Metastable Atom-Activated Dissociation (MAD): A Novel Dissociation Method
Employed within a Quadrupole Ion Trap Mass Spectrometer

A dissertation presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

Shannon L. Cook
March 2012
©2012 Shannon L. Cook. All Rights Reserved.
This dissertation titled

Metastable Atom-Activated Dissociation (MAD): A Novel Dissociation Method

Employed within a Quadrupole Ion Trap Mass Spectrometer

by

SHANNON L. COOK

has been approved for

the Department of Chemistry and Biochemistry

and the College of Arts and Sciences by

______________________________

Glen P. Jackson

Associate Professor of Chemistry and Biochemistry

______________________________

Howard D. Dewald

Interim Dean, College of Arts and Sciences
Metastable Atom-Activated Dissociation (MAD): A Novel Dissociation Method
Employed within a Quadrupole Ion Trap Mass Spectrometer

Director of Dissertation: Glen P. Jackson

Recent advances in mass spectrometry have provided unprecedented investigative power for biomedical and clinical researchers. The long-term goal of this project is to catalyze the process of biomedical research by developing new and superior mass spectrometric techniques, thereby aiding in disease diagnosis, prognosis, and biomarker discovery. The traditional dissociation method typically employed in mass spectrometers, collision induced dissociation (CID), produces mainly b-/y-type ions with abundant neutral losses of the post-translational modification and generating incomplete sequencing information. Newer techniques which rely on radical-induced chemistry, electron capture and transfer dissociations (ECD/ETD), have become the preferred way to analyze peptides and proteins. However they are inapplicable to singly-charged cations and anions as well as ineffective on peptides of lower-charge states.

Metastable atom-activated dissociation (MAD) induces fragmentation through the interaction of isolated ions with a high kinetic energy beam of metastable atoms. Extensive back-bone fragmentation resulting in a-, b-, c-, x-, y-, and z-type ions is observed of singly- and multiply-charged peptide cations, multiply-charged anions, and lipids through their interaction with a high kinetic-energy beam of argon or helium metastable atoms in a modified quadrupole ion trap mass spectrometer. The ability to determine post-translational sites, especially highly labile nitrosylation modifications, is
demonstrated as well as the ability to distinguish between leucine and isoleucine residues and to cleave two covalent bonds of the proline ring resulting in z- and w-type ions. The fragmentation spectra indicate that fragmentation occurs through non-ergodic radical ion chemistry akin to ECD and ETD mechanisms. However, MAD demonstrates three apparent benefits to ECD and ETD: 1) the ability to fragment singly-charged precursor ions, 2) the ability to fragment negatively charged ions, and 3) the ability to cleave the proline ring, which requires the cleavage of two covalent bonds. Reaction times less than 250 ms and efficiencies approaching 5% are compatible with on-line fragmentation, as would be desirable for bottom-up proteomics applications.

Approved: _____________________________________________________________

Glen P. Jackson

Associate Professor of Chemistry and Biochemistry
Dedicated to my mom and to the memory of my father for giving their unwavering love
and support for all my endeavors no matter how great or small
ACKNOWLEDGMENTS

First and foremost I would like to express my gratitude to my advisor, Glen Jackson, for spending countless hours mentoring with patience and respect. Under his guidance I have matured into an independent chemist and have developed invaluable skills. His style of ‘hands on’ teaching and mentoring not only in the laboratory but also in the classroom will be one I carry with me and implement in future endeavors.

At Ohio University many people have been instrumental in encouraging and teaching me throughout my journey. Dr. Olivier Collin spent his last year teaching me everything he knew, which was a lot, about every instrument we had in the department. To his credit, my diversity and depth of instrument knowledge can be linked back to him. I had the great pleasure to collaborate with Dr. Ünige Laskay and through her I learned the basic fundamentals of the quadrupole ion trap mass spectrometer. Her knowledge of mass spectrometry fundamentals was extensive and I relished coming in each day to pick her brain. Without the help of Bascom French I believe the many ‘mishaps’ I had in my research would have been more detrimental. I appreciate his patience and tutelage. Dr. Shadrick Paris spent many hours helping me strengthen my fundamental chemistry background and was a great scientist to bounce ideas off, many thanks for all the informative lunches. I would also like to thank Paul Schmittauer, Rollie Merriman, Dr. Zeland Muccio, Dr. Carolyn Zimmermann (who collected data for Chapter 5), Xiaobo Sun, and the rest of the Jackson group and the Harrington group for the countless talks, advice, and wonderful memories.
I must acknowledge the instrumental person who encouraged me to pursue my doctorate in the first place, Sridhar Varadarajan, my research advisor at the University of North Carolina at Wilmington. Sridhar saw the potential in me before I did and to him I will always be grateful.

I would also like to thank my dissertation committee: Dr. Hao Chen, Dr. Shiyong Wu, and Dr. Saw-Wai Hla. I appreciate the time each one of you has given to read through both my proposal and dissertation as well as for my presentations. Additionally for all of the beneficial suggestions and comments put forth to strengthen my final defense. I would like to acknowledge the funding support from: the Department of Chemistry and Biochemistry at Ohio University, Center for Intelligent Chemical Instrumentation (CICI), NSF grant number 0649757 through the Division of Biological Infrastructure (DBI), and the NSF CAREER Award CHE 0745590.

Several collaborations have been instrumental in my career at Ohio University which I would like to mention. Protea Biosciences has generously given numerous peptide samples which I have been able to utilize successfully in my research. I will be forever grateful to Dr. Kari Green-Church and The Ohio State University who loaned the Jackson group the use of a quadrupole ion trap mass spectrometer at a time when most needed. I would like to thank Dr. Ralf Hoffman and his group, who collaborated with us on Chapter 5 and who also donated tau phosphopeptides in addition to other peptides.

On a more personal note I would like to thank my entire family, who has been my grounding rock. Each and every one of them has contributed to this accomplishment through encouragement, support, love, and understanding. Without them I would not be the person I am today and my world would not be as bright. To Bear, who never once
asked why I was home so late, yet instead greeted me every night with warmth, love, and a chin full of water. At last, I must thank Mike Carlson, who has made the last couple of years not only enjoyable but unforgettable. You have provided a shoulder when needed, an ear when necessary, and your support constantly.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>3</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>5</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>6</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>13</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>14</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>20</td>
</tr>
<tr>
<td><strong>CHAPTER 1: INTRODUCTION</strong></td>
<td>25</td>
</tr>
<tr>
<td>1.1 Goals</td>
<td>25</td>
</tr>
<tr>
<td>1.2 Project Overview</td>
<td>26</td>
</tr>
<tr>
<td>1.3 Quadrupole Ion Trap Mass Spectrometry</td>
<td>28</td>
</tr>
<tr>
<td>1.3.1 Instrumentation</td>
<td>29</td>
</tr>
<tr>
<td>1.3.2 Operation</td>
<td>30</td>
</tr>
<tr>
<td>1.3.2.1 Ion Trapping and Ion Stability</td>
<td>30</td>
</tr>
<tr>
<td>1.3.2.2 Resonant Excitation</td>
<td>33</td>
</tr>
<tr>
<td>1.4 Tandem Mass Spectrometry for Peptides and Proteins</td>
<td>34</td>
</tr>
<tr>
<td>1.4.1 Collision Induced Dissociation</td>
<td>35</td>
</tr>
<tr>
<td>1.4.2 Ion/Ion Reactions</td>
<td>39</td>
</tr>
<tr>
<td>1.4.3 Electron Capture Dissociation</td>
<td>40</td>
</tr>
<tr>
<td>1.4.4 Electron Transfer Dissociation</td>
<td>44</td>
</tr>
<tr>
<td>1.4.5 Negative-Ion Mode Electron Transferring Methods</td>
<td>45</td>
</tr>
<tr>
<td>1.5 Metastable Atom-Activated Dissociation</td>
<td>46</td>
</tr>
<tr>
<td>1.5.1 Background</td>
<td>46</td>
</tr>
<tr>
<td>1.5.2 MIES/PIES</td>
<td>49</td>
</tr>
<tr>
<td>1.5.3 Previous Research</td>
<td>50</td>
</tr>
<tr>
<td>1.5.4 Metastable Atom Production</td>
<td>52</td>
</tr>
<tr>
<td><strong>CHAPTER 2: METASTABLE ATOM-ACTIVATED DISSOCIATION MASS SPECTROMETRY (MAD-MS): LEUCINE/ISOLEUCINE DIFFERENTIATION AND RING CLEAVAGE OF PROLINE RESIDUES</strong></td>
<td>53</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>53</td>
</tr>
<tr>
<td>2.2 Experimental</td>
<td>59</td>
</tr>
</tbody>
</table>
2.2.1 Instrumentation ................................................................. 59
2.2.2 Reagents .......................................................... 63
2.2.3 Method ........................................................... 63
2.3 Results and Discussion ...................................................... 64
  2.3.1 Bradykinin: 1+ and 2+ Charge States .......................... 64
  2.3.2 Substance P: 1+ and 2+ Charge States ......................... 67
  2.3.3 Backbone Cleavage of Proline Residues ....................... 71
  2.3.4 Differentiating Leucine and Isoleucine ......................... 73
  2.3.5 Phosphorylated Peptides ........................................... 75
    2.3.5.1 Angiotensin II .............................................. 75
    2.3.5.2 Cholecystokinin (peptide 10-20) .......................... 76
  2.3.6 Glycosylated Peptides ............................................. 78
  2.3.7 Peptide Anions: 2- Charge State ............................... 80
  2.3.8 Kinetic Study of 1+ and 2+ Charge States of Bradykinin ... 81
  2.3.9 Proposed Mechanism of Metastable-Atom Activation ........ 83
2.4 Conclusion .................................................................. 86

CHAPTER 3: CHARACTERIZATION OF TYROSINE NITRATION AND CYSTEINE NITROSYLATION MODIFICATIONS BY METASTABLE ATOM-ACTIVATION DISSOCIATION MASS SPECTROMETRY .............................................. 88
  3.1 Introduction .......................................................... 88
  3.2 Experimental .......................................................... 93
    3.2.1 Instrumentation .................................................... 93
    3.2.2 Preparation of Peptides ......................................... 94
    3.2.3 Method ........................................................... 95
      3.2.3.1 CID .......................................................... 95
      3.2.3.2 MAD ......................................................... 95
  3.3 Results and Discussion ................................................. 96
    3.3.1 Tyrosine Nitrated Peptides ..................................... 96
    3.3.2 Cysteine Nitrosylated Peptides ................................ 101
    3.3.3 Effect of Modification on Peptide Backbone Cleavage ...... 104
    3.3.4 Effect of Charge State on Peptide Backbone Cleavage ...... 113
    3.3.5 S-Nitrosylated and Nitrated Peptide Anions ............... 116
CHAPTER 6: METASTABLE ATOM-ACTIVATED DISSOCIATION MASS SPECTROMETRY OF SINGLY-CHARGED SPHINGOMYELIN AND PHOSPHATIDYLCHOLINE (16:0-18:1).................................................................................. 174

6.1 Introduction.................................................................................................................. 174
6.2 Experimental.............................................................................................................. 176
6.3 Results and Discussion ............................................................................................ 177
6.4 Conclusion .................................................................................................................. 186

CHAPTER 7: CONCLUSIONS AND FUTURE WORK................................................... 187

REFERENCES ................................................................................................................. 190
Table 1.1. Characteristics of Metastable Atoms of Noble Gases.\textsuperscript{a} ................................................................. 48

Table 2.1. Comparison of Demonstrated Activation Techniques for Peptide and Protein Precursor Ion. ........................................................................................................................................................................ 54

Table 3.1. Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD and CID methods on: (a) [DRVnYIHFP+2H]\textsuperscript{2+}, (b) [VFDARDnCRSAQ+2H]\textsuperscript{2+}, and (c) [EMFTYInCNHIK+2H]\textsuperscript{2+}. .................................................. 110

Table 3.2. Comparison of the Type and Charge of the Fragmentation Ions Produced After He MAD of the Modified and Unmodified Peptides: (a) [DRVYIHFP+2H]\textsuperscript{2+}, (b) [VFDARDCRSAQ+2H]\textsuperscript{2+}, and (c) [EMFTYICNHIK+2H]\textsuperscript{2+}. .................................................. 113

Table 3.3. Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD of the Singly-, Doubly-, and Triply-Charged Modified Peptides: (a) DRVnYIHFP, (b) EMFTYInCNHIK, and (c) GPLEnYGFAKGPLAK. ................................................................................................................................. 115

Table 3.4. List of the Neutral Losses Observed from the Charge-Reduced Species, [M-2H]\textsuperscript{-}, After He MAD of the 2-Peptide Ions. .................................................................................................................................................. 118

Table 4.1. Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD on the [M-2H]\textsuperscript{2-} and [M-3H]\textsuperscript{3-} Precursor Ions of the Following Peptides: (a) IKNLQSLDPpSH, (b) DFNKFHpTFPQTAIGV, and (c) DRVnYIHFP. .............................................. 136

Table 4.2. Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD on the [M+2H]\textsuperscript{2+} and [M-2H]\textsuperscript{2-} Precursor Ions of the Following Peptides: (a) IKNLQSLDPpSH, (b) DFNKFHpTFPQTAIGV, and (c) DRVnYIHFP. .............................................. 138

Table 5.1 A Comparison of the Percent Sequence Coverage for CID, ETD, and MAD of Each Tau Phosphopeptide. .............................................................................................................................................. 159

Table 5.2. List of Neutral Losses Observed Through CID, ETD, and MAD from the Precursor Ion, Charge-Reduced Ion, and Penning Ionized Ion. .................................................................................................................. 164
**LIST OF FIGURES**

**Figure 1.1.** Schematic of the QIT illustrating the hyperbolic cross section and the dimensions $r_0$ and $z_0$. .......................................................... 29

**Figure 1.2.** Mathieu Stability Diagram for ions within the QIT. Ions with a $q_z$ value between 0 and 0.908 are stable within the trap. .......................................................... 32

**Figure 1.3.** Nomenclature for peptide sequence ion observed in tandem mass spectrometry: (a) a-, b-, c-, x-, y-, and z-ions and (b) d-, v-, and w- ions. ...................... 35

**Figure 1.4.** Illustration of the mobile proton model, with hydrogen propagation to the thermodynamically less stable amide nitrogens. .......................................................... 38

**Figure 1.5.** The Grotian diagram for helium highlighting the $2^1S_0$ and $2^3S_1$ metastable states.......................................................... 47

**Figure 2.1.** Schematic representation of the instrument used in this study. .................. 59

**Figure 2.2.** Schematic representation of electronic components used to power the FAB gun for pulsed operation. .......................................................... 61

**Figure 2.3.** MAD spectra of bradykinin: (a) He$^m + [M+H]^+$ (b) He$^m + [M+2H]^{2+}$, (c) Ar$^m + [M+H]^+$ (d) Ar$^m + [M+2H]^{2+}$. (Key for peptidesequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; green line = product ions observed in both 1+ and 2+ charge states; precursor is indicated by a red arrow; side chain fragments are circled). .......................................................... 65

**Figure 2.4.** MAD spectra of substance P: (a) He$^m + [M+H]^+$ (b) He$^m + [M+2H]^{2+}$, (c) Ar$^m + [M+H]^+$ (d) Ar$^m + [M+2H]^{2+}$. (Key for peptidesequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; green line = product ions observed in both 1+ and 2+ charge states; precursor is indicated by a red arrow; side chain fragments are circled). .......................................................... 68

**Figure 2.5.** Comparison of fragmentation observed through CID and MAD of substance P (RPKPQQFFGLM) and bradykinin (RPFGFPFR). .......................................................... 71

**Figure 2.6.** MAD spectra of (a) bradykinin and (b) the PHPRL peptide. Highlighted are the $z_3$-2H, $z_3$-CH, and $w_3$ ($z_3$-C$_2$H$_4$) ions produced via the cleavage of two covalent bond bonds in the five-membered ring of the proline residue at position 3 in PHPRL and position 7 in bradykinin (red P indicates proline residue of interest). The two covalent bond cleavages necessary to produce the $z$-2H, $z$-CH, and $w$ ($z$-C$_2$H$_4$) ions are illustrated in (c). .......................................................... 72
Figure 2.7. MAD spectra of the two synthetic peptides PHPRL and PHPRI: (a) He$^m + [M+H]^+$, showing several neutral losses of 49Da, (b) He$^m + [M+H]^+$, showing several neutral losses of 29Da, (c) confirming the leucine neutral loss from the z$_2$ and z$_4$ ions which are not present in the isoleucine spectrum, (d) confirming the isoleucine neutral loss from the z$_2$ and z$_4$ ions which are not present in the leucine spectrum. (Key for peptidesequencing: black line = product ions observed in 1+ charge state; green line = product ions observed in both 1+ and 2+ charge states; precursor is indicated by a red arrow; side chain fragments are circled).

Figure 2.8. MAD spectrum of singly-protonated phosphorylated angiotensin II with a He metastable atom beam. (Key for peptidesequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; red * = b$_6$, b$_5$, a$_5$, b$_4$, and a$_4$ without the phosphate group; precursor is indicated by a red arrow; side chain fragments are circled).

Figure 2.9. MAD spectra of singly-protonated phosphorylated cholecystokinin (peptide 10-20) using a He metastable atom beam, (a) average of 2500 spectra and (b) average of 5 spectra (a single data point), collected in ~1 second. (Key for peptidesequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; precursor is indicated by a red arrow; side chain fragments are circled).

Figure 2.10. He MAD spectra of [M+2H]$^{2+}$ glycosylated peptides: (a) of Rnase B with a n-linked beta GlcNAc and (b) a synthetic octapeptide with an o-linked beta GlcNAc. (Key for peptidesequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; precursor is indicated by a red arrow; side chain fragments are circled).

Figure 2.11. MAD spectrum of doubly-deprotonated substance P using a He metastable atom beam.

Figure 2.12. Fragmentation intensity of a$_3$+H ion (324.3 m/z), c$_5$ ion (573.4 m/z), and y$_8$ ion (904.4 m/z) using 7 keV after 50, 100, 150, 200, and 250 ms metastable atom beam exposures on the [M+H]$^+$ species of bradykinin.

Figure 2.13. Relationship of how He metastable atom pulse time and voltage applied to FAB gun anode effects fragmentation of singly- and doubly-charged bradykinin: (a) fragmentation intensity of the z$_4$+H ion at 50, 100, 150, 200, and 250 ms metastable atom exposure using 6, 7, 8, 9, and 10 keV on the [M+H]$^+$ species.

