ion trap CID spectra of protonated TITLEVEPSDTIENVK and the modified peptide anion (i.e., \([M + H]^+\) and \([M + \text{H}]^+\), respectively) are compared in Figure 3. Collisional activation of \([M + \text{H}]^+\) leads to 9 of 15 amide bond cleavages, producing 11 fragment ions (Figure 3b). Fragmentation of the singly protonated peptide produces limited sequence-informative ions as well as non-informative neutral losses. The product ion spectrum produces relatively large contributions from the fragment ions \(y_8\) and \(y_9\). The fragment ions \(y_8\) arises from the well-established C-terminal cleavage of aspartic acid,\(^{52}\) while the fragment ion \(y_9\) originates from the well-established N-terminal cleavage of the proline residue.\(^{55}\) Dominance of low-energy fragmentation pathways has been frequently observed in tandem MS experiments with MALDI-derived peptide cations.\(^{29}\) When compared to b-ions, the y-ions clearly dominate the fragmentation spectrum of the unmodified cation. Collisional activation of the peptide cation produced only four b-ions compared to seven y-ions. Limited N-terminal information (e.g., b-ions) from the collisional activation of \([M + H]^+\) can be explained by the presence of a C-terminal basic lysine residue as reported by Biemann.\(^{20}\) Collisional activation of modified TITLEVEPSDTIENVK (Figure 3a) produces distinct dissociation behavior compared to the unmodified spectrum. In the product ion spectrum, the most abundant peaks are associated with modified b- and y-ions, which are represented as \(b\text{⧫}\) or \(y\text{⧫}\). The nomenclature of modified fragment anions is adapted from the peptide literature for protonated peptides.\(^{23}\) The \(b\text{⧫}\) ions are interpreted to arise from a covalent modification of the N-terminus, while the \(y\text{⧫}\) ions are interpreted to arise from the covalent modification of the \(\varepsilon\text{-NH}_2\) of lysine. Collisional activation of the modified peptide anion produces a higher degree of fragmentation than the unmodified peptide cation, where 12 of 15 amide bonds are...
cleaved, generating a total of 16 fragment ions and a significant increase in N-terminal information. In the spectrum of the modified ion, seven b• ions are produced compared to four b-ions in the spectrum of the unmodified peptide. We note that previous studies have clearly shown that [M + •]- ions yield significantly more sequence information upon CID than the corresponding [M − H]− ions. The incorporation of the two sulfonate groups into the peptide is expected to have a significant impact on proton mobility within the anion, which influences the favored dissociation pathways. This may favor charge-remote processes, or it may require the ions to be elevated to higher energies in order to promote intramolecular transfer. In any case, the disulfonic acid modification to the peptide has been shown to lead to consistently greater sequence coverage than singly charged versions of the unmodified peptide (i.e., [M + H]+ or [M − H]−). Furthermore, modification at the N-terminus gives rise to much greater contributions from anionic b• ions, which are not readily observed without the modification. Ion trap CID of [M + •]− also appears to have less selective cleavage along the peptide backbone compared to the peptide cation, which has been observed in previous studies.

Another ubiquitin tryptic peptide with a C-terminal lysine, TLSDYNIQK, produced results similar to those noted for the peptide TITLEVEPSDTIENVK. Ion trap CID of modified TLSDYNIQK (i.e., [M + H]+) produced a high degree of sequence information, where a cleavage at every amide bond is observed (Figure 4a). The presence of many b• and y• ions is consistent with the covalent modification of the N-terminus and ε-NH2 of lysine. The high relative contribution of b1• in the product ion spectrum is attributed to the labile C-terminal cleavage of aspartic acid. The presence of b1• is particularly noteworthy because the b1-ion is not typically observed in the collisional activation of unmodified protonated and unprotonated peptides. Ion trap CID of the singly protonated peptide generates a product ion spectrum with seven of eight amide bond cleavages (Figure 4b); however, many less informative fragment ions are also observed in the spectrum (e.g., internal fragments and neutral losses). The product spectrum of the unmodified peptide cation is dominated by the C-terminal cleavage of aspartic acid, generating the highly abundant y5-ion. Conversely, the modified anion appears to fragment more uniformly. Dissociation of the peptide cation is dominated by the generation of y-ions, while the presence of b-ions is limited. The modified peptide anion produces a significant number of b• ions and an even higher number of y-type ions due to the presence of y1• and y2•. With a near-doubled increase of y- and b-type ions (i.e., 13 vs 7 fragment ions), the modified peptide ion clearly produced more sequence information compared to the unmodified peptide.