Figure 3.1. He MAD spectra of nitrated angiotensin II (DRVnYIHPF): (a) [M+H]$^+$, (b) [M+2H]$^{2+}$, (c) [M+3H]$^{3+}$, and He MAD spectra of nitrated GPLEnYGFAKGPLAK: (d)[M+H]$^+$, (e) [M+2H]$^{2+}$, (f) [M+3H]$^{3+}$. The precursor is indicated by an arrow and
the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 3.2.** He MAD spectra of S-nitrosylated EMFTYInCNHIK: (a) [M+H]\(^+\), (b) [M+2H]\(^{2+}\), (c) [M+3H]\(^{3+}\), and He MAD spectra of S-nitrosylated VFDARDnCRSAQ: (d) [M+2H]\(^{2+}\), (e) [M+3H]\(^{3+}\). The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 3.3.** Comparison of the 2+ charged peptide DRVYIHPF: (a) MAD of non-nitrated DRVYIHPF, (b) MAD of nitrated DRVnYIHPF, and (c) CID of nitrated DRVnYIHPF. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 3.4.** Comparison of the 2+ charged VFDARDCRSAQ: (a) MAD of non-nitrosylated VFDARDCRSAQ, (b) MAD of S-nitrosylated VFDARDnCRSAQ, and (c) CID of S-nitrosylated VFDARDnCRSAQ. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 3.5.** Comparison of the 2+ charged EMFTYICNHIK: (a) MAD of non-nitrosylated EMFTYICNHIK, (b) MAD of S-nitrosylated EMFTYInCNHIK, and (c) CID of S-nitrosylated EMFTYInCNHIK. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 3.6.** Venn diagrams comparing the different types of fragment ions produced by He MAD and CID: (a) [DRVnYIHPF+2H]\(^2+\), (b) [VFDARDnCRSAQ+2H]\(^2+\), and (c) [EMFTYInCNHIK+2H]\(^2+\).

**Figure 3.7.** Venn diagrams comparing the number of fragmentation ion types produced after He MAD of the modified and unmodified peptides: (a) [DRVYIHPF+2H]\(^2+\), (b) [VFDARDCRSAQ+2H]\(^2+\), and (c) [EMFTYICNHIK+2H]\(^2+\).

**Figure 3.8.** Venn diagrams comparing the number of fragment ion types produced by He MAD of the 1+, 2+, and 3+ charge states of the modified peptides: (a) DRVnYIHPF, (b) EMFTYInCNHIK, and (c) GPLEnYGFAKGPLAK.

**Figure 3.9.** He MAD spectra of douly-deprotonated S-nitrosylated: (a) EMFTYInCNHIK, (b) VFDARDnCRSAQ, and (c) GPLEnYGFAKGPLAK. The small inserts compare the actual and expected (vertical lines) isotopic distribution. The precursor is indicated by an arrow, neutral losses from the charge-reduced species are indicated by a dot and the neutral loss of modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
Figure 4.1. Comparison of He MAD and CID spectra of cholecystokinin and calcitonin in the 2- charge state: (a) MAD of non-phosphorylated IKNLQSLDPpSH, (b) MAD of phosphorylated IKNLQSLDPpSH, (c) CID of phosphorylated IKNLQSLDPpSH, (d) MAD of non-phosphorylated DFNKFHpTFPQTAIGV, (e) MAD of phosphorylated DFNKFHpTFPQTAIGV, and (f) CID of phosphorylated DFNKFHpTFPQTAIGV. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

Figure 4.2. Comparison of He MAD and CID spectra of different phosphorylated peptides in the 3- charge state: (a) MAD and (b) CID of phosphorylated cholecystokinin (IKNLQSLDPpSH), (c) MAD and (d) CID of phosphorylated calcitonin (DFNKFHpTFPQTAIGV), (e) MAD and (f) CID of phosphorylated angiotensin II (DRVpYIHPF). An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

Figure 4.3. Venn diagrams comparing the number of fragmentation ion types produced after He MAD and CID of the phosphorylated peptides: (a) [IKNLQSLDPpSH -3H]3−, (b) [DFNKFHpTFPQTAIGV -3H]3−, and (c) [DRVpYIHPF -3H]3−.

Figure 4.4. Venn diagrams comparing the number of fragment ion types produced after He MAD of the 2- and 3- phosphorylated peptides: (a) cholecystokinin (IKNLQSLDPpSH), (b) calcitonin (DFNKFHpTFPQTAIGV), and (c) angiotensin II (DRVpYIHPF).

Figure 4.5. Venn diagrams comparing the number of fragment ion types produced after He MAD of the 2+ and 2- phosphorylated peptides: (a) cholecystokinin (IKNLQSLDPpSH), (b) calcitonin (DFNKFHpTFPQTAIGV), and (c) angiotensin II (DRVpYIHPF).

Figure 4.6. The fragment ions produced by He MAD on the following doubly-protonated phosphorylated peptides: (a) cholecystokinin, (b) calcitonin, and (c) angiotensin II.

Figure 4.7. Tandem mass spectra comparing He MAD and CID of sulfonated cholecystokinin and hirudin in the 2- charge state: (a) MAD of non-sulfonated DYMGWMD, (b) MAD of sulfonated DsYMGWMD, (c) CID of sulfonated DsYMGWMD, (d) MAD of non-sulfonated DFEIEPEEYLQ, (e) MAD of sulfonated DFEIEPEEsYLQ, and (f) CID of sulfonated DFEIEPEEsYLQ. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
Figure 4.8. Tandem mass spectra comparing He MAD and CID of sulfonated cholecystokinin and hirudin in the 3- charge state: (a) MAD of sulfonated DsYMGWMDF, (b) CID of sulfonated DsYMGWMDF, (c) MAD of sulfonated DFEEPEEsYLQ, and (d) CID of sulfonated DFEEPEEsYLQ. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. ............................... 144

Figure 4.9. He MAD spectra of sulfonated angiotensin II and leucine enkephalin in the 1+ charge state: (a) DsYMGWMDF and (b) sYGGFL. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. .................................................. 146

Figure 5.1. Comparison of the dissociation of the di-phosphorylated Tau peptide P1a SRpTPpSLPTPPTREPK (Tau210-224) generated of the 2+ charge state through (a) CID, (b) ETD, (c) MAD, and of the 3+ charge state through (d) CID, (e) ETD, and (f) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (○) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts. ........................................................................... 146

Figure 5.2. Venn diagrams comparing the number of product ions types produced for CID, ETD, and MAD of both the 2+ and 3+ charge states of the di-phosphorylated Tau peptides P2b VAVVRpTPPpSPSSAK (Tau226-240) and P3a RSGYSpSPGSPGpTPGSRSR (Tau194-210). ........................................................................... 147

Figure 5.3. Comparison of the dissociation of the 3+ charge state of the mono-phosphorylated Tau peptide P2a VAVVRTPPKpSPSSAK (Tau226-240) through (a) CID, (b) ETD, (c) MAD, and of the di-phosphorylated Tau peptide P2b VAVVRpTPPpSPSSAK (Tau226-240) through (d) CID, (e) ETD, (f) and MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (○) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts. ........................................................................... 148

Figure 5.4. Comparison of He MAD and CID of phosphorylated cholecystokinin in the 3- charge state. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. ........................................................................... 149

Figure 5.5. Comparison of the dissociation of the 3+ charge state of the di- phosphorylated Tau peptide P3b RSGYSSPGpSPGpTPGSRSR (Tau194-210) through (a) CID, (b) ETD, (c) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to
98 Da (\(^\ddagger\)) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 5.6.** Comparison of the dissociation of the 3+ charge state of the monophosphorylated Tau peptide P5 TDHGAIEIVKSPVSVGDTSPR (Tau386-406) through (a) CID, (b) ETD, (c) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (\(^\ddagger\)), phosphorylation modification corresponding to 98 Da (\(^\ddagger\)) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

**Figure 5.7.** Charts plotting the total frequency of occurrence of the product ion types for CID, ETD, and MAD during this study.

**Figure 5.8.** Venn diagrams comparing the number of product ions types produced for CID, ETD, and MAD of 3+ charge states of the three Tau peptides (a) p2a VAVVRTPPKSPpSSAK (Tau226-240), (b) P3b RSGYSSPGpSPGpTPGSRSR (Tau194-210), and (c) P5 TDHGAIEIVKSPVSVGDTSPR (Tau386-406).

**Figure 6.1.** Comparison of He MAD and CID of the [M+H]\(^+\) charged species of sphingomyelin. The precursor is indicated by an arrow.

**Figure 6.2.** Comparison of He MAD and CID of the [M+Na]\(^+\) charged species of sphingomyelin. The precursor is indicated by an arrow.

**Figure 6.3.** Comparison of He MAD and CID of the [M+H]\(^+\) charged phosphatidylcholine (16:0/18:1) species. The precursor is indicated by an arrow.

**Figure 6.4.** Comparison of He MAD and CID of the [M+Na]\(^+\) charged phosphatidylcholine (16:0/18:1) species. The precursor is indicated by an arrow and the neutral loss of the sodium is indicated by an asterisk.

**Figure 6.5.** 3-D model of protonated phosphatidylcholine. The ester-trimethylammonium interaction is 1.7 kcal/mol over the (b) ester-phosphate interaction. Calculations performed by Dr. E. Masson using the B3LYP density functional method and 6-31 G (d) basis set (fatty acid chains were shortened for the calculations).

**Figure 6.6.** 3-D model of sodiated phosphatidylcholine. The ester-Na\(^+\)-phosphate interaction (a) is 11 kcal/mol over the (b) ester-trimethylammonium interaction. Calculations performed by Dr. E. Masson using the B3LYP density functional method and 6-31 G (d) basis set (fatty acid chains were shortened for the calculations).
LIST OF SCHEMES

Scheme 1.1. Fragmentation pathway for the formation of b-/y-type ions for a doubly-charged peptide through CID. ........................................................................................................................................ 37

Scheme 1.2. Formation of c-/z-type ions through ECD by the cleavage of N–Cα bonds: A radical c-ion and z+H ion is observed when the radical is directed towards the N-terminus and a radical z-ion and c+H ion is observed when the radical is directed towards the C-terminus. ........................................................................................................ 41

Scheme 1.3. The schematic illustrates the formation of c-/z-type ions through the Cornell mechanism which involves a radical hydrogen atom transfer from protonated sites to amide oxygen atoms to form an aminoketyl radical. ........................................................................ 42

Scheme 1.4. The schematic illustrates the formation of c-/z-type ions through the Washington-Utah mechanism where initial electron capture occurs in amide π* orbitals generating a superbase which can abstract a proton from a protonated site and induce N–Cα backbone cleavage. ........................................................................................................ 43

Scheme 2.1. Possible outcome from Penning ionization (PI) of dialanine [AA+H]+ ...... 84

Scheme 2.2. Alternative outcome from Penning ionization (PI) of dialanine [AA+H]+. 85

Scheme 6.1. Possible outcome from Penning ionization (PI) on the first oxygen atom directly connected and two carbon atoms away from the trimethylamine portion of the head group of both the sodiated PC and SM lipids. Dissociation generates cleavage between the nitrogen and carbon atoms. ..................................................................................... 186
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Ar</td>
<td>Argon</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>ASMS</td>
<td>American Society for Mass Spectrometry</td>
</tr>
<tr>
<td>BIRD</td>
<td>Blackbody infrared radiative dissociation</td>
</tr>
<tr>
<td>CAD</td>
<td>Collision activated dissociation</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>dc</td>
<td>Direct current</td>
</tr>
<tr>
<td>DCID</td>
<td>Dynamic collision induced dissociation</td>
</tr>
<tr>
<td>EA</td>
<td>Electron affinity</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron capture dissociation</td>
</tr>
<tr>
<td>EDD</td>
<td>Electron detachment dissociation</td>
</tr>
<tr>
<td>EED</td>
<td>Electron excitation dissociation</td>
</tr>
<tr>
<td>EID</td>
<td>Electron induced dissociation</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ETcaD</td>
<td>Electron transfer dissociation with supplemental activation</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron transfer dissociation</td>
</tr>
<tr>
<td>ETnoD</td>
<td>Electron transfer without dissociation</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast atom bombardment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform-ion cyclotron resonance</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>HASTE</td>
<td>High amplitude short time excitation</td>
</tr>
<tr>
<td>He</td>
<td>Helium</td>
</tr>
<tr>
<td>HECD</td>
<td>Hot electron capture dissociation</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IRMPD</td>
<td>Infrared multiphoton dissociation</td>
</tr>
<tr>
<td>IT</td>
<td>Ion trap</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tag for relative and absolute quantitation</td>
</tr>
<tr>
<td>LMCO</td>
<td>Low mass cut off</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MAD</td>
<td>Metastable atom-activated dissociation</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>MIDI</td>
<td>Metastable induced dissociation of ions</td>
</tr>
<tr>
<td>MIES</td>
<td>Metastable induced electron spectroscopy</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS^n</td>
<td>Multi-stage mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>Ne</td>
<td>Neon</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>niECD</td>
<td>Negative ion electron capture dissociation</td>
</tr>
<tr>
<td>NECD</td>
<td>Negative electron transfer dissociation</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>PAPS</td>
<td>Phosphoadenosine phosphosulfate</td>
</tr>
<tr>
<td>PEEK</td>
<td>Polyether ether ketone</td>
</tr>
<tr>
<td>PI</td>
<td>Penning ionization</td>
</tr>
<tr>
<td>PIES</td>
<td>Penning ionization electron spectroscopy</td>
</tr>
<tr>
<td>PQD</td>
<td>Pulsed Q dissociation</td>
</tr>
<tr>
<td>PqDCID</td>
<td>Pulsed q dynamic collision induced dissociation</td>
</tr>
<tr>
<td>PSD</td>
<td>Post source decay</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>QIT</td>
<td>Quadrupole ion trap</td>
</tr>
<tr>
<td>QTOF</td>
<td>Quadrupole time of flight</td>
</tr>
<tr>
<td>ReTOF</td>
<td>Reflectron time of flight</td>
</tr>
<tr>
<td>rf</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>SID</td>
<td>Surface-induced dissociation</td>
</tr>
<tr>
<td>Th</td>
<td>Thomson, unit of mass/charge ratio in mass spectrometry</td>
</tr>
<tr>
<td>TMN</td>
<td>TrimethylNitrate</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>TOF/TOF</td>
<td>Tandem time of flight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>TTL</td>
<td>Transistor-transistor logic</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>Xle</td>
<td>Leucine and isoleucine</td>
</tr>
</tbody>
</table>
1.1 Goals

The main focus of this dissertation is the development of a novel radical-induced dissociation method in a quadrupole ion trap mass spectrometer (QIT). The overarching goal of this study was to obtain the fundamental understanding of metastable atom-activated dissociation (MAD) and to establish its capabilities for characterizing peptidic and non-peptidic species. Current electron transferring dissociation methods, such as electron capture and electron transfer dissociation (ECD/ETD), fail to produce information where we propose MAD can deliver.

In addition to the dissociation of multiply-charged cationic peptides, we plan to extend MAD to singly-charged cations. Due to MAD’s unique mechanism of Penning ionization (PI), dissociation can occur throughout the peptide backbone following ionization at the lone pairs of electrons on the carbonyl oxygen and amide nitrogen atoms. For cations, PI is not dependent on charge state or location of charge and therefore produces similar ions for 1+ or 1- precursor ions. This is in stark contrast to both ECD and ETD which rely on a positive charge to initiate the radical transfer process. Additionally, MAD will be extended to multiply-charged anionic peptides.

Collision induced dissociation (CID), the most widely used dissociation method, notably undergoes neutral losses of labile post-translational modifications (PTMs). We propose that MAD is a nonergodic dissociation method similar in nature to ECD and ETD. Therefore the excess energy after the electron transferring process would be directed to the dissociation of the bonds in the direct vicinity of the site of ‘impact’. This
nonergodic energy transfer will allow for the retention of highly labile PTMs such as nitrosylation.

CID, ECD, and ETD in many instances must rely on multiple MS/MS stages or an additional dissociation method to generate complete sequencing and modification information. Therefore a major goal with MAD is to achieve complete sequencing and modification information within one MS/MS stage.

1.2 Project Overview

To start the project, the primary objective was to modify a FAB gun, our metastable atom source, and an existing QIT mass spectrometer to allow a direct line-of-sight passage for the metastable atoms to the center of the QIT. To achieve MAD, a beam of metastable atoms was generated during the traditional collisional activation period through the aid of a function/arbitrary waveform generator and power amplifier. Complete instrument modification and pulse production details are in sections 1.5.4 and 2.2.1.

Our next step was to induce dissociation of isolated precursor peptide ions through MAD. Doubly- and singly-charged peptides were successfully dissociated providing extensive sequencing information. One main advantage of MAD is that other radical type dissociation methods are ineffective on singly-charged ions. A comparison of the degree and type of fragment ions produced when employing helium (He) versus argon (Ar) metastable atoms was also conducted. Additionally, isobaric amino acids such as leucine and isoleucine can undergo different neutral losses of their respective side chains. Several peptides containing leucine and isoleucine were studied to determine if
MAD had the ability to distinguish between these isobaric amino acids. Through these studies it was observed that MAD could also cleave two covalent bonds and produce z-ions on the N-terminal side of proline. All of these studies are explained in detail in Chapter 2.

Our objectives progressed to the study of post-translationally modified peptides, specifically nitrated and nitrosylated. The nitration modification tends to be a more stable modification, however the extremely labile nature of the nitrosylation modification proves it hard to analyze. Recent research has also shown that the NO₂ modification has deleterious effects on ECD and ETD, resulting in little dissociation of doubly-charged peptides. Several different singly-, doubly-, and triply-charged peptides in their non-modified and modified forms were compared to assess the effect of the modification and charge states on the backbone cleavage observed. Comparison of MAD and CID were also conducted. The nitration and nitrosylation modifications present were also observed in the negative ion mode. All of these studies are explained in detail in Chapter 3.

To date, little study has been devoted to the analysis of post-translational modified peptide anions, which became our next objective. Phosphorylation and sulfation modifications are by nature negatively charged and give rise to superior signal in negative ion mode. We studied several phosphorylated peptide anions in the 2- and 3-charge state through MAD to evaluate the effect of the modification and charge state on the observed backbone cleavage. Additionally, the same peptides were studies using CID to compare these methods. Several sulfonated peptide anions in the 2- and 3-charge states were evaluated based on degree of dissociation and type of ions produce through
MAD in comparison to CID. The study also extended to singly-charged sulfonated peptides which tend to be highly acidic and ineffectively dissociated by ECD and ETD. All of these studies are explained in detail in Chapter 4.

In order to have a direct comparison of MAD with another radical induced type dissociation method we collaborated with Dr. Hoffman at Leipzig University. A total of eleven phosphorylated tau peptides were dissociated through CID, ETD, and MAD. Sequence coverage percentages, fragment ion type frequencies, and the ability to unambiguously determine PTM site location were all studied and compared. These studies are summarized in detail in Chapter 5.

The last objective was to extend MAD to non-peptidic species, such as singly-charged lipids. The study investigated MADs ability to provide primary structural characterization of lipids. The conformation sensitivity and resulting dissociation of sodiated versus protonated adducts was investigated and compared. Additionally, the results obtained with MAD were compared with CID results. Conformational sensitivity is indicated when comparing the MAD fragmentation behavior of the sodiated versus protonated lipids. These studies are summarized in detail in Chapter 6.

1.3 Quadrupole Ion Trap Mass Spectrometry

The QIT was first introduced by Paul and Steinwedel in 1953, which lead to the their receipt of the Nobel Prize in Physics in 1989 [1]. Linear ion traps, miniature cylindrical ion traps, and toroidal ion traps have all built upon improvements of the QIT instrument design. QIT mass spectrometers have become a versatile tool for biomolecular
analysis. Ease of use, high sensitivity, low cost, and multi-stage tandem mass spectrometric capabilities have contributed to the rise of QIT utilization worldwide.

1.3.1 Instrumentation

The QIT separates ions based on their mass to charge ratio \((m/z)\) and their stability within the trap. The three dimensional trap consists of three electrodes, a central ‘donut’ shaped ring electrode and two adjacent end caps, all with hyperbolic cross sections. The entrance end cap contains a small hole which allows ions to enter the QIT, whereas the exit end cap contains multiple openings to allow ions to be ejected to the detector. Figure 1.1 illustrates the geometry of the QIT cut in half showing the dimensions \(r_0\).

*Figure 1.1. Schematic of the QIT illustrating the hyperbolic cross section and the dimensions \(r_0\) and \(z_0\). (Reprinted in part from March, R. E.; Todd, J. F. J. Quadrupole Ion Trap Mass Spectrometry. 2nd ed.; New Jersey, Wiley-Interscience, 2005.)*
which is the radius of the ring electrode, and \( z_0 \), which is the distance from the center of the ring electrode to the end cap electrode. The QIT geometry needs to satisfy a relationship of

\[
\frac{r_0^2}{z_0^2} = 2
\]

(1)
to successfully manipulate the ions motion in the r- and z-directions and produce an ideal trapping field [1]. Ions maintain a secular frequency in both the r-(radial) and z- (axial) direction. The entire trap typically is the size of a tennis ball allowing QITs to be relatively cost efficient in comparison to other mass spectrometers.

1.3.2 Operation

The quadrupole field is produced by applying an rf potential between the two grounded endcap electrodes and the ring electrode. The basic principle of the QIT is creating stable and unstable trajectories for wanted and unwanted ions of a specific \( m/z \), respectively, through the manipulation of the quadrupole field. With the invention of soft ionization techniques, electrospray ionization (ESI) [2, 3] and matrix assisted laser desorption ionization (MALDI) [4], the ions are typically generated outside of the trap and guided through the entrance endcap to the center of the trap [5].

1.3.2.1 Ion Trapping and Ion Stability

Ions are successfully trapped and stored when their trajectories in the r- and z-directions are stable. The fundamental rf applied to the ring electrode typically is set to a precise frequency of about 1 MHz while the amplitude varies to manipulate the ions stability [1]. Qualitatively, this stability can be described by the Mathieu equations

\[
a_z = \frac{-16zU}{m(r_0^2 + 2z_0^2)\Omega^2}
\]

(2)
\[ q_z = \frac{8zV}{m(r_0^2 + 2z_0^2)\Omega^2} \]  

(3)

where \( a_z \) and \( q_z \) are the stability coordinates in the z-direction, \( m \) and \( z \) are the mass and charge of the ion, respectively, at a given dc potential (U) and ac potential (V) with an angular rf frequency (\( \Omega \)) [1]. However, as stated earlier, the endcaps are usually electrically grounded and only an rf potential on the ring electrode is applied. Therefore, the only parameter needed to determine stability is \( q_z \). Trapped ions are also characterized by their secular frequencies in the axial direction and their stability within the trap and is defined by the equation

\[ \beta_z = \sqrt{(a_z + \frac{1}{2}q_z^2)} \]  

(4)

where \( q_z \) must fall between a \( \beta_z \) value of 0 and 1 to be trapped [1]. The overlapping region of radial and axial stability is plotted in the Mathieu stability diagram in Figure 1.2.

To successfully trap ions, several conditions must be taken into account. These conditions include the density of the stored ions and the kinetic energy required to trap the ions. These conditions can be taken into account with the Dehmelt pseudopotential well model which describes the strength at which ions are stored. When \( q_z < 0.4 \), ions are trapped within a parabolic potential well [1]. The well depth is determined by the equation

\[ D_z = \frac{mq_z^2\Omega^2z_0^2}{16e} \]  

(5)

where larger \( q_z \) values are trapped in a deep pseudopotential well and require more kinetic energy to be ejected [6].
Once ions are gated into the QIT and trapped inside the quadrupole field their varying oscillation frequencies will produce a large ion cloud that will inherently produce broader peaks in mass spectra [1, 7]. Interactions with an inert bath gas to reduce the kinetic energy of the stored ions thereby focuses the ion cloud to the center of the trap which improves mass resolution and is known as collisional cooling. Additionally, QITs have a size constraint therefore the ion accumulation period must be optimized to allow for maximum signal while minimizing space-charge effects. Coulombic repulsion of too
many ions in the trap leads to a distortion within the quadrupole field and can appear as mass shifts and broad peaks in the mass spectra [1].

1.3.2.2  *Resonant Excitation*

Resonance conditions occur when the applied supplemental ac potential on the endcaps are matched to the secular frequency of the ions motion in the z direction. This process of resonance excitation allows selected ions to accumulate additional kinetic energy, which can be used to achieve resonant ejection or resonant excitation (collision-induced dissociation), depending on the amplitude of excitation.

To collect a mass spectrum, ions have to be scanned out of the ion trap in a mass-selective manner. Mass-selective instability scanning is accomplished by linearly ramping the rf amplitude applied on the ring electrode to increase the secular frequencies of the trapped ions. When ions of different \( m/z \) reach a \( q_z \) value of 0.908, corresponding to the boundry \( \beta_z = 1 \), they become unstable and are subsequently ejected from the trap and detected using an electron multiplier [1]. Resonant ejection, or axial modulation, is carried out by applying a high amplitude supplemental ac potential on the endcaps in conjunction with the rf amplitude on the ring electrode. The supplemental potential is applied at an rf frequency at a pre-selected \( q_z/\beta_z \) value which is slightly lower than the desired ejection \( q_z/\beta_z \) value [1]. This supplemental potential increases the ions kinetic energy and as the ions secular frequencies are swept in the z direction from a low to high \( m/z \) value they are resonantly ejected when they exceed the stability boundry \( (q_z > 0.908 \text{ and } \beta_z > 1) \). This will allow the ions secular frequencies to come into resonance with the applied supplemental frequency resulting in a tighter ion packet at ejection and enhanced ejection efficiencies producing sharper mass spectral peaks [1, 8].
Applying a low amplitude supplemental ac potential on the endcaps, which increases the kinetic energy of the trapped ions enabling fragmentation instead of inducing ejection, is another form of resonance excitation [1, 8]. Ions are typically generated externally and are guided to the QIT where they are trapped within the quadrupole field. A precursor ion with a specific m/z is isolated and all other ions are ejected through mass-selective instability or resonance ejection. The isolated precursor ions kinetic energies are dampened and the ion cloud is focused to the center of the trap. The applied supplemental potential increases the isolated precursor ions secular frequencies and kinetic energies thereby inducing contact with the bath gas. Inelastic collisions with the bath gas allow the ions to convert kinetic energy to internal energy, and thereby build enough internal energy to overcome the threshold for fragmentation. This process of resonant excitation is a form of tandem mass analysis known as collision induced dissociation (CID).

1.4 Tandem Mass Spectrometry for Peptides and Proteins

Tandem mass spectrometry (MS/MS), a form of resonance excitation, has given the field of proteomics a valuable tool to sequence and characterize peptides and proteins as well as a variety of other non-peptidic species. Determining structural change can give vital information concerning the discovery and detection of biomarkers, the prognosis of disease, and pharmacological responses. QITs are tandem in time instruments providing multi-stage MS (MS^n) capabilities which allows for the “re-fragmentation” of product ions [9]. This capability can provide additional structural information, especially when simple neutral losses are dominant in the original MS/MS spectrum.
Common nomenclature for peptide fragmentation was proposed by Roepstorff and Fohlman in 1984 and based on the letters of the alphabet [10]. When peptide dissociation occurs and the charge is retained on the N-terminus products, a-, b-, and c-ions are observed. Alternatively x-, y-, and z-ions are observed when the charge is retained on the C-terminus, as indicated in Figure 1.3.a. The \( C_\alpha-C \) backbone bonds are cleaved to produce a-/x-ions, C–N bonds to produce b-/y-ions, and N–C\(_\alpha\) bonds for the c-/z-ions. Johnson et al. proposed additional nomenclature to include the cleavage between the beta and the gamma carbons on the amino acid side chains [11]. Figure 1.3.b shows that the d-ion originates from a neutral loss of the side chain from the a-ion, the v-ion from a y-ion, and the w-ion from a z-ion.

![Figure 1.3](image)

*Figure 1.3. Nomenclature for peptide sequence ion observed in tandem mass spectrometry: (a) a-, b-, c-, x-, y-, and z-ions and (b) d-, v-, and w- ions.*

1.4.1 Collision Induced Dissociation

The most commonly employed MS/MS technique within mass spectrometers is CID. CID is considered a “slow-heating” and low-energy dissociation method when
employed within rf trapping instruments [12]. Dissociation occurs through multiple (hundreds or thousands) of low-energy collisions, as described previously, where the conversion of kinetic to internal energy is continually redistributed throughout the entire peptide as vibrational energy. When the internal energy exceeds the necessary energy to break the peptidic covalent bonds, dissociation occurs. Low-energy CID leads to dissociation or fragmentation of the weaker C–N peptide backbone bonds and produces b-/y-type ions, as illustrated in Scheme 1.1. [13]. Other low energy pathways, such as neutral losses of water, ammonia, carbon dioxide, and labile PTMs are also in competition with the C–N backbone cleavages. All of these pathways of dissociation can lead to complex spectra, which makes interpretation difficult. Database search algorithm scores are usually lower (i.e. worse) for complicated spectra. Also, complete sequencing information of whole proteins is rarely obtained.

An important aspect of CID is the mobile proton model (Figure 1.4) [14]. When ions are generated via ESI, protonation occurs at basic amino acids such as arginine (Arg), lysine (Lys), histidine (His), and at the N-terminus. When a peptide is multiply protonated the high proton affinity of the basic amino acids will preferentially sequester protons first. Any additional protons residing at the N-terminus can propagate along the peptide backbone, either to carbonyl oxygen or amide nitrogen atoms. During the slow collisional heating process this process induces fragmentation. This model has been able to aid the understanding and help to predict how CID will favor certain pathways based
Scheme 1.1. Fragmentation pathway for the formation of b-/y-type ions for a doubly-charged peptide through CID. (Reproduced from Kinter, M.; Sherman, N. E. Protein Sequencing and Identification Using Tandem Mass Spectrometry; New York, Wiley-Interscience, 2000.)
Figure 1.4. Illustration of the mobile proton model, with hydrogen propagation to the thermodynamically less stable amide nitrogens.

on peptide sequence. Preferential cleavage adjacent to acidic amino acids has been attributed to mobile protons originating from the acidic amino acid or from acid-base interactions [15]. Precursor ion charge states has also been shown to influence fragmentation behavior such as increased cleavage on the N-terminal side of proline and the C-terminal side of aspartic acid [16, 17, 18, 19]. Regardless, high amounts of preferred dissociation pathways at specific amino acids can also result in poor algorithms scores for CID.

An inherent disadvantage of CID within QITs is the tradeoff between CID efficiency and the low mass cutoff (LMCO). At smaller $q_z$ values (<0.25) the trapping pseudopotential well depth is shallow and isolated ions must be resonantly excited with lower voltages to avoid unwanted ejection. However, these low voltages may not produce ample internal energy to induce dissociation. At larger $q_z$ values, more low-mass product ions fall outside the stability boundary and reduce the collection efficiency. Typically, CID is performed around a $q_z$ of 0.25 which leads to the common “1/3 rule” of ion
stability [20]. Fragment ions with \( m/z \) values smaller than \( \sim 1/3 \) the \( m/z \) of the precursor ion will fall outside the stability boundary and will be lost. These low mass ions are usually useful and necessary to aid in the determination of specific amino acids and PTMs [21, 22]. Furthermore, low mass quantitative reporter ions commonly used in iTRAQ [23] between the \( m/z \) of 114-117 are also readily lost when fragmented in this conventional manner. Therefore, valuable information obtainable through low mass ions are difficult to utilize without the use of software modified methods such as HASTE [24], PQD [25], and PqDCID [26].

1.4.2 Ion/Ion Reactions

With the invention of ESI, which has made the generation of multiply charged ions readily available, a new area of study has emerged. For well over a decade McLuckey and co-workers have been studying ion/ion reactions to investigate the behavior and the reaction mechanisms of multiply-charged ions with oppositely charged ions. Reactions involve interactions between multiply-charged cations with singly-charged anions (the latter of which is the most common reaction studied to date) or multiply-charged anions with multiply- or singly-charged cations. Proton transfers from the multiply-charged cations to the anions, or anions to cations, are the most dominant reactions resulting in a charge reduction of the precursor ions as seen in Eq. 5.

\[
(M + nH)^{n+} + Y^- \rightarrow (M + (n - 1)H)^{(n-1)+} + HY
\]

(5)

With few exceptions, little to no fragmentation is observed from proton transfer reactions, regardless of the high exothermicities associated with reactions of oppositely charged ions [27]. One reason for lack of fragmentation is thought to be due to collisional cooling,
i.e. the removal of kinetic energy from the product ions through collisions with He bath gas atoms. [27]. Regardless, ion/ion reactions remain an efficient way to reduce precursor charge state thereby reducing the complexity of MS spectra. Additionally, ion/ion reactions within a QIT facilitate the ability to perform “ion parking” or selectively inhibit a specific m/z from undergoing ion/ion reactions [28]. By applying a supplemental frequency to accelerate ions of a specific m/z ratio, the analyte signal that was originally distributed over many charge states can be focused into one charge state.

Unlike simple charge reduction, extensive fragmentation of relatively small deprotonated anions has been observed when exposed to rare gas cations through an ion/ion electron transferring type reaction as written below [27, 29]:

\[(M - nH)^{n-} + Y^{+} \rightarrow (M - nH)^{(n-1)-} + Y \rightarrow \text{fragments}\]  

(6)

The observed fragmentation is attributed to the small ion size of the anion and to the high reaction exothermicity. McLuckey and co-workers weren’t able to successfully demonstrate electron transfer reactions of peptides cations until after Hunt and co-workers implemented ETD in 2004 [30]. A detailed discussion on ETD of peptide cations is included in section 1.4.4.

### 1.4.3 Electron Capture Dissociation

In 1998 McLafferty and co-workers developed a new fragmentation technique, ECD, which induces radical chemistry through the interaction of low-energy electrons with polycations [31]. Since its conception, ECD has become a leader in peptide and protein sequencing and characterization, especially in top-down sequencing approaches and PTM identification and localization [32]. However, ECD is not applicable to
negatively-charged ions and singly-charged cations. The electron capturing process generates ≤7 eV of energy which can be directed to fragmentation [33]. Multiply-protonated peptides and proteins capture low-energy (<eV) electrons leading to a charge neutralization and N–Cα backbone cleavage resulting in either c’/-z’- or c’/-z’-type ions (’ denotes a hydrogen addition and a • denotes a radical), the first of which is most

Scheme 1.2. Formation of c’/-z’-type ions through ECD by the cleavage of N–Cα bonds: A radical c-ion and z+H ion is observed when the radical is directed towards the N-terminus and a radical z-ion and c+H ion is observed when the radical is directed towards the C-terminus.
dominant. The use of free electrons requires this technique to be employed within expensive Fourier transform ion cyclotron resonance (FT-ICR) instruments. Scheme 1.2 illustrates the fragmentation mechanism which leads to the c/z-type ions which is complementary to the b/y-type ions CID produces.

To date the initial electron capture and radical hydrogen transfer is not fully understood. Two different mechanisms have been proposed; the Cornell mechanism (Scheme 1.3) and the Utah-Washington mechanism (Scheme 1.4). The original mechanism described by McLafferty and co-workers, later became known as the Cornell mechanism, propose initial electron capture to form high Rydberg states with the protonation sites (i.e. protonated amines, guanidines, and imidazole rings) [31, 34, 35]. A radical hydrogen (H') is then ejected when the Rydberg radical drops to lower energy Rydberg states through internal conversions. If the H’ is ejected in the right direction and contains enough energy it can covalently bind with a carbonyl oxygen atom and form the

Scheme 1.3. The schematic illustrates the formation of c/z-type ions through the Cornell mechanism which involves a radical hydrogen atom transfer from protonated sites to amide oxygen atoms to form an aminoketyl radical.
Scheme 1.4. The schematic illustrates the formation of c-/z-type ions through the Washington-Utah mechanism where initial electron capture occurs in amide $\pi^*$ orbitals generating a superbase which can abstract a proton from a protonated site and induce N–Cα backbone cleavage.

C–OH radical or aminoketyl radical. This recapturing process must overcome an energy barrier of 40-68 kJ/mol [36]. The entire process, including N–Cα backbone cleavage, is believed to occur at rates faster than it would take for the energy to redistribute throughout the entire peptide or protein and is known as a non-ergodic process [31, 34, 35].