The peptides TITLEVEPSDTIENVK and TLSDYNIQK are illustrative of the synthetic peptide GLSDGEWQQVLNVWGK. Collisional activation of the modified and unmodified GLSDGEWQQVLNVWGK is provided in the Supporting Information (Figure S-1). Ion trap CID of [M + •]− produces a high order of b• and y• ions (Supporting Information Figure S-1a), while the protonated peptide produces limited b- and y-ions (Supporting Information Figure S-1b). The modified peptide anion again produced a higher order of sequence information than the unmodified peptide cation. However, the aforementioned lysine-containing peptides are not necessarily illustrative of low m/z peptides, such as MQIFVK (Figure 5) and LIFAGK (Supporting Information Figure S-2).

Figure 4. Ion trap CID product ion spectra of (a) [M + •]− derived from M = TLSDYNIQK.

Figure 5. Ion trap CID product ion spectra of (a) [M + •]− derived from M = MQIFVK.

Ion trap CID of modified MQIFVK (i.e., [M + •]−) produces mainly y• ions at four of the five amide bond cleavages (Figure 5a) and only one ion in the b• ion series (i.e., the b1•-ion), which is distinct from the observation of many more b• ions from the large modified peptide ions (i.e., TITLEVEPSDTIENVK, TLSDYNIQK, and GLSDGEWQQVLNVWGK). Ion trap CID of the unmodified peptide cation produces many b- and y-ions, which account for four of the five amide bond cleavages (Figure 5b). Smaller peptide ions tend to yield more extensive sequence information in general. While similar amide bonds are cleaved in the modified and unmodified peptide ions, there is a degree of complementarity from the two peptide ion types. The modified anion produces lower m/z y• ions, while the [M + H]+ produces mainly b-ions. Also, the relative contributions of the fragment ions in the product ion spectra are markedly different. The modified peptide ion produces a highly abundant y1•-ion, and the peptide cation generates a highly abundant internal fragment ion, showing the distinct dissociation behavior. The results from the peptide MQIFVK are illustrative of the peptide LIFAGK (Supporting Information Figure S-2). Ion trap CID of [LIFAGK + •]− produces mainly modified y•-ions, which is consistent with the covalent modification of the ε-NH2 of...
lysine. Collisional activation of the unmodified peptide cation produces a, b-, and y-ions. Ion trap CID of both the modified and unmodified versions of LIFAGK displays a degree of complementarity, much like MQIFVK.

Arginine-Terminated Tryptic Peptides. Previous studies of the ion trap CID of modified arginine-containing peptides [i.e., angiotensin II (DRVYIHPF) and ubiquitin tryptic peptides] demonstrated product ion spectra consistent with two product populations: Schiff base formation and non-covalent binding of the reagent to the guanidinium side chain of arginine.39,45 Product ion spectra showed the appearance of y ions; however, the only primary amine in the peptide capable of engaging in Schiff base formation was the N-terminus. The y ions were determined to arise from [M + H] ions composed of an electrostatic interaction with the arginine guanidinium side chain. The water loss leading to the nominal [M + H] − ions originated from elsewhere in the peptide (i.e., it was unrelated to the reagent). The presence of y + FBDSA ions also provided evidence of an electrostatic rather than a covalent interaction. Model systems (i.e., YGGFLX) further demonstrated strong interactions between the sulfonate groups and peptides containing an arginine residue and one or more carboxyl groups (e.g., C-terminus) in the gas phase.39 These noncovalent interactions with FBDSA dianions are sufficiently strong that covalent bond cleavage can compete with reagent detachment upon collisional activation.39,45