The Utah-Washington mechanism was proposed independently by Simons and co-workers [36, 37, 38] and Tureček and co-workers [39, 40], respectively, describing a one-step hydrogen migration versus a two-step elimination-recapture described in the Cornell mechanism. The initial electron capture is proposed to directly attach to amide $\pi^*$ orbitals. This creates a strong amide base, referred to as an amide superbase, with a high proton affinity which can effectively abstract an H· from a protonated site to form an aminoketyl radical, which is the identical end product proposed in the Cornell
mechanism, followed by N–Cα backbone cleavage. However, N–Cα backbone cleavage can be induced prior to hydrogen migration which produces an imine superbase as an intermediate (Scheme 1.4). This mechanism, regardless of pathway, explains how dissociation can be observed at distances greater than 20 Å away from the protonation site and H⁺ ejection, as noted by Marshall and co-workers [41]. More evidence shows that a combination of these two mechanisms leads to the observed results of ECD.

1.4.4 Electron Transfer Dissociation

In 2004, Hunt and co-workers introduced ETD, which utilizes a radical fluoranthene anion species as opposed to free electrons to induce ETD-like fragmentation [30] (Eqn 7).

\[
(M + nH)^{n+} + (C_{16}H_{10})^- \rightarrow (M + nH)^{(n-1)+} + C_{16}H_{10} \rightarrow \text{fragments}
\]  

(7)

However, unlike ECD, the ion/ion chemistry of ETD can be employed within cheaper rf trapping instruments [30, 42], quadrupole time of flight (QTOF) [43, 44], and hybrid linear ion trap-orbitrap mass spectrometers [45, 46]. An additional difference between ECD and ETD is that electron transfer from an anion to a polycation is a less exothermic process than ECD [36, 47]. The initial electron transfer and subsequent N–Cα backbone cleavage are the same mechanisms as proposed in ECD, the Cornell and Utah-Washington mechanisms (Schemes 1.2, 1.3, 1.4). Therefore ETD is not applicable to singly-charged cations or negatively-charged ions.

McLuckey and co-workers studied the characteristics of a range of radical reagent anion species to determine the likelihood of electron- versus proton-transfer [42]. Proton transfer is energetically less favorable to induce fragmentation in comparison to electron
transfer. It was deduced that competition between these two ion/ion reactions is dependent on the electron affinity and favorable Frank–Condon factors associated with the transition from anion to neutral of the reagent anion [42, 44]. Additionally, McLuckey and co-workers examined the degree of electron transfer that occurs with subsequent dissociation (ETD) and without (ETnoD) when employing different radical reagent anion. Radical azobenzene and fluoranthene proved to be the most efficient electron transfer reagent anion and they produced 48 and 37% ETD, respectively, when involved in ion/ion reaction with a triply-charged cation [42, 44]. Regardless, with ETD, larger proteins tend to undergo charge reduction reactions with little to no dissociation or ETnoD [48, 49].

### 1.4.5 Negative-Ion Mode Electron Transferring Methods

The fragmentation of negatively-charged ions is achieved using ion/electron and ion/ion reactions in similar manners to ECD and ETD, respectively. Electron detachment dissociation (EDD) bombards multiply-charged anions with electrons (>10 eV), to detach electrons from the negative sites (Eqn 8) producing dominate Cα–C backbone cleavages, a mechanism similar to positron capture [50].

\[
(M - nH)^{n-} + e_{\text{fast}}^- \rightarrow (M - nH)^{(n-1)-} + 2e^- \rightarrow \text{fragments} \tag{8}
\]

When the anion is ionized it creates a positive radical charge or “hole”. The “hole” is then mobilized toward the negative charges due to Coulombic attractions which can initiate covalent bond cleavage. Ionization typically occurs at amide N atoms through hydrogen radical abstraction that leads to α-/x-type ions [51]. EDD favors C-terminal ions, illustrating EDD as a complimentary method to ECD which typically favors the
production of N-terminal and c-type ions [51]. Conversely, the fragmentation efficiency for EDD varies between 2-20% depending on the mass spectrometer (QIT or FT-ICR) [51] and activation times can be as long as several seconds [52, 53].

ETD of peptide anions or negative electron transfer dissociation (NETD) was first implemented by McLuckey and co-workers on small oligonucleotides [29] as mentioned in sectioned 1.4.2. Hunt and co-workers utilized Xe⁺⁺ to abstract an electron from phosphorylated anions [54]. Similar to EDD, backbone dissociation occurs dominantly at Cα–C backbone bonds facilitated through hydrogen radical abstraction at amide N atoms located throughout the peptide backbone. Huzarska et al. employ a radical fluoranthene cation, (C_{16}H_{10})⁺⁺, as opposed to Xe⁺⁺ to reduce abundant neutral losses observed by Hunt and co-workers [54, 55]. Additionally, Huzarska et al. determined that proton transfer ion/ion reactions directly between the reagent ion and the isolated peptide ion only account for less than 20% of the total product ions observed [55]. However, proton transfer reactions were very prevalent when using the Xe⁺⁺ cation. They proposed that Xe⁺⁺ ionized with other molecules within the ion trap which then reacted with the isolated ions to transfer a proton.

1.5 Metastable Atom-Activated Dissociation

1.5.1 Background

When an electron in an atomic orbital of He is excited to another bound state, its spin can either be antiparallel to the ground electron (parahelium, S=0) or parallel (orthohelium, S=1). Figure 1.5 is the Grotrian diagram or the electron energy diagram for helium. As noted, the orthohelium excited energy states are lower in energy compared to
the parahelium states. Usually, the excited electrons can radiatively decay back to the ground state through the process of emission. However, no decay from the $2^1S_0$ and $2^3S_1$ energy levels is allowed because of quantum-mechanical transition rules: such forbidden transitions gives rise to long-lived or metastable states [56]. Therefore, a metastable atom contains one of its valence electron in an excited s-state which is forbidden to photo-relax to the ground state as seen in Figure 1.5 for helium. Table 1.1,

*Figure 1.5.* The Grotrian diagram for helium highlighting the $2^1S_0$ and $2^3S_1$ metastable states. (Reprinted in part from Alberty, R. A.; Silbey, R. J. Physical Chemistry. 2nd Ed.; New York, John Wiley & Sons, Inc., 1997.)

adapted from Siska [57], lists the characteristics of the noble gas metastable atoms. As noted, metastable atoms can exist for several seconds in the absence of collisions giving them amble time to travel through a substantial distance to interact with an isolated ion
cloud stored within a MS. The ionization potential, which is the amount of energy needed to remove an electron, varies between 2.68-4.95 eV depending on noble gas and energy state [57]. However, if an electron is excited to a higher excited state such as a Rydberg energy level, the ionization potential theoretically would be less [58]. In the gas phase, metastable atom (denoted by an *) collisions with neutral atoms or surfaces can lead to elastic (Eqn 9) and inelastic reactions (Eqns 10, 11) [59].

\[
M^* + B \rightarrow M^* + B \quad (9)
\]

\[
M^* + B \rightarrow M + B^+ + e^- \quad (10)
\]

\[
M^* + B \rightarrow M + B^{**} \quad (11)
\]

The latter two mechanisms occur when electronic excitation transfer from the metastable atom through collisions induces either ionization, Penning ionization, (Eqn 10) or an

Table 1.1.

*Characteristics of Metastable Atoms of Noble Gases.*

<table>
<thead>
<tr>
<th>Atom</th>
<th>State</th>
<th>Excitation Energy (eV)</th>
<th>Ionization Potential (eV)</th>
<th>Lifetime(^a) (s)</th>
<th>Polarizability (Å(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>He</td>
<td>(^2)S(_0)</td>
<td>20.62</td>
<td>3.97</td>
<td>\textasciitilde 0.02</td>
<td>&gt;9</td>
</tr>
<tr>
<td></td>
<td>(^2)S(_1)</td>
<td>19.82</td>
<td>4.77</td>
<td>&gt;7900</td>
<td>46.9</td>
</tr>
<tr>
<td>Ne</td>
<td>(^3)P(_0)</td>
<td>16.72</td>
<td>4.85</td>
<td>430</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(^3)P(_2)</td>
<td>16.62</td>
<td>4.95</td>
<td>24.4</td>
<td>27.8</td>
</tr>
<tr>
<td>Ar</td>
<td>(^3)P(_0)</td>
<td>11.72</td>
<td>4.04</td>
<td>44.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(^3)P(_2)</td>
<td>11.55</td>
<td>4.21</td>
<td>55.9</td>
<td>47.9</td>
</tr>
<tr>
<td>Kr</td>
<td>(^3)P(_0)</td>
<td>10.56</td>
<td>3.43</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(^3)P(_2)</td>
<td>9.92</td>
<td>4.08</td>
<td>85</td>
<td>50.7</td>
</tr>
<tr>
<td>Xe</td>
<td>(^3)P(_0)</td>
<td>9.45</td>
<td>2.68</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(^3)P(_2)</td>
<td>8.32</td>
<td>3.81</td>
<td>150</td>
<td>63.6</td>
</tr>
</tbody>
</table>


\(^b\)In the absence of collisions
electronically excited neutral or a Rydberg state (Eqn 11) [59]. The highly excited neutral, $B^{**}$, can then further undergo dissociation through electron emission. Penning ionization (Eqn 10) was first discovered by F. M. Penning in 1927 when he noticed a decrease in the breakdown voltage in rare gas cells when impurity gases were introduced [60]. He proposed that fast electrons could electronically excite the rare gas atoms but didn’t have enough energy to ionize them at the lower voltages which lead to Eqn 10. This reaction is allowed when the excitation energy of the metastable atom is greater than the ionization potential of the neutral ($B$), or in our case biomolecules [57, 59]. The difference in energy between the metastable atom state and the ionization potential of the biomolecule determines the exothermicity of the reaction. Any excess energy can be dispersed as kinetic energy, internal energy in the products, or carried away with the ejected electron.

1.5.2 MIES/PIES

Metastable induced electron spectroscopy (MIES) and Penning ionization electron spectroscopy (PIES) are two techniques that utilize metastable atoms to study the interactions and reaction dynamics of molecules and surfaces. Metastable atoms have weakly bound outer electrons which are at farther distances from the nucleus. Therefore at the molecular level metastable atoms behave as positive ions. Research shows that metastable atoms are attracted to regions of high electron density when they are in close proximity and exhibit reaction preference to groups with lone-pairs of electrons and electric dipoles [61, 62, 63, 64, 65, 66].
When the excess energy from Penning Ionization is carried as kinetic energy by the free electron, measurement of the kinetic energy can provide information about the molecular orbital from which an electron was abstracted. Ohno et al. used He*(2\(^3\)S) PIES to show that the sp hybridization of oxygen atoms within molecules containing C=O groups had high activity and were preferentially ionized when exposed to the metastable atoms [65]. Additional attractive interactions have been noted for C=S groups with weaker attraction for NH\(_2\) groups [62]. Recently, PIES has been utilized to study the interaction of He*(2\(^3\)S) with the amino acid glycine by Ohno and co-workers [66]. Nonbonding orbitals of N and O atoms (NH\(_2\) and C=O groups, respectively) produce intense bands demonstrating strong attractions between the electron density of the groups and the metastable atoms. The strongest interaction was noted to be between the N orbital of the NH\(_2\) group and the metastable atom. Therefore one can postulate that Penning ionization within peptides and proteins will most likely occur at the lone pairs of electrons on the carbonyl oxygen and nitrogen atoms along the peptide backbone and amino acid side chains.

1.5.3 Previous Research

Dissociating peptide multiply-charged ions using metastable atom noble gases was first implemented by Zubarev and co-workers in 2005, inducing N–C\(\alpha\) backbone cleavage and disulfide bonds for cations and C\(\alpha–C\) backbone cleavage for anions [58]. The authors referred to the process as metastable-induced dissociation of ions (MIDI) and proposed the mechanisms of charge reduction for cations, the same mechanism for
ECD/ETD (Eqn 12), and Penning ionization for anions, the same mechanism for EDD (Eqn 13).

\[
(M + nH)^{n+} + M^* \rightarrow (M + nH)^{(n-1)+} + M + e^- \quad (12)
\]

\[
(M - nH)^{n-} + M^* \rightarrow (M - nH)^{(n-1)-} + M + e^- \quad (13)
\]

In both of the proposed mechanisms the direct generation of a radical on the peptide backbone leads to the observed dissociation. He and Ar MIDI were explored on substance P showing that He metastable atoms produce more fragment ions and a less abundant charge reduced species than Ar metastable atoms. Additionally, He MIDI was successfully applied to multiply-charged cations and anions, phosphopeptides, and peptides containing disulfide bridges [58]. Even though modification retention was achieved, complete sequence coverage wasn’t always observed. Zubarev and co-workers utilized a Bruker Esquire QIT-MS and employed a FAB gun to generate metastable atoms, a similar set-up to ours.

Since the first publication, Berkout and co-workers as well as Jackson and co-workers have extended the applications of metastable atom induced dissociation of peptides in the last several years to include singly-charged cations which demonstrated Penning ionization of cations [67, 68]. Berkout and co-workers used a glow discharge to produce a low kinetic energy beam of metastable atoms which was placed over the last quadrupole region to introduce the metastable atom beam between the quadrupole rods to interact with ions as they passed through the linear trap-TOF-MS. In addition to He metastable atoms, Berkout also explored neon and krypton metastable atoms, which produced dominant a- and b-type ions after exposure to singly-charged angiotensin II [69]. Additionally, Berkout and co-workers demonstrated dissociation of polyanions,
phosphorylated peptides, and the ability to distinguish between leucine and isoleucine residues [67, 69].

1.5.4  Metastable Atom Production

To produce metastable atoms, a saddle field Ion Tech FAB gun is used, which is considered a cold cathode discharge device. A potential in the range of 6-10 kV is applied to the anode. This generates a gas discharge between the anode and grounded cathode with the noble gas that is flowing into the discharge chamber. This discharge can be sustained as long as the working pressure is within that of the operating pressure of the MS (~3 x 10⁻⁵). The electrostatic saddle potential field provides the electrons ample oscillatory paths to ionize gaseous atoms [58, 70]. Additionally, metastable atom species are formed when neutrals and ions within the discharge collide:

\[ \text{A}^+ \cdot \text{(fast)} + \text{A} \rightarrow \text{A}^+ \text{ (fast)} + \text{A}^+ \]  \hspace{1cm} (14)

Additionally, neutrals and ions can be excited through inelastic collisions with surfaces forming metastable atoms in the process. The beam of ions, electrons, and metastable atoms exit the FAB gun through a 1 mm orifice. A thorough description of the mass spectrometer instrument modification and metastable atom pulse production are detailed in Chapter 2, Section 2.2.1.
CHAPTER 2: METASTABLE ATOM-ACTIVATED DISSOCIATION MASS SPECTROMETRY (MAD-MS): LEUCINE/ISOLEUCINE DIFFERENTIATION AND RING CLEAVAGE OF PROLINE RESIDUES


2.1 Introduction

Recent advances in mass spectrometry have provided unprecedented investigative power for biomedical and clinical researchers. In tandem mass spectrometry, a mass-to-charge relationship is established between the product ions of a reaction and the precursor ions from which they originated and there are three fundamental processes by which precursor gas-phase ions can be made to fragment: 1) collisions with atoms, molecules, or surfaces [71, 72]; 2) photodissociation [73, 74, 75, 76]; and 3) dissociative-recombination with an oppositely charged species [29, 31]. Table 2.1, adapted from reference [77], summarizes these methods, including the fragmentation method promulgated in this work: metastable atom-activated dissociation (MAD).

Of these activation methods, collision-induced dissociation (CID), also called collision-activated dissociation (CAD), is the most widely used method [71, 77]. CID of peptides and small proteins has been extensively studied and the major fragmentation pathways are well known [13, 78, 79, 80]. CID in RF-trapping instruments is classified as a “slow-heating” excitation method [12]. The fragmentation process in these instruments is a result of multiple collisions of the ions with neutral gas atoms and peptide cleavage...
Table 2.1.


<table>
<thead>
<tr>
<th>Activation method</th>
<th>Energy range</th>
<th>Ion charge</th>
<th>Instruments</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSD</td>
<td>Low</td>
<td>+/-</td>
<td>ReTOF</td>
<td>Metastable decay caused by excess internal energy from ionization.</td>
</tr>
<tr>
<td>CID (CAD)</td>
<td>Low</td>
<td>+/-</td>
<td>IT, FT-ICR, QqX, TOF/TOF, sectors</td>
<td>Controlled-energy (1-100 eV&lt;sub&gt;lab&lt;/sub&gt;) collisions with inert gases. Same, but keV energies.</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>+/-</td>
<td>TOF/TOF, sectors</td>
<td></td>
</tr>
<tr>
<td>SID</td>
<td>Low</td>
<td>+/-</td>
<td>XqQ, IT, FT-ICR</td>
<td>1-100 eV&lt;sub&gt;lab&lt;/sub&gt; collisions between ions and a metal or SAM surface. Same, but at keV energies.</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>+/-</td>
<td>TOF/TOF, sectors</td>
<td></td>
</tr>
<tr>
<td>IRMPD</td>
<td>Low</td>
<td>+/-</td>
<td>IT, FT-ICR</td>
<td>IR laser slowly raises internal energy of ions above dissociation thresholds</td>
</tr>
<tr>
<td>BIRD</td>
<td>Low</td>
<td>+/-</td>
<td>IT, FT-ICR</td>
<td>Heated systems used as IR source to achieve similar consequences as IRMPD.</td>
</tr>
<tr>
<td>UV-laser</td>
<td>High</td>
<td>+/-</td>
<td>IT, FT-ICR</td>
<td>Absorption of UV photon leads to significant fragmentation of peptide backbone.</td>
</tr>
<tr>
<td>ETD</td>
<td>Low or High</td>
<td>&gt;2+</td>
<td>IT, FT-ICR</td>
<td>Positively charged precursor ions capture an electron from reagent anions; radical ion chemistry follows.</td>
</tr>
<tr>
<td>ECD</td>
<td>Low</td>
<td>&gt;2+</td>
<td>FT-ICR, QqX, IT</td>
<td>Capture of low energy electrons by positive ions; radical ion chemistry follows.</td>
</tr>
<tr>
<td>EDD EID</td>
<td>High</td>
<td>+</td>
<td>QIT, FT-ICR</td>
<td>Electron-ion fragmentation of anions or cations.</td>
</tr>
<tr>
<td>MAD/MIDI</td>
<td>Low or High</td>
<td>&gt;2+,+ , &lt;2-</td>
<td>IT</td>
<td>Metastable atoms used to add electrons to polycations or to remove electrons from singly-charged cations or polyanions. Radical ion chemistry follows electron ejection/transfer.</td>
</tr>
</tbody>
</table>

PSD = post-source decay; CID = collision-induced dissociation; CAD = collision-activated dissociation; SID = surface-induced dissociation; ECD = electron capture dissociation; IRMPD = infrared multiphoton dissociation; BIRD = blackbody infrared radiative dissociation; ETD = electron transfer dissociation; ECD = electron capture dissociation; EDD = electron detachment dissociation, EID = electron ionization dissociation; MAD = metastable atom-activated dissociation; MIDI = metastable induced dissociation of ions; ReTOF = reflectron time-of-flight; IT = linear or quadrupole ion trap; FT-ICR = fourier transform ion cyclotron resonance; QqX = mass selective quadrupole followed by rf-only multipole followed by any mass analyzer; TOF/TOF = tandem time of flight; XqQ = any mass selective device followed by an rf-only multipole followed by a mass selective quadrupole; SAM = self-assembled monolayer.
occurs mostly at the weakest bonds, forming mainly b-/y-type ions. The charge state of proteins undergoing CID also plays a significant yet unpredictable role in determining the fragmentation pathways [16, 81, 82]. Although whole proteins can often be identified using CID, complete sequence coverage is rarely obtained. Post-translational modifications such as phosphorylation are often not pinpointed in CID because they are often lost during fragmentation [83].

The capture of low kinetic energy electrons by multiply-charged peptide cations has been demonstrated to result in effective cleavage of the peptide backbone [31]. This approach, called electron capture dissociation (ECD), is the new preferred method of sequencing peptides in expensive FT-ICR instruments [31, 84] and, more recently, in an off-axis linear ion trap by Hitachi [85]. One advantage of ECD is the extensive fragmentation along the amide backbone [86] resulting in mainly c-/z-type ions which has been shown to generate more complete amino-acid sequence coverage of peptides than conventional, collisional activation. A drawback of ECD is that it sometimes favors certain fragmentation pathways such as on the C-terminal side of tryptophan [35, 87]. Hot ECD (HECD) has the ability to impart more kinetic energy into peptide fragmentation leading to more fragmentation than is achieved in conventional ECD and can sometimes differentiate between isoleucine and leucine [33, 88]. The major downsides to ECD are that it has only been developed on rather expensive instrument platforms, and that ECD is not applicable to singly-charged or negatively charged precursor ions. Recently, ECD, including HECD and EDD have been developed in the linear ion trap time-of-flight mass spectrometer, but the techniques are still in development [89, 90].
To overcome the limitation of trapping low-mass electrons in rf-operated ion traps, it has been demonstrated that certain radical anions can serve as effective vehicles for electron transfer reactions that result in fragmentation of the polycationic precursor ions [30, 91]. Electron transfer dissociation (ETD) has been proven to work very effectively for small to medium size polycationic peptides [92, 93]. Larger bio-ions tend to undergo simple charge reduction reactions instead of fragmentation when reacted with xenon cations or small organic anions [48, 94]. In 2005, the orbitrap mass analyzer was made commercially available by Thermo, rivaling the high resolution and high mass-accuracy of the FT-ICR instruments [95]. The orbitrap has recently been adapted with a linear ion-trap to achieve ETD, which results in c-/z-type fragmentation and preserves PTMs [46].

The ability to sequence anions as well as cations can be an important advantage when analyzing highly acidic or modified peptides. Electron-detachement dissociation (EDD) achieves peptide fragmentation through electronic excitation of anions from electron-anion collisions, leading to radical formation and inter-residue N–Cα and Cα–C bond cleavage [51]. Although a-/x-type ions are predominately detected, there are some fragments which have yet to be detected, such as d-type ions [50]. Electron excitation dissociation (EED) [33] and electron ionization dissociation (EID) [96] use a high-kinetic-energy beam of electrons to excite or ionize precursor peptide ions and induce fragmentation. EED fragmentation is similar to UV photodissociation and yields mainly a-type ions whereas EID achieves side chain and backbone fragmentation, mainly in the form of c-/z-type ions and a-/x-type ions [96].
Leucine and isoleucine differentiation has been thoroughly examined over the past few decades, by high and low energy CID [97, 98], fast atom bombardment (FAB) [99], HECD [88], and through the formation of metal complexes [100, 101]. FAB and low energy CID, which can be implemented on linear and quadruple trap instruments, requires MS\(^n\) of the low mass, labile immonium ion (86 \(\text{m/z}\)) of Xle (Leu or Ile) residues in order to differentiate the two amino acids [97, 98]. To date, only small peptides containing one Xle residue or those peptides with Xle as the N-terminal residue have been readily characterized. High energy CID and HECD are able to produce high energy fragments, such as d- and w-type ions, which can differentiate the Xle residue [88]. However, commercial access to these dissociation methods require expensive sector or FT-ICR instruments, respectively.

It is widely know that both ECD and ETD induces secondary fragmentation leading to side chain fragments and Xle differentiation, yet cleavage within the proline amino acid residue is rarely seen [102]. Proline is the only naturally occurring amino acid which forms a five-membered ring with its three carbon alkyl chain and the peptide backbone. Peptide backbone cleavage of the proline residue to produce a-/x- and b-/y-type ions occurs through a single covalent bond cleavage and y-ions are indeed readily observed with low-energy dissociation methods such as CID and IRMPD photodissociation [103, 104]. However, for the formation of c-/z-type ions, two covalent bonds must be broken, one among the peptide backbone and the other within the proline ring structure itself. To our knowledge, this type of side chain cleavage resulting in z- and w-type ions has been observed only once by Cooper et al. using ECD and HECD [102].
MAD now offers an alternative method for the generation of not only d- and w-type ions from the Xle residue, but z- and w-type ions from proline residues.

In 2005/6, several groups proposed the use of metastable atoms as a method for imparting energy to precursor ions to induce fragmentation [58, 67, 105]. Misharin et al. noted that the exposure of precursor peptide ions to the beam of a fast atom bombardment (FAB) source within a quadrupole ion trap lead to effective c-/z-type fragments of peptide cations and a-/x-type fragments of peptide anions [58]. For peptide cations, the mechanism proposed is electron transfer from fast moving metastable atoms to the polypeptide cations, resulting in ETD-like fragmentation. For peptide anions, the proposed mechanism is electron detachment caused by collisions with metastable atoms (e.g. Penning ionization). In both cases, the generation of a radical on the peptide backbone led to the observed fragmentation spectra. Berkout et al. [106] obtained similar results on a linear ion trap coupled to a time-of-flight mass spectrometer using a low kinetic beam of metastable atoms in the range of 0.05-0.1 eV. They showed that the number of c-/z-type products was superior to ETD reactions on FT-ICR instruments, especially for peptides in the 2+ charge state. More recently, Berkout and Doroshenko [67] confirmed our proposed mechanisms [107] for the Penning ionization of singly-protonated precursor peptide ions, and subsequent fragmentation. This most recent work demonstrates a unique capability for metastable atom-activation over ECD and ETD; the ability to fragment singly-charged precursor ions. In contrast to work by Berkout et al., our work is performed in a modified quadrupole ion trap mass spectrometer and with a high kinetic energy beam of metastable atoms in the range of 6-10 keV. The results agree with former observations and hypotheses and demonstrate that the efficiencies and data
acquisition rates are quite compatible with those required for on-line use with liquid-phase separations such as HPLC. We note a new observation for MAD-MS; the ability to distinguish between isoleucine and leucine and to generate w and z ions from proline residues.

2.2 Experimental

2.2.1 Instrumentation

All experiments were performed on a modified Bruker EsquireLC QIT MS (Bruker Daltonics, Bremen, Germany) as shown in Figure 2.1. The trap was modified in a similar manner to that reported elsewhere [58, 107]. A 2 mm hole was drilled in the ring

![Diagram](image-url)

*Figure 2.1. Schematic representation of the instrument used in this study. (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. *J. Mass Spectrom.* 2009, 44, 1211-1223.)*
electrode to permit metastable atoms to enter the trap. The rf-matching network did not require retuning after this modification, but the mass axis did require recalibration using a standard tune mix solution (Agilent, Santa Clara, CA). An Ion Tech (P50 PSU, Teddington, UK) FAB gun was used as the metastable atom source. The FAB gun was removed from its original housing, leaving the existing 1 MΩ resistor attached to the anode. A collar spacer was fabricated in house to attach the FAB gun to the vacuum chamber lid of the mass spectrometer. The exit orifice of the FAB gun was approximately 2 cm above the ring electrode. Two flat-ended 4-40 screws, used as deflection electrodes, were mounted in tapped holes in a PEEK (polyether ether ketone) sleeve, which was mounted with a set screw on the bottom of the FAB gun. The electrodes were positioned ~1 cm downstream of the exit orifice of the FAB gun, orthogonal to the FAB beam, with an inter-electrode spacing of ~5 mm. One electrode was grounded and the other was biased to ~800 V using a high voltage power supply (EL 03R 15L, Glassman High Voltage Inc., High Bridge, NJ).

Before use each day, the FAB gun was turned on for 20-30 minutes to ‘burn’ away residual contaminants such as pump oil. This helped decrease background ion signals. The Ion Tech power supply was modified slightly to permit operation at 110 V instead of the default 240 V. The presence of an active beam was measured at all times using the Ion Tech power supply through the E_mon connector. For pulsed operation, the anode power supply was replaced with a 10 kV high voltage amplifier (ANT 10B10, Matsusada Precision Inc., Shiga, Japan), as shown in Figure 2.2. The rise and fall time of this amplifier are specified as >360 V/ms, yet oscilloscope readings demonstrated considerably faster rates than this; the rise and fall times were greater than 7 kV/ms. A
trigger was taken from pin 15 of connector J522 of the Bruker EsquireLC and fed to a function/arbitrary waveform generator (33250A, Agilent, Santa Clara, CA) through 1MΩ termination. The function generator was used to generate either a single, 50-300 ms pulse or a burst of multiple 5 ms (50% duty cycle) pulses to the high voltage power amplifier. The delay time and pulse width of the FAB gun were continually compared with the timing of the scan functions of the ion trap exit end-cap. The FAB gun was timed to be on during the section of the scan function that is normally reserved for collisional activation. To accomplish metastable atom activation at any chosen low mass cut-off (LMCO) value, the fragmentation amplitude was set to zero volts after ion isolation and the ‘fragmentation’ or exposure time could be made as long as desirable (550 ms was the longest studied) at the desired LMCO value.

The FAB gun and auxiliary deflection electrodes were also characterized off-line by positioning the exit orifice of the FAB gun 3 cm above an electron multiplier (5900 Magnum, Burle, Sturbridge, MA) in a custom-modified vacuum chamber (Teledyne, Thousand Oaks, CA). Preliminary results showed that approximately 10-20% of the ion signal on the electron multiplier could be removed with a deflection voltage of 400 V/cm,
with no additional reduction in ion signal at higher deflection voltages (up to 3000 V/cm). This observation is consistent with theoretical calculations for the deflection of Ar ions from the beam, which show this electric field strength to be more than capable of deflecting 8 keV Ar ions to a non-transmitting angle. This study suggests that Ar ions make up a small proportion of the species capable of producing secondary electrons at the surface of the electron multiplier. We assume that the remaining 80-90% of the active species detected in this off-line study are either metastable or Rydberg states, as proposed by Misharin and coworkers [58]. The FAB gun and deflection plates were also characterized on-line with the ion trap with the electrospray ionization source (ESI) not in use. With a LMCO set at 18 m/z, many low mass ions could be generated from the neutral beam with a pulse as short as 2 ms. The ions observed in this experiment are formed as a result of Penning ionization of residual gases and pump oil within the trap. Low mass negative ions were also observed in negative ion mode with the FAB gun on. These ions could also be actively removed from the trap by employing a LMCO value above 150 m/z. The background signal was larger when using He as the excitation source rather than Ar, consistent with the fact that He metastable atoms have greater potential energy and can Penning ionize more substances. By raising the LMCO to 250 m/z, the background noise could be reduced to consistently low/negligible levels. The subsequent reduced intensity of the background peaks enabled the observation of product ions above the set LMCO. Turning the FAB gun on for prolonged periods before use significantly reduced the background signals.
2.2.2 Reagents

Bradykinin, substance P, and fibrinopeptide were purchased from Sigma-Aldrich (St. Louis, MO). The phosphorylated and non-phosphorylated standards of angiotensin II and cholecystokinin (10-20) were provided by Protea Biosciences Inc. (Morgantown, WV). The synthetic peptides, PHPRI and PHPRL, were donated from Dr. Ralf Hoffmann (University of Leipzig, Germany). Methanol (HPLC grade) and glacial acetic acid were also purchased from Sigma-Aldrich (St. Louis, MO). All peptides were reconstituted in a 1:1 mixture of methanol and water with 1% acetic acid to provide solutions between 10-25 μM of the individual peptides. Ultra high purity He and Ar (Airgas, Parkersburg, WV) were further purified during use with a noble gas purifier (HP2, VICI, Houston, TX).

2.2.3 Method

The singly- and doubly-protonated species of each peptide were generated through ESI at a flow rate of 0.4 mL/min, provided with an electronic syringe pump (BM-1000, Protea Bioscience Inc, Morgantown, WV). A mass isolation window of 1 m/z was used to isolate the precursor ions before exposing them to the metastable atom beam at a low mass cutoff value of 200-250 m/z. A pulsed metastable beam was applied for 200-550 ms, depending on the charge state of the precursor ion and the type of metastable atoms used. The pressure in the vacuum chamber, outside the ion trap, was kept at ~1.3 mbar with He as the bath gas (leaking out of the trap) and an additional 2 mbar (uncorrected) of the noble gas from the metastable atom source. The anode in the FAB gun was powered at 7 keV, unless otherwise stated, giving an instantaneous feedback current reading on the monitor electrode of ~0.2 mA. Fragmentation data was typically
acquired in 1-5 minute intervals. In addition to the MAD-MS spectra of each peptide, background spectra and negative controls were also obtained for each peptide. These included 1) positive ion mode with FAB gun on and ESI source off, 2) negative ion mode with FAB gun on and ESI source off, and 3) positive ion mode with FAB source off and ESI and isolation on, to ensure fragmentation does not occur in the absence of metastable atoms and to provide a reference precursor ion signal from which MAD-MS efficiencies could be calculated. All fragment identifications and assignments were manually determined based on predicted fragmentation patterns and were within ±0.8 m/z of the expected product ions. Peaks were only assigned if the intensities were at least 3 times the signal-to-noise ratio. Doubly-charged ions were identified according to the expected m/z value and the presence of an isotope envelope peak at +0.5 m/z.

2.3 Results and Discussion

2.3.1 Bradykinin: 1+ and 2+ Charge States

Figure 2.3 shows four different MAD-MS fragmentation spectra of bradykinin. Each charge state, whether dissociated with He or Ar metastable atoms achieved at least 100% sequence coverage.† MAD fragmentation efficiencies were calculated based on the relative intensities of the product ions and isolated precursor ion relative to the precursor

† 100% coverage is defined as one cleavage between every amino acid residue. Since, in theory, it is possible to cleave each residue in six different ways (a, b, c, x, y, z), it is possible to achieve 100% sequence coverage in $6^{n-1}2^m$ different permutations, where $n$ is the number of amino acids and $m$ is the number of proline residues not on the N-terminus (proline cannot ordinarily give c- or z-type ions). Because it is also possible to obtain complete sequence coverage of a peptide using independent permutations of cleavages (i.e. all a-type cleavages or all z-type cleavages), it is hypothetically possible to achieve 100% sequence coverage up to six times over.
ion signal, as described by Yost et al. [108]. For bradykinin the efficiencies ranged from 0.9-1.3%. Combining the fragmentation products for the 1+ and 2+ charge states gave a total of thirty-three a-, b-, c-, x-, y-, and z-type fragments out of a possible forty-eight fragments with three different side chain fragments. MAD of the 2+ charge state using a He metastable atom beam resulted in a variety of ions (a, b, c, x, y, and z) and a relatively low abundance of the charge-reduced species, [M+2H]+. MAD of the 1+ charge state of bradykinin yielded a higher number of fragment ions, mainly increasing the number of a-type ions detected. Cα–C and N–Cα cleavages were more prominent than cleavage of the

Figure 2.3. MAD spectra of bradykinin: (a) He+m + [M+H]+ (b) He+m + [M+2H]+2+, (c) Ar+m + [M+H]+ (d) Ar+m + [M+2H]+2+. (Key for peptide sequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; green line = product ions observed in both 1+ and 2+ charge states; precursor is indicated by a red arrow; side chain fragments are circled) (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)
low-energy C–N bonds, which are the favored cleavage points in CID, PSD and IRMPD.

The residual charge had a slightly higher preference for the C-terminus product ions for the 1+ precursor ion, but was evenly distributed between the N- and C-terminus product ions for the 2+ charge state precursor ion. Neutral losses of OH or NH$_3$, CH$_4$N$_2$, CH$_5$N$_3$, and C$_4$H$_{10}$N$_3$ (indicated with a red asterisk in Figure 2.3) were observed for both the singly- and doubly-charged precursor ions. The latter three neutral losses are known to be indicative of side chain losses from arginine [32, 109]. Singly- and doubly-charged precursor ions of bradykinin also revealed the side losses of serine, indicated by the cleavage of a hydroxyl radical group [110] and the loss of 17 Da from the a$_6$ product ion resulting in the d$_6$ ion (indicated with a circle). A variety of hydrogen gains in the form of a+1, x+1, and z+1 ions were observed for both charge states, which was similarly observed in the latest metastable atom dissociation research of Berkout and Doroshenko in the case of a+1 ions [67]. In one case, a hydrogen loss resulting in an x-1 ion was observed for the 1+ charge state of bradykinin. Hydrogen rearrangement is commonly observed and well characterized in ECD and can contribute to the generation of an aminoketyl radical which can then propagate along the peptide backbone [86, 111]. N–C$_\alpha$ or C$_\alpha$–C bond cleavage on the N-terminal side of the aminoketyl radical can then lead to the predominantly observed x+1 and z+1 even-electron species.

It is important to note the presence of the 2+ product ions upon exposure of the 1+ precursor ions to metastable atoms states of He or Ar. The doubly-charged product ions support the proposed mechanism of activation of singly-charged peptide ions via Penning ionization [58, 107]. Two fragmentations at the first proline (y$_8$ and x$_8$ ions), two fragments at the second proline (y$_7$ and b$_2$ ions), and seven fragments at the third proline
(x₃, y₃, w₃, z₃-2H,z₃-CH₆,a₆, and b₆ ions) were observed for the singly- and doubly-charged precursor ions. One a+1 ion and one b+1 ion were observed for the 1+ charge state and one z+1 ion in the 2+ charge state of bradykinin.