Ion trap CID of modified EGIPPDQQR is displayed in Figure 6a. Collisional activation of the modified anion [M + H] − derived from M = EGIPPDQQR is the generation of a b-ion at each peptide bond (Figure 7a), including the b-ion. Collisional activation of [M + H] − produces a combination of [M + H] − and y + FBDSA ions. The product ion spectrum shows a high contribution from the well-established N-terminal cleavage of the proline residue (i.e., b − H); however, more uniformity of cleavage among amide bonds is observed compared to the unmodified cation. While the ion trap CID of [M + H] − (Figure 7b) and [M + H] − produces similar m/z y-type product ions, the modified anion also produces lower m/z y-type ions that the unmodified version lacks.

Ion trap CID of the modified and unmodified synthetic tryptic peptide, APPGFSPFR, is illustrated in Figure 7. The modified anions produces cleavages at all eight amide bonds, and the most notable observation in the product ion spectrum is the generation of a b-ion at each peptide bond (Figure 7a), including the b-ion. Collisional activation of [M + H] − produces a combination of y − and y + FBDSA ions. The product ion spectrum shows a high contribution from the well-established N-terminal cleavage of the proline residue (i.e., b − H); however, more uniformity of cleavage among amide bonds is observed compared to the unmodified cation. While the ion trap CID of [M + H] − (Figure 7b) and [M + H] − produces similar m/z y-type product ions, the modified anion also produces lower m/z y-type ions that the unmodified version lacks.

Figure 6. Ion trap CID product ion spectra of (a) [M + H] − derived from M = EGIPPDQQR.

Figure 7. Ion trap CID product ion spectra of (a) [M + H] − derived from M = APPGFSPFR.
Analytical Chemistry

fragment ion information. Collisional activation of the peptide cation produces only three b-ions, while the modified anion produces eight b'-ions. The presence of the b'-ions significantly increases the observed sequence information. The result of EGPDPDQQR and APPGSPFR are illustrative of ubiquitin trypptic peptide ESTLHLVLR (Supporting Information Figure S-3). Ion trap CID of [ESTLHLVLR + H]+ produces a mixture of y-ions and y+ FBDSA ions along with higher m/z b'-ions. The unmodified peptide cation produces similar b- and y-type ions; however, the modified anion additionally produces lower m/z modified y-ions, i.e., y+ FBDSA.

We note that many of the singly protonated peptides derived by MALDI and subjected to ion/ion reactions in this work have also been derived via electrospray and subjected to reactions with the same reagent. Very similar product ion spectra were derived from the same peptide ions derived by these two ionization methods. Not surprisingly, the main difference in the two approaches was that singly protonated peptides dominated in all cases in the MALDI experiment, whereas multiply protonated peptides were the major ions noted for the larger peptides in the electrospray experiment.

CONCLUSIONS

Covalent and electrostatic modification of MALDI-derived tryptic peptide cations via gas-phase ion/ion reactions with doubly deprotonated FBDSA has been demonstrated. Covalent modification is observed at the N-terminus and ε-NH₂ of lysine for lysine-terminated tryptic peptides. Covalent and electrostatic modification is observed for arginine-terminated tryptic peptides, where the covalent modification occurs at the N-terminal primary amine and electrostatic modification occurs at the C-terminal arginine residue. Both modified lysine- and arginine-terminated tryptic peptides have shown to result in increased sequence information upon collisional activation compared to the unmodified version in high m/z peptides (>1000 Th). Ion trap CID of the modified anions generally shows an increase in the relative contribution of b-type ions, where unmodified peptide anions have shown limited b-ions. In many cases, the modified anion produces sequence-informative y-ions of lower m/z that are often not observed from the unmodified peptide cation. In general, modified anions have shown more uniform fragmentation compared to the unmodified cations, which often show dominant cleavages due to low-energy CID pathways. This study demonstrates the first example of gas-phase ion/ion reactions involving MALDI-derived ions (other than those that may occur inherently in the MALDI process). In this case, we demonstrate the gas-phase modification of the ions to improve the MS/MS performance of MALDI-derived tryptic peptides without recourse to solution phase chemistry.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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