Activation of bradykinin using the Ar metastable atom beam provided at least one type of fragment ion at every amino acid for both the 1+ and 2+ precursor charge states of bradykinin. The fragmentation spectra for the two charge states were similar to the fragmentation achieved with He in that all ion type fragments resulted. However, approximately 35% fewer fragments were observed when using Ar metastable atoms. The fragment ion c₃ is the only new fragment observed in the 2+ charge state using Ar metastable atoms. When fragmented with Ar metastable atoms, the charge on the product ion had a slightly higher tendency to be retained on the N-terminal side of the peptide. For the doubly-charged precursor ion, the charge-reduced species, [M+2H]⁺⁺ was produced with a higher abundance when using Ar metastable atoms as opposed to He. Similar side chain neutral losses were observed for Ar metastable activation as for He. The loss of the serine side chain, 17 Da, was also observed using Ar metastable atoms but at a much lower intensity.

2.3.2 Substance P: 1+ and 2+ Charge States

Figure 2.4 shows fragmentation spectra of substance P collected in four different modes. The 2+ charge state, whether dissociated with He or Ar metastable atoms achieved 100% sequence coverage, yet the cleavage between the glycine and leucine was elusive when fragmenting the singly-charged precursor ion using He metastable atoms. Only five fragment ions were observed for the singly-charged precursor ion upon
exposure to Ar metastable atoms, indicating that Penning ionization with this relatively low-energy metastable atom species (11.55 or 11.72 eV) does not provide as much energy as the He metastable atoms for inducing fragmentation. MAD fragmentation efficiencies ranged from 0.7-3.6% for substance P, with a total of thirty-six combined (both charge states and metastable gases) fragments out of fifty-eight total possible fragments ($c_1$, $c_3$, $z_8$ and $z_{10}$ are not normally possible because of the proline residues).

*Figure 2.4. MAD spectra of substance P: (a) He$^m$ + [M+H]$^+$ (b) He$^m$ + [M+2H]$^{2+}$, (c) Ar$^m$ + [M+H]$^+$ (d) Ar$^m$ + [M+2H]$^{2+}$. (Key for peptide sequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; green line = product ions observed in both 1+ and 2+ charge states; precursor is indicated by a red arrow; side chain fragments are circled) (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. *J. Mass Spectrom.* 2009, 44, 1211-1223.*
Fragmentation of the 2+ charge state using a He metastable atom beam resulted in a variety of ion types (a, b, c, x, y, and z) and a relatively low abundance charge-reduced species [M+H]+ and [M+2H]+. These observations were similar to those observed for bradykinin. MAD of the 1+ charge state of substance P yielded a similar number of product ions in comparison to the 2+ charge state, but with slightly more a-type ions and slightly fewer c-type ions. The small insert in Figure 2.4a shows the characteristic isotopic distribution of not only a singly charged b5 ion with a 1 m/z difference, but two doubly charged ions with a 0.5 m/z difference. Due to the basicity of the arginine residue on the N-terminus of substance P, the product ions had a two-fold higher preference for a-, b-, and c-type ions over x-, y- and z-type ions. The neutral losses observed for substance P were not as extensive as those observed for bradykinin; only OH or NH3 and CH4N2 losses were observed (indicated with a red asterisk in Figure 2.4). However, the side-chain loss of 58 Da, C2H4NO, from the glutamine residue [110] at position 5 resulting in the d5 ion (indicated with a circle) is apparent in Figure 2.4a. Substance P contains two proline residues, resulting in the a3 and b3 ions via the fragmentation of the singly- and doubly-charged precursor ions with He metastable atoms. To our knowledge, such product ions for proline residues are not common in ECD or ETD. Again, a series of a+1, x+1, and z+1 ions were observed for both charge states, with one y+1 ion in the 2+ charge state and one z-1 ion for the 1+ charge state of substance P.

MAD of substance P using Ar metastable atoms results in fragmentation that is not as extensive as observed with He metastable atoms, especially for the singly-charged precursor ion. The small insert in Figure 2.4c shows the isotopic distribution of the [M+H]2+• ion. The doubly-charged precursor ion produced a complete c-type ion series,
lacking only c₁ and c₃ which are unlikely due to the proline residues at these positions. MAD using metastable Ar atoms produces ions not observed in the doubly-charged peptide fragmentation with He such as z₉, and one which is not observed in either charge states for helium, y₉. For the doubly-charged precursor ion, the charge is retained almost exclusively on the N-terminus side of the product ions after fragmentation.

For bradykinin and substance P, we observed that Ar metastable atom exposure generates more charge-reduced species, such as [M+2H]⁺⁺ and [M+H]⁺, than He metastable atom exposure. This was also observed in the work of Misharin et al. and is thought to be related to the fact that He metastable atom states are closer to the ionization potential and therefore result in more exothermic electron transfer reactions–which results in more fragmentation. The doubly-charged ion experienced similar neutral losses as seen with bradykinin, losing OH or NH₃, CH₄N₂, and CH₅N₃ (indicated with a red asterisk in Figure 2.4). Again, the latter two neutral losses indicate the presence of arginine. When Ar metastable atoms were used for the dissociation of substance P, no a+1, x+1 etc ions were observed, and only one y-1 radical ion for the 2+ charge state was observed. Ar metastable atoms therefore appeared to be less capable of promoting the migration of H atoms between product ions.

Figure 2.5 compares the dissociation achieved when employing CID versus MAD for both bradykinin and substance P. MAD produces twenty-eight different cleavage points from doubly-charged substance P. CID of the same peptide produced eleven fragments. Eight fragments were common to both methods. MAD produced thirty different cleavage points in the singly-charged peptide bradykinin compared to eight through CID. Six fragments were common to both methods.
2.3.3 Backbone Cleavage of Proline Residues

Fragmentation resulting in a mixture of a-, b-, c-, w-, x-, y-, and z-type ions from proline, as observed here with MAD, is not at all characteristic of ECD and ETD dissociation. However, the cleavage of the N–C_\text{a} bond of proline has once been observed in ECD and HECD experiments by Cooper et al [102]. A similar z ion was observed here using MAD dissociation on the 7th amino acid proline residue of bradykinin (Figure 2.6a). This type of fragmentation occurs through the cleavage of two covalent bonds, one on the peptide backbone (N–C_\text{a} bond) and one within the proline side chain (Figure 2.6c), resulting in the ions: z_3-2H, z_3-CH, and w_3 (z_3-C_2H_4). Figure 2.6a shows the three ion fragments indicative of cleavage of the proline residue in the third position of bradykinin which are observed at 402, 391, and 377 m/z respectively. The combined observations of a, b, w, x, y and z ions at this residue indicate that the mechanism of fragmentation is not a simple electron transfer process, like ECD or ETD, but is instead unique to metastable
atom activation. These ions were observed following MAD of either precursor charge state of bradykinin when using He metastable atoms (Figure 2.3a, b). In addition to bradykinin, cleavage of the proline ring was again detected in both the PHPRI and PHPRL spectra resulting in the $a_2$, $b_2$, $c_2$, $x_3$, $y_3$, $z_3$-2H, $z_3$-CH, and $w_3$ ions for each peptide (Figure 2.6b). The three fragments indicative of the proline ring cleavage were observed at the same $m/z$ for both the peptides, PHPRI and PHPRL: $w_3$ at 342.3 $m/z$, $z_3$-2H, $z_3$-CH, and $w_3$.
CH at 357.3 \textit{m/z}, and \textit{z}_3\text{-2H} at 368.3 \textit{m/z}, as seen in Figure 2.6a and 2.6b. Figure 2.5b shows the mass spectrum of PHPRL, highlighting the \textit{z}_3\text{-2H}, \textit{z}_3\text{-CH}, and \textit{w}_3 (\textit{z}_3\text{-C}_2\text{H}_4) ions.

### 2.3.4 Differentiating Leucine and Isoleucine

The singly-charged species of peptides PHPRL and PHPRI were fragmented by He metastable atoms, resulting in fifteen different fragment ions of all ion types and a small Penning product ion, [M+H]^{2++}. The MAD spectrum of PHPRL shown in Figure 2.7a has four particular peaks of interest at 229.4, 342.3, 506.3, and 463.3 \textit{m/z} representing a loss of 43 Da from the \textit{z}_2+1, \textit{y}_3, \textit{x}_4+1, and \textit{z}_4+1 ions, respectively. These ions are indicative of a neutral loss of \textbullet\text{CH(CH}_3\text{)}\text{2} from the leucine side chain [88]. Figure 2.7b is the spectrum of PHPRI, which has five diagnostic peaks at 243.4, 357.4, 383.3, 520.6, and 477.4 \textit{m/z}. These peaks correspond to the loss of 29 Da from the \textit{z}_2+1, \textit{x}_3+1, \textit{y}_3,\textit{x}_4+1, and \textit{z}_4+1 ions, respectively, which is indicative of the neutral loss of \textbullet\text{CH}_2\text{CH}_3 from the isoleucine side chain [88]. Similar to ECD, MAD activation may also permit radical ion migration and fragment at more than one covalent bond. Another alternative reason for the observation of cleavage at two disparate covalent bonds is the possibility of multiple metastable atom collisions, especially at long exposure times. The \textit{d}_5 ion (indicated with a circle) is present in both the PHPRL and PHPRI mass spectra which corresponds to the neutral loss of either the leucine (43 Da) or isoleucine (29 Da) side chains. Figure 2.7c verifies the leucine residue in the peptide by the presence of a peak at 531.4 \textit{m/z} in the PHPRL spectrum, corresponding to a neutral loss of 43 Da from the \textit{a}_5 ion, which is absent in the PHPRI spectrum. Likewise, Figure 2.7d shows a peak at 545.4
$m/z$ in the PHPRI spectrum detecting the neutral side chain loss from isoleucine which is not present in the PHPRL spectrum. To our knowledge this is the first report to provide evidence that MAD can distinguish between isoleucine and leucine, indicating a unique and highly beneficial capability.

Figure 2.7. MAD spectra of the two synthetic peptides PHPRL and PHPRI: (a) $\text{He}^m + [\text{M}+\text{H}]^+$, showing several neutral losses of 49 Da, (b) $\text{He}^m + [\text{M}+\text{H}]^+$, showing several neutral losses of 29 Da, (c) confirming the leucine neutral loss from the $z_2$ and $z_4$ ions which are not present in the isoleucine spectrum, (d) confirming the isoleucine neutral loss from the $z_2$ and $z_4$ ions which are not present in the leucine spectrum. (Key for peptide sequencing: black line = product ions observed in 1+ charge state; green line = product ions observed in both 1+ and 2+ charge states; precursor is indicated by a red arrow; side chain fragments are circled) (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)
2.3.5 *Phosphorylated Peptides*

2.3.5.1 *Angiotensin II*

Phosphorylated angiotensin II has a phosphorylated tyrosine residue at position 4. The singly-charged phosphopeptide ion of 1127 \textit{m/z} was isolated and the fragmentation spectrum following exposure to He metastable atoms is shown in Figure 2.8. The dissociation resulted in mostly a-, b-, and z-type ions. Figure 2.8 shows that the phosphorylation site remains mostly intact during cleavage of the amide backbone, and thereby enables phosphorylation-site determination. Tandem mass spectrometry and CID of phosphopeptides is well characterized, showing that phosphotyrosine loses HPO$_3$ (80 Da) and phosphoserine loses H$_3$PO$_4$ (98 Da) [112]. Phosphotyrosine is different from phosphoserine in that cleavage tends to occur between the phosphorus and highly stable phenolic oxygen resulting in the loss of HPO$_3$. Our data is consistent with these reports, detecting several neutral losses of 80 Da from the phosphotyrosine peptide fragment ions (labeled with a red asterisk), which correspond to the loss of HPO$_3$. However, we do detect a neutral loss of 98 Da from the precursor ion, which is assigned to a sequential loss of HPO$_3$ and H$_2$O [112]. The preservation of the labile phosphate group during fragmentation is also possible with ECD and ETD, but ECD and ETD are not applicable to singly-charged precursor ions, as demonstrated here for MAD activation. In addition to phosphate site determination, the isoleucine residue at position five is confirmed and differentiated from leucine by the presence of a w$_4$ (indicated with a circle) ion which is the result of a neutral loss of 29 Da from the z$_4$ ion.
Figure 2.8. MAD spectrum of singly-protonated phosphorylated angiotensin II with a He metastable atom beam. (Key for peptide sequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; red * = b₆, b₅, a₅, b₄, and a₄ without the phosphate group; precursor is indicated by a red arrow; side chain fragments are circled) (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)

2.3.5.2 Cholecystokinin (peptide 10-20)

Phosphorylated cholecystokinin (Figure 2.9) has a phosphorylated serine residue at position 5. Fragmentation of the isolated 1+ charge state (1332 m/z) by He metastable atoms are shown in Figure 2.9a. Extensive fragmentation in the form of a-, b-, c-, x-, y-, and z-type ion fragments are again observed. As with angiotensin II, the phosphate group remains mostly intact after MAD dissociation, thereby enabling phosphorylation-site determination. Again our results are consistent with previous phosphoserine research [112], showing several neutral losses of phosphoric acid (98 Da) which are indicated in
Figure 2.9. MAD spectra of singly-protonated phosphorylated cholecystokinin (peptide 10-20) using a He metastable atom beam, (a) average of 2500 spectra and (b) average of 5 spectra (a single data point), collected in ~1 second. (Key for peptide sequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; precursor is indicated by a red arrow; side chain fragments are circled) (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)
Figure 2.9 with a red asterisk. A number of side-chain cleavages were also observed, including the w₅ and d₇ ions, which confirmed the presence of the leucine residue at position 7, and the v₄ ion (indicated with a circle), which is a neutral loss of CO₂H, or 45 Da, from aspartic acid. The spectrum shown in Figure 2.9a is generated from an average of 50 scans, each of which is composed of 5 averaged spectra (representing 3.8 minutes of data acquisition). The spectrum shown in Figure 2.9b is generated from a single scan of 5 averaged spectra, which took less than 1 second to collect. This single scan spectrum contains 54% of the identified peaks in the spectrum obtained from 50 scans, and all the information required to determine the phosphorylation site.

2.3.6 Glycosylated Peptides

Glycosylated RNase B (Figure 2.10) contains an N-linked beta N-Acetylglucosamine (GlcNAc) modification on the asparagines residue at position 3. The doubly-charged glycopeptide ion at m/z 476 was isolated and the fragmentation spectrum following exposure to He metastable atoms is shown in Figure 2.10a. Extensive fragmentation in the form of a-, b-, c-, x-, y-, and z-type ions are observed with the GlcNAc modification intact. Multiple cleavages occurred between each amino acid which allows complete sequence coverage. A charge-reduced species is observed, [M+2H]⁺⁺, which also undergoes a water loss. Only six out twenty-eight fragment ions underwent a neutral loss of the modification (203 Da) which are indicated with asterisks. Six different fragment ions, a₃, b₃, c₃, y₄, z₄, and [z₄]²⁺, provided unambiguously identify of the GlcNAc modification to the asparagine residue.
Figure 2.10. He MAD spectra of [M+2H]$^{2+}$ glycosylated peptides: (a) of Rnase B with an n-linked beta GlcNAc and (b) a synthetic octapeptide with an o-linked beta GlcNAc. (Key for peptide sequencing: black line = product ions observed in 1+ charge state; red line = product ions observed in 2+ charge state; precursor is indicated by a red arrow; side chain fragments are circled)
The glycosylated octapeptide with an O-linked α N-Acetylglucosamine (GlcNAc) modification on the serine residue at the 5th position is illustrated in Figure 2.10b. The doubly-charged precursor ion at m/z 527 was isolated and exposed to He metastable atoms to produce extensive backbone cleavages. A total of thirty-two fragments ions were produced with cleavage between each residue. Again, complete sequence coverage was achieved. As observed for the Rnase B glycopeptides, a charge-reduced species was also observed, however in this case, no neutral loss of the sugar were observed from this species. Even though nine fragment ions lost the GlcNAc modification during dissociation ample fragment ions allowed for unambiguous modification site determination to the Ser-5 residue.

2.3.7 Peptide Anions: 2- Charge State

When the 2- charge state of bradykinin was exposed to a He metastable atom beam, the product ion spectrum included eleven different ions and a large charge reduced product ion. The most predominant ions observed in Figure 2.11 were a series of x-type ions (x4-x7, and x9), which were also observed by Misharin et al. [58]. However, we also observed two y- and two z-type ions as well as one a- and one b-type ion. A neutral loss of 43 Da from the charge reduced species, [M-2H]+, is the loss of •CH(CH₃)₂ from Leu-10 residue (indicated with an asterisk in Figure 2.11). Fragmentation efficiencies for MAD of anionic substance P exceeded 7%, almost double the efficiencies observed for cationic MAD. Similar results were observed for fibrinopeptide B (data not shown); a series of x-type ions (x₆-x₉ and x₁₁-x₁₃), one a-type ion, and the charged reduced ion were produced from the precursor ion, [M-2H]²⁻.
Figure 2.11. MAD spectrum of doubly-deprotonated substance P using a He metastable atom beam. (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)

2.3.8 Kinetic Study of 1+ and 2+ Charge States of Bradykinin

Internal energy deposition during MAD was explored using both the singly- and doubly-charged ion states of bradykinin. He metastable atom pulse lengths from 50 to 250 ms generated at kinetic energies between 6 and 10 keV were used. As expected, with increasing metastable atom pulse length and increasing kinetic energy, the precursor ion intensity of both the singly- and doubly-charged states decreased (data not shown), indicating that precursor ions are indeed reacted away during the experiments. Figure 2.12a and 2.12b show the effect of pulse length and kinetic energy of the metastable atom pulse on the observed intensity of the z_4^+H ion for the singly- and doubly-charge
Figure 2.12. Fragmentation intensity of $a_3$H ion (324.3 m/z), $c_5$ ion (573.4 m/z), and $y_8$ ion (904.4 m/z) using 7 keV after 50, 100, 150, 200, and 250 ms metastable atom beam exposures on the [M+H]$^+$ species of bradykinin. (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)

states, respectively. Longer pulse lengths are optimal at lower metastable atom kinetic energies producing the greatest number of fragments, while shorter lengths are optimal at higher kinetic energies. Regardless of pulse length, kinetic energies above 9 and below 6 keV produce spectra with low signal to noise ratio (data not shown), with limited detectable fragmentation. Figure 2.13 shows the $a_3$, $c_5$, and $y_8$ product ion intensities at different pulse widths using varying kinetic energies. The singly-charged species was optimal with pulse lengths above 200 ms, yet the doubly-charged species (data not shown) required a shorter pulse length (on average between 100-150 ms) for maximum fragmentation intensity. The intensity of the Penning product ion is highest using kinetic energies of 8 keV at pulse widths between 200-250 ms. The charge reduced ion (data not shown) had the highest intensity at kinetic energies between 6 and 7 keV, which is 1 keV lower than the optimal kinetic energy of the fragment product ions generated from the singly-charge state. The data generated from the $a_3$, $b_4$, $z_4$, $c_5$, $x_6$, and $y_8$ ions (not all data
shown) showed that signals for the C–α–C, N–Cα, and C–N backbone bond cleavages are optimized at approximately the same conditions.

Figure 2.13. Relationship of how He metastable atom pulse time and voltage applied to FAB gun anode effects fragmentation of singly- and doubly-charged bradykinin: (a) fragmentation intensity of the z4+H ion at 50, 100, 150, 200, and 250 ms metastable atom exposure using 6, 7, 8, 9, and 10 keV on the [M+H]+ species. (Reproduced with permission from Cook, S. L.; Collin, O. L.; Jackson, G. P. J. Mass Spectrom. 2009, 44, 1211-1223.)

2.3.9 Proposed Mechanism of Metastable-Atom Activation

Misharin et al. have already proposed an electron transfer reaction mechanism for charge-reduction of polyprotonated peptide cations with metastable atoms [58]. Our group [107] and Berkout and Doroshenko [67] have also proposed a mechanism for the Penning ionization of polyanionic peptide ions. Our results are consistent with these proposed mechanisms. Reactions between metastable atoms and singly-protonated
peptide cations, as observed here and elsewhere [67], result in several possible outcomes, one of which is the Penning ionization [57, 60, 113] of the protonated peptide ion (P),

\[
[P + nH^+]^{n+} + M^* \rightarrow [P + nH^+]^{(n+1)+} + M + e^-
\]  

(1)

Where M is a noble gas atom. This reaction leads to the generation of a radical cation, which is known to be more reactive than even-electron cations. This type of process has been shown to be accompanied by electronic excitation, rearrangement and subsequent fragmentation when neutrals are used as the precursors [67, 107]. Ohno et al. [65] have also shown that neutral targets of Penning ionization are preferentially ionized at surface-exposed regions of high electron density, such as the lone pairs of electrons on carbonyl oxygen atoms.

Scheme 2.1, shows a McLafferty rearrangement [114] for protonated dialanine as an example of the type of fragmentation that can follow Penning ionization of singly-charged peptide cations. This reaction occurs via hydrogen transfer from the γ-position

![Scheme 2.1. Possible outcome from Penning ionization (PI) of dialanine [AA+H]^{1+}.](image)

(Note: PI leads to a radical cation on the carbonyl oxygen atom which follows a McLafferty rearrangement to provide β-cleavage of the amide backbone to create c- and z-type fragments. Only one outcome is shown, but the original charging proton is independent of the reaction chemistry and may reside on either of the product ions.)
followed by bond cleavage of the β-bond, thus creating c- or z-type ions. The reaction is simplified as a concerted mechanism in the scheme. Scheme 2.2 shows another potential pathway for the rearrangement/fragmentation of radical cations. This fragmentation rearrangement/fragmentation pathways following metastable atom-activation result in cleavage of the amide backbone, with the possible generation of a-/x- and c-/z-type ions, in addition to the commonly observed rearrangement b-/y-type products observed through collisional activation. Another occurrence is the charge-reduction of doubly-protonated peptide cations from an electron transfer through ion-metastable atomoccurs

Scheme 2.2. Alternative outcome from Penning ionization (PI) of dialanine [AA+H]\(^{1+}\).

Note: PI leads to a radical cation on the carbonyl oxygen atom, which fragments through α-cleavage of the amide backbone to create a-/x- or b-/y-type fragments. b-/y-type ions can also form through the more-common cyclization mechanism (not shown) [114]. The original charging proton is independent of the reaction chemistry and may reside on either of the product ions, thereby affecting the observed product ions.
via α-cleavage. These schemes show that several different possible collisions resulting in $[\text{M+2H}]^+$. Subsequently, the ejection of a radical hydrogen atom forms the $[\text{M+H}]^+$ charge-reduced product ion, as seen with substance P. Our product ion spectra show all types of product ions (a-, b-, c-, d-, x-, y-, z-, w-, and v-) indicating that an extensive variety of competing mechanisms are also observable.

2.4 Conclusion

MAD of bradykinin and substance P resulted in abundant levels of a-, b-, c-, x-, y- and z-type ions for both precursor charge states regardless of the type (Ar or He) of metastable atoms used. Substance P contains two basic residues close to the N-terminus, which favors the production of a-, b-, and c-type ions over C-terminal ions. Bradykinin showed no specific preference for C-terminal and N-terminal product ions. A series of x-type ions were produced for both anionic substance P and fibrinopeptide B. MAD of the two phosphopeptide cations, angiotensin II and cholecystokinin, produced an extensive amount of ions while leaving the labile phosphate group mostly intact after dissociation which readily-enabled phosphorylation-site determination. Leucine and isoleucine were successfully differentiated in several peptides through the neutral loss of their side chain, resulting in several different distinct fragment ions for either leucine or isoleucine. We have successfully shown that MAD-MS can result in over 100% sequence coverage with MAD fragmentation efficiencies approaching 4% for cationic peptides and 7% for anionic peptides and provide a variety of a-, b-, c-, x-, y-, and z-type ions with frequent side chain fragmentation. This novel dissociation method therefore appears to be an interesting and complementary alternative to ECD, ETD, and CID, especially for singly-
charged precursor ions. Another distinct advantage is the ability to utilize relatively low cost ion trap instrumentation to perform these extensive fragmentations, as opposed to expensive FT-ICR instruments which are commonly employed for ECD fragmentation.
3.1 Introduction

Elucidating how a PTM can alter a protein’s structure and biological function is an integral part of proteomics. The ability to identify and localize these modifications, in particular nitration and S-nitrosylation, can aid in biomarker discoveries for neurological diseases [115, 116], cardiovascular diseases [117, 118], lung diseases [119, 120], and diabetes [121]. Tyrosine and cysteine amino acid residues are highly susceptible to oxidation by one or more forms of reactive nitrogen species such as nitric oxide radicals [122]. For example, reaction of a nitrogen dioxide radical with tyrosine can produce a covalently bound –NO₂ to the tyrosine side chain thereby producing 3-nitrotyrosine [123]. Cysteine nitrosylation can occur through a reaction with nitric oxide radicals resulting in the addition of –NO [124]. Developing rapid and accurate mass spectrometry (MS)-based methods for direct detection of both nitration and S-nitrosylation can aid in the understanding of how the reactive nitrogen species plays a role in certain diseases and can provide new diagnostic tools.
MS has become a leading tool in proteomics because of its ability to sequence peptides and proteins and characterize PTMs. The most widely used MS techniques for studying nitrotyrosine and nitrosylated cysteine PTMs in peptides and proteins are matrix assisted laser desorption/ionization time-of-flight MS (MALDI-TOF) and electrospray ionization (ESI) MS/MS [123]. UV-MALDI has been noted to lead to a high degree of photodegradation or in-source decay of nitrated or S-nitrosylated peptides, thereby rendering the modified peptides weak or absent [123, 125, 126]. The dissociation method traditionally employed in ESI MS/MS is CID, and CID tends to preferentially cleave side chain PTMs instead of the peptide backbone. The preference for side-chain cleavages leads to poor sequencing information and PTM characterization. Biotin-switching, in which the labile nitrosylated cysteines are derivatized to a more stable biotinylated cysteine, was developed to combat these issues [127]. However, the precise control of the chemical reactions has proven to be difficult and can result in false positives [126, 128]. Methods such as precursor ion scanning of the nitrotyrosine immonium ions [129] and selected reaction monitoring combined with UV absorption [130] have both shown to be promising techniques to characterize nitrotyrosine when analyzing complex samples.

Alternative types of peptide dissociation methods that utilize radical chemistry have been recently introduced to provide both accurate sequencing and PTM information. ECD, first introduced in 1998 by McLafferty and co-workers, has proven to effectively fragment the peptide backbone in Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR-MS) [31]. This method preferentially cleaves the more energetic peptide backbone N–Cα bonds resulting in mainly c-/z-type ions, in contrast to the “slow-heating” dissociation methods such as CID and IRMPD [12]. The latter activation
methods tend to cleave the lower energy C–N bonds and produce b-/y-type ions. ETD, introduced by Hunt and co-workers, uses ion-ion reactions to transfer electrons from a radical anion to polycations resulting in ECD like fragmentation [30]. Successful PTM site determinations of phosphorylation [30, 131], sulfation [132, 133], and glycosylation [134, 135, 136], among others, have been reported for ECD and ETD. However, traditional ECD and ETD are not applicable to singly protonated or negatively charged precursor ions such as the more acidic peptides. Recently new techniques have been developed to apply these two methods, NETD and niECD, to negative ions [54, 55, 137].

There have been only a few reports applying ECD and ETD to nitrated and S-nitrosylated peptides. It has been shown that in ECD and ETD, backbone fragmentation is completely inhibited in peptides containing specific tags with electron affinities (EA) larger than 1 eV. Such tags are termed electron predators and examples include 3-nitrobenzyl and 3,5-dinitrobenzyl moieties [138]. Beauchamp and co-workers proposed that the propagation of the captured electron can relax through space or through bond (π) to high EA predators (tags) which competes with the traditional electron transfer to the amide π* orbital. A hydrogen atom from a separate site on the peptide can then be transferred to the electron predator radicals due to their high H-atom affinity. Tureček, on the other hand, proposes intermolecular H-atom transfer from the aminoketyl radical of the charge-reduced species to the nitrobenzyl moieties instead of direct electron sequestering [139]. In this mechanism, backbone fragmentation is inhibited based on the high H-atom affinity of the nitrobenzyl groups. Regardless of the exact mechanism, the presence of nitrobenzyl moieties inhibits traditional ECD and ETD N–Cα backbone cleavage. Recently, nitrotyrosine residues have been shown to hinder backbone cleavage
during ECD because of the electron predator or H-atom trap effect [140]. These results demonstrate that ECD is not the method of choice to characterize nitrated tyrosine residues of 2+ and 3+ charge states of peptides [140]. However, top-down analysis of multiply charged, larger nitrotyrosine-containing proteins using ECD has shown promise when combined with other “slow heating” methods to identify PTM sites [141].

Few studies have concentrated on direct MS detection of cysteine nitrosylation, probably due to its labile nature. S-nitrosylated bovine insulin was explored with ETD resulting in only a select number of backbone cleavages and fragment ions retaining the modification, thereby hindering site determination [133]. The major dissociation pathway observed in ECD was charge reduction (electron transfer without fragmentation) with and without losses of the NO [133]. ETD was therefore unable to identify the location of the PTM.

Recently, a new type of dissociation method using metastable atoms as the electron vehicle or potential energy source has been explored in RF ion trapping instruments, which we term metastable atom-activated dissociation (MAD). The interaction of isolated precursor ions with a high kinetic energy beam of argon or helium metastable atoms produces a high degree of peptide backbone cleavages resulting in a-, b-, c-, x-, y-, and z-type ions while retaining PTMs [68]. To date, comprehensive dissociation has been demonstrated to fragment multiply charged cations and anions [58, 68, 106], 1+ cations [67, 68, 69], phosphorylated cations [58, 67, 68], disulfide bonds [58], to cleave the amide ring structure of proline [68] and to differentiate isoleucine from leucine [68, 69].
Unlike ECD and ETD, there is evidence for two competing dissociation mechanisms responsible for the type and degree of fragment ions observed in MAD. The first is Penning ionization, which has been well characterized with neutral precursor molecules and occurs when the potential energy of the metastable atom is greater than the ionization potential of the neutral [57] or biomolecular ion. It has been well documented that metastable atoms are attracted to regions of high electron density such as the lone pairs of electrons on carbonyl oxygen atoms. Penning ionization at these sites leads to the generation of radicals at the lone pair [64]. The second mechanism observed in MAD is a similar mechanism proposed for ECD and ETD and is referred to as charge reduction. The charge reduction mechanism occurs when an electron from the metastable atom is transferred to the polycation of interest. The specific electron capture mechanism in ECD and ETD of peptides and proteins is still in debate [34, 37, 40, 86]. However, all mechanisms agree that the electron transfer process generates a radical bio-ion that can rearrange, propagate along the peptide backbone, and subsequently fragment.

In this work, we investigated the ability of He MAD to characterize nitrated and S-nitrosylated peptides. We conducted a comparison of MAD and CID on the 1+, 2+, 3+, and 2- charge states of two nitrated peptides, DRVnYIHPF and GPLEnYGFAKGPLAK, and two S-nitrosylated peptides, VFDARDnCRSAQ and EMFTYInCNHIK as well as the respective unmodified peptides. S-nitrosylated peptides were extremely labile and often difficult to isolate. However, when combining the MAD fragmentation information from different precursor ion charge states, modification site location was achieved. We demonstrate that MAD is a viable alternative for the direct analysis of nitrated and S-nitrosylated peptides and provides radical-like fragmentation where ETD and ECD fail to
deliver. The unique chemistry and structural information obtained through MAD can complement CID, ETD, and ECD methods of analysis.

3.2 Experimental

3.2.1 Instrumentation

All experiments were performed on a modified Bruker EsquireLC QIT MS (Bruker Daltonics, Bremen, Germany) as described previously [68]. An Ion Tech FAB gun (P50 PSU, Teddington, UK) was used as the metastable atom source as reported elsewhere [58, 68]. A deflection electrode consisting of two flat-ended 4-40 screws mounted within a PEEK (polyether ether ketone) sleeve was mounted on the bottom of the FAB gun to prevent ions and free electrons from entering the trap. The electrodes were positioned 1 cm downstream of the exit orifice of the FAB gun, orthogonal to the FAB beam, with an inter-electrode spacing of 5 mm. One electrode was grounded and the other was biased to +800 V using a high voltage power supply (EL 03R 15L, Glassman High Voltage Inc., High Bridge, NJ). For pulsed operation of the FAB gun, the anode power supply was replaced with a 10 kV high voltage amplifier (ANT 10B10, Matsusada Precision Inc., Shiga, Japan). A trigger was taken from the Bruker EsquireLC at pin 15 of connector J522 and fed to a function/arbitrary waveform generator (33250A, Agilent, Santa Clara, CA) which generated a square pulse with a set pulse width and delay time depending on precursor mass. Complete pulse operation and generation has been previously described [68]. The FAB gun was placed approximately 2 cm above a 2 mm-hole in the ring electrode which enabled the metastable atoms to traverse to the center of the QIT and interact with the isolated biomolecular ions of interest. Background ions
were observed in both positive and negative modes due to Penning ionization of residual pump oils which could be actively removed from the trap by employing a trapping voltage corresponding to a LMCO value above \( m/z \) 200 for positive mode and \( m/z \) 300 for negative mode. Before use each day, the FAB gun was turned on for 30 mins to ‘burn’ away residual contaminants such as pump oil. This helped decrease background ion signals.

3.2.2 Preparation of Peptides

The S-nitrosylated and non-nitrosylated forms of two endothelial nitric oxide synthase (eNOS) peptides (194-204; 205-215) and non-nitrated angiotensin II were provided by Protea Biosciences Inc. (Morgantown, WV). Nitrated GPLEnYGFAKGPLAK was purchased from Alta Bioscience (Birmingham, UK). Trimethylnitrate (TMN), AccuGENE Tris-Borate buffer, methanol (HPLC grade), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). All peptides were reconstituted in a 1:1 mixture of methanol and water with either 1% acetic acid or ammonium hydroxide (depending on desired charge state) to provide solutions between 20-40 µM of the individual peptides. Ultrahigh-purity He and Ar (Airgas, Parkersburg, WV) were further purified on-line with a noble gas purifier (HP2, VICI, Houston, TX). Angiotensin II was nitrated by incubating for 1 hr at 37°C with 11 mM TNM in 5 mM Tris-Borate (pH 8.3), as described by Lee et al [142]. Samples were desalted by using C_{18} SpinTips Sample Prep Kit, using protocol 2 (Protea Biosciences Inc., Morgantown, WV).
3.2.3 Method

All peptides were directly injected using an electronic syringe pump (BM-1000, Protea Bioscience Inc, Morgantown, WV) at a flow rate of 200 μL/hr. Isolation windows varied between \( m/z \) 1-6 depending on the labile nature of the modified precursor ion. Ion accumulation was set to 15-25 ms to reach an ion target of 20,000. Precursor ion intensity ranged from \( 8 \times 10^4 \) – \( 4 \times 10^5 \) AU depending on peptide and precursor charge state. All fragment ion identifications and assignments were manually determined based on predicted fragmentation patterns and were within ±0.8 \( m/z \) of the expected product ions. Peaks were only assigned if the intensities were at least 3 times the signal-to-noise ratio. The 2+ and 3+ ions were identified according to the expected \( m/z \) value and the presence of an isotope envelope peak at \( m/z +0.5 \) and \( m/z +0.3 \), respectively.

3.2.3.1 CID.

All cationic and anionic peptides were fragmented using the “SmartFrag” option in the Bruker EsquireLS NT 4.5 software, which applied a sweeping collisional energy starting at 20% and ending at 150% of a set value. The fragmentation time was set to 20 ms in all experiments. The collisional energy was set to 0.4-1.2 V depending on precursor mass and compound stability. CID MS/MS data was acquired in 1 min intervals, and consist of approximately 200 averaged spectra.

3.2.3.2 MAD.

1+, 2+, 3+, and 2- precursor ions were exposed to a beam of high kinetic energy He metastable atoms with the FAB anode powered at 6 kV. A pulsed metastable atom beam was applied for 75-150 ms depending on the charge state of the precursor ion. The pressure in the vacuum chamber, outside the ion trap, was kept at 1.8 mbar (uncorrected)
with He leaking out of the trap and an additional 1.0 mbar (uncorrected) of the noble gas from the metastable atom source. MAD MS/MS spectra were acquired in ~2min intervals and consist of between 300-450 averaged spectra. MAD MS/MS data were background subtracted using a spectrum obtained with identical conditions as above, but with the ESI source off.

3.3 Results and Discussion

3.3.1 Tyrosine Nitrated Peptides

Figure 3.1a-f shows MAD-MS spectra of the 1+, 2+, and 3+ charge states of nitrated angiotensin II (DRVnYIHPF) and the synthetic peptide, GPLEnYGFAKGPLAK. A lower case ‘n’ indicates the amino acid directly to the right is modified. Both peptides had a precursor ion mass increase of 45 Da relative to the unmodified peptides, confirming the addition of an –NO₂ group. Peptide sequence inserts in Figure 3.1 omit those fragment ions that have undergone a neutral loss of the PTM modification.

Combining the fragmentation products for the 1+, 2+, and 3+ charge states of angiotensin II (Figure 3.1a-c, respectively) a total of twenty-six distinct a-, b-, c-, x-, y-, and z-type fragment ions out of forty-two possible backbone cleavages are observed. At least one backbone cleavage is observed between each amino acid residue for the 1+ and 2+ charge states resulting in complete sequence coverage. MAD of the 1+ precursor ion (Figure 3.1a) yielded twenty-one total fragment ions with seven 2+ product ions and a dominant Penning ionized species, [M+H]²⁺. The production of 2+ product ions from 1+ precursor ions is direct evidence for Penning ionization (PI) [57, 68] and is unique to MAD in comparison to CID, ECD, and ETD. The small insert in Figure 3.1a is the
Figure 3.1. He MAD spectra of nitrated angiotensin II (DRVnYIHPF): (a) [M+H]$^+$, (b) [M+2H]$^{2+}$, (c) [M+3H]$^{3+}$, and He MAD spectra of nitrated GPLEnYGFAKGPLAK: (d) [M+H]$^+$, (e) [M+2H]$^{2+}$, (f) [M+3H]$^{3+}$. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)
isotopic distribution of the PI product ion species showing that the most intense peak is
the 1+ species. Figure 3.1a of the 1+ precursor shows eight C–N, 6 Cα–C and only two
N–Cα bond cleavages. The figure also shows that the nitration modification remains
mostly intact during exposure to He metastable atoms, which enables PTM site
determination. In total, seven different product ion-types undergo a neutral loss of the
PTM modification (indicated by * in the spectrum), however five of these seven ions are
also observed with the modification intact. A neutral loss of 18 Da (-H2O) from both the
\([\text{M}+\text{H}]^{2+}\) and the \([\text{M}+\text{H}-\text{NO}_2]^{2+}\) species was observed.

Figure 3.1b is a MAD spectrum of the 2+ precursor ions. MAD resulted in
twenty-five product ions and cleavage between every amino acid. Of note in Figure 3.1b
is the neutral loss of a hydrogen radical from the charge-reduced ions, \([\text{M}+2\text{H}]^{+}\), which
is more abundant than the PI product species, \([\text{M}+2\text{H}]^{3+}\). Again the most dominant
fragmentation pathway was cleavage of the C–N bonds, with nine total b-/y-type ions.
However, a large number of a- and c-type ions were also observed. The neutral loss of 29
Da from the side chain of the z4 ion produced the w4 ion and enables the definitive
identification of an isoleucine residue instead of the more ambiguous, isobaric isoleucine
residue. Isoleucine undergoes a neutral loss of •CH2CH3 (29 Da) whereas leucine
undergoes a neutral loss of •CH(CH3)2 (43 Da) [88]. The majority of the product ions,
sixteen out of a possible eighteen, contain the nitrated tyrosine modification. Neutral
losses of 18 Da (-H2O) were observed from the \([\text{M}+2\text{H}]^{+}\) species and the precursor ion,
\([\text{M}+2\text{H}]^{2+}\), respectively. Figure 3.1c is a fragmentation spectrum of the 3+ precursor ions
using MAD. In total, only twelve product ions were observed and were mostly C–N bond
cleavages. The most intense product ion peaks observed are the \([\text{M}+2\text{H}]^{2+}\) species,
presumably from a hydrogen radical loss from the charge-reduced product ion, and the neutral loss of H₂O from the [M+3H]²⁺ species. The minimal amount of backbone cleavages in the electron transfer from the metastable atom to the polyprotonated precursor is consistent with observations in ETD, although MAD is inhibited to a smaller extent than ETD [140]. The nitration modification was retained in all cases when dissociating the 3+ precursor. The hydrogen radical loss from the charge-reduced species, [M+3H]²⁺, is the most intense peak within the charge-reduced isotope envelope in Figure 3.1c; in fact this is the case for all positively charged peptides in this study. However, when evaluating the isotopic distribution of the charge-reduced species, electron transfer is still apparent by the observation of the [M+3H]²⁺ peak (Figure 3.1c). A mass isolation window of m/z 1 was possible in this case because of the stable nature of the nitrated peptides studied. The smaller isolation window reduced the contribution of ¹³C to the intensity of the charge-reduced peak. Hydrogen radical neutral loss from the charge-reduced species is also noted in ECD [140, 143]. In each of the three charge states studied, the nitration modification is unambiguously identified to be bound to the fourth amino acid residue.

Figure 3.1d-f shows comprehensive backbone cleavage for the 1+, 2+, and 3+ precursor ions of the synthetic peptide GPLEnYGFAKGPLAK. A total of forty-two distinct a-, b-, c-, x-, y-, and z-type fragment ions, out of a total of seventy-eight, are produced and complete peptide sequencing is achieved when combining different charge state product ions. MAD of the 1+ precursor ion (Figure 3.1d) produced twenty-five product ions as well as three 2+ product ions and the PI product ion species, [M+H]²⁺. The major fragmentation pathway tends to be the Cα–C bonds with eleven total a-/x-type
ions. An additional nine C–N bond cleavages and four N–Cα bond cleavages were also observed. The observation of the w3 ion, a neutral loss of 43 Da from the z3 ion, allows for the differentiation of leucine from isoleucine at position 12. Out of ten possible fragment ions containing the nitrated tyrosine, all ten retained the modification thereby allowing for PTM site determination. The most notable feature in the MAD spectrum of the 2+ species shown in Figure 3.1e are the neutral losses from the charge-reduced species [M+2H]⁺. The four neutral losses are 18, 36, 53, and 70 Da, which correspond to [M+ 2H - H2O]⁺, [M+ 2H - 2(H2O)]⁺, [M+ 2H - 2(H2O) -NH3]⁺, and [M+ 2H - 3(H2O)-NH3]⁺, respectively. The first two ions (loss of 18 and 36 Da) are more abundant than the [M+H]⁺ species which corresponds to a neutral loss of a hydrogen radical from the charge-reduced species, [M+2H]⁺. Similar neutral losses are also observed when dissociating the same nitrated peptide through ECD [140]. For MAD of the 2+ precursor, a total of twenty-four product ions are produced with backbone cleavage between every amino acid residue with the exception of the first two, as illustrated in Figure 3.1e. Again, the w3 ion was observed, thereby enabling the 12th residue to be confirmed to be leucine. Similar to the 1+ precursor for this peptide (Figure 3.1d), no neutral losses associated with the loss of the nitrated PTM were observed and eleven fragment ions containing the nitration modification were observed. The major fragmentation pathway observed was cleavage of the C–N bonds, producing thirteen b-/y-type ions.

MAD of the 3+ species, Figure 3.1f, produced twenty-eight different product ions and two charge-reduced species, [M+3H]²⁺ and [M+2H]⁺. The [M+2H]⁺ product presumably results from two subsequent charge reduction reactions. The most intense peaks within the charge-reduced isotopic distributions correspond to the neutral loss of a
hydrogen radical producing the $[M+2H]^{2+}$ and $[M+H]^+$ species. Again neutral losses of 18 and 36 Da were observed for both the $[M+3H]^{2+}$ and $[M+2H]^{3+}$ species and a neutral loss of 53 Da from the $[M+2H]^{3+}$ species. No neutral losses of the modification were observed, so thirteen different PTM-containing fragment ions were available for nitration site determination. The $w_3$ ion is again observed, thereby enabling the 12$^{th}$ residue to be confirmed to be leucine. The majority of the backbone cleavages produced b-/y-type ions as similarly noted for MAD of the 2+ species. Again, using MAD, characterization of the nitration modification was unambiguously assigned to the 5$^{th}$ amino acid, tyrosine.

3.3.2 *Cysteine Nitrosylated Peptides*

Figure 3.2a-e shows MAD spectra of the 1+, 2+, and 3+ charge states of the S-nitrosylated peptide EMFTYInCNHIK (205-215 of eNOS) and the 2+ and 3+ charge states of the S-nitrosylated peptide VFDARDnCRSAQ (194-204 of eNOS). Isolation of the latter 1+ peptide was not possible because of the labile nature of this PTM. The precursor mass of each of the peptides had an increase of 29 Da relative to the unmodified peptides, confirming the addition of an $–NO$ group. Again, the fragments with a neutral loss of the PTM modification were omitted from the peptide sequence inserts in Figure 3.2. Due to the extremely labile nature of the S-nitrosylation [144, 145], more gentle MS parameters and wider $m/\zeta$ windows of 5-6 were required to isolate the precursor ions of the modified peptides. A higher percentage of S-nitrosylated neutral losses (30 Da) were detected relative to nitrated tyrosine containing peptides. The disruption of the S–NO bond has been observed to easily break under the most modest gas phase conditions [144].
When combining the product ions observed for the 1+, 2+, and 3+ charge states of the EMFTYInCNHIK peptide, MAD produced thirty-seven, out of a possible sixty, distinct fragment ions (Figure 3.2a, b, and c). MAD of the 1+ precursor, illustrated in Figure 3.2a, produced eleven fragment ions and an intense PI product species, [M+H]^{2+}, with neutral losses of the S-nitrosylation from both the precursor ion and the PI product ion. Forty five percent of the backbone cleavages in Figure 3.2a were between the C\textsubscript{\alpha}–C bonds producing a-/x-type ions and 36% cleaved the N–C\textsubscript{\alpha} bond (c-/z-type ions). Of the six detected fragments which contained the modified cysteine residue, only three retained the S-nitrosylation group. S-nitrosylation PTM site determination was therefore difficult or unambiguous with the [M+H]\textsuperscript{+} precursor. More comprehensive dissociation was achieved for the 2+ precursor of the peptide (Figure 3.2b), producing thirty product ion types. However, sixteen of the nineteen fragment ions that could potentially give modification site information didn’t retain the S-nitrosylation, thereby hindering PTM location. For MAD of the 2+ precursor (Figure 3.2b), complete peptide sequencing was achieved but the site of the PTM could not be unambiguously assigned. For the 2+ precursor 37% of the fragment ions cleaved the C–N bonds, with the majority being y-type ions. N–C\textsubscript{\alpha} and C\textsubscript{\alpha}–C bond cleavages are equally abundant at about 31% each. MAD of the 3+ precursor ion also achieved complete sequencing information by producing thirty-five different product ions (including those which lost the S-nitrosylation) as shown in Figure 3.2c. In contrast to the dissociation of the 1+ and 2+ species, ten
Figure 3.2. He MAD spectra of S-nitrosylated EMFTYInCNHIK: (a) [M+H]$^+$, (b) [M+2H]$^{2+}$, (c) [M+3H]$^{3+}$, and He MAD spectra of S-nitrosylated VFDARDnCRSAQ: (d) [M+2H]$^{2+}$, (e) [M+3H]$^{3+}$. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)
different product ions retained the labile S-nitrosylation, thereby successfully determining the modification site on the cysteine as opposed to the tyrosine or threonine residues. A larger percentage of product ions retain the C-terminus than the N-terminus because of the two basic amino acid residues at the C-terminus.

When combining the dissociation of both the 2+ and 3+ precursors of the VFDARRnCRSAQ peptide (Figure 3.2d and e), at least one cleavage between each amino acid is observed resulting in twenty-one distinct product ions in total. Cleavage of the Cα–C bond is the main backbone fragmentation observed when dissociating the 2+ species (Figure 3.2d) with nine a-/x-type ions or 42% of the total product ions. The main dissociation pathway observed is the neutral loss of 30 Da from both the PI, [M+2H]^{3+*}, and the [M+2H]^{2+*} species, corresponding to the loss of NO from the modification site. Figure 3.2e shows the dissociation of the 3+ species with nineteen evenly distributed backbone cleavages. The two main peaks are the [M+2H]^{2+*} species (the charge-reduced species with a hydrogen radical loss) and the neutral loss of the NO from the [M+3H]^{2+*} species. For the 2+ and 3+ precursor ions, less than half of the product ions containing the nitrosylated cysteine residue retained the modification.

3.3.3 Effect of Modification on Peptide Backbone Cleavage

There is evidence that PTMs can have an adverse effect on the fragmentation efficiency of some electron-induced dissociation methods. Cooper and co-workers show that phosphorylation modifications can interact with basic residues decreasing the amount of ECD backbone cleavages in 2+ species [146]. Therefore, determining how a certain modification affects fragmentation efficiencies and type of fragment ions observed needs
to be further explored. Jones et al. provide evidence that ECD of nitrotyrosine-containing peptides severely inhibits N–Cα backbone cleavages [140]. In fact, for 2+ species, little backbone dissociation—except for small neutral losses from the charge-reduced species—is observed. More cleavages are noted for ECD of the 3+ species, most likely due to coulombic repulsion extending the peptide conformation when not in close proximity to the modification [140]. This phenomenon is explained in two separate studies by Sohn et al. and Tureček which have shown that ECD and ETD backbone fragmentation is completely inhibited when high EA (>1 eV) nitrobenzyl moieties are present [138, 139]. S-nitrosylation has also been shown to hinder the efficiency of ETD. ETD of S-nitrosylated insulin chain B was investigated by Mikesh et al. with very little backbone cleavage and PTM retention observed [133]. Mikesh et al. concluded that the neutral loss of the NO from the charge-reduced species could act as its own proton transfer reagent, which ultimately decreases fragmentation yields.

Comparison of MAD and CID spectra of the nitrated and S-nitrosylated peptides, DRVnYIHPF, VFDARDnCRSAQ, and EMFTYInCNHIK, and their unmodified equivalents in the 2+ charge state are shown in Figures 3.3-3.5 respectively. MAD of the modified peptides produced enough dissociation to confer complete sequencing information, which has not been demonstrated for ECD and ETD. When comparing the amount and type of backbone cleavages observed with MAD and CID (Figure 3.6 and Table 3.1) we note that CID alone is unable to obtain enough information to sequence the peptides and determine unambiguously the modification site locations. In fact, MAD produces almost two times more fragment ion types than CID that contain modification
Figure 3.3. Comparison of the 2+ charged peptide DRVYIHPF: (a) MAD of non-nitrated DRVYIHPF, (b) MAD of nitrated DRVnYIHPF, and (c) CID of nitrated DRVnYIHPF. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)
Figure 3.4. Comparison of the 2+ charged VFDARDCRSAQ: (a) MAD of non-nitrosylated VFDARDCRSAQ, (b) MAD of S-nitrosylated VFDARDnCRSAQ, and (c) CID of S-nitrosylated VFDARDnCRSAQ. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)
Figure 3.5. Comparison of the 2+ charged EMFTYICNHIK: (a) MAD of non-nitrosylated EMFTYICNHIK, (b) MAD of S-nitrosylated EMFTYInCNHIK, and (c) CID of S-nitrosylated EMFTYInCNHIK. The precursor is indicated by an arrow and the neutral loss of the modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)
unmodified and modified peptides. We observe a slight decrease in the number of information. Figure 3.7 and Table 3.2 compares the total number and types of fragment ions for the spectra in Figures 3.3-3.5 produced for the 2+ charge state of the backbone cleavage types when either modification is present (nitrosylated cysteine or nitrated tyrosine), with reductions between 5 and 42%. Nonetheless, due to the ample dissociation of the modified peptides, these reductions do not undermine the ability to sequence the peptides independent of the PTM.

![Venn diagrams comparing the different types of fragment ions produced by He MAD and CID: (a) [DRVnYIHPF+2H]2+, (b) [VFDARDnCRSAQ+2H]2+, and (c) [EMFTYInCNHIK+2H]2+. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)](image)

A direct comparison of MAD of the non-nitrated and nitrated species of the peptide GPLEYGFAKGPLAK was unattainable; however we are still able to make some conclusions based on the MAD spectra of the nitrated form of this peptide species (Figure 3.1d, e, and f). Regardless of precursor charge state, extensive backbone cleavage is observed including cleavage in and around the vicinity of the modification. When taking into account those fragments which undergo a neutral loss of the modification, all of the
modified peptides studied contain at least two and as many as five backbone cleavage ion types directly adjacent to the modified residue. In contrast, fragmentation is inhibited near the site of nitration or S-nitrosylation in ECD and ETD [133, 140]. MAD fragmentation appears to not suffer the same inhibitions as ECD and ETD. There are two possible explanations for this contrasting behavior between MAD and ETD/ECD. The first explanation relates to the unique nature of metastable atom chemistry. Metastable atoms at the molecular level behave like positive ions and are known to be attracted to regions of high electron density or polar groups [62, 65]. In MAD, radicals can be

Table 3.1.

*Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD and CID methods on: (a) [DRVnYIHPF+2H]^{2+}, (b) [VFDARDnCRSAQ+2H]^{2+}, and (c) [EMFTYInCNHIK+2H]^{2+}. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)*

<table>
<thead>
<tr>
<th>a) Product ions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAD</td>
</tr>
<tr>
<td>CAD</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b) Product ions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAD</td>
</tr>
<tr>
<td>CAD</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c) Product ions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAD</td>
</tr>
<tr>
<td>CAD</td>
</tr>
</tbody>
</table>

...
directly generated anywhere along the peptide backbone where there is a polar group or lone pair of electrons, such as carbonyl oxygen atoms [68]. In ECD and ETD, radical migration must instead occur from original sites of protonation through long-range interactions with the carbonyl oxygen atoms along the backbone producing aminoketyl radicals. Whether the radical or hydrogen atoms are transferred first in ETD and ECD, these techniques are apparently more easily hindered by high electron- or hydrogen-affinity groups than MAD [139].

The second explanation for the difference between MAD and ETD/ECD fragmentation behavior may be due to high levels of secondary reactions in MAD. It was shown in our previous work that longer exposure times and more energetic He metastable atom exposure causes an increase in the relative abundance of low mass ions at the expense of high mass ions, most likely due to secondary reactions [68]. Although primary metastable atom reactions with the oxygen atom lone pairs of the –NO or –NO\(_2\) modifications could result in radical sequestering without fragmentation, a second metastable atom interaction with the same peptide at a different location could induce the observed dissociation.

Figure 3.7 and Table 3.2 compares the different amount and types of fragment ions produced for the modified and unmodified peptides DRVYIHPF, VFDARDCRSAQ, and EMFTYICNHMK after He MAD exposure. Even though, in Figures 3.3-3.5, we observe backbone cleavage adjacent to the modified residues, a greater abundance of these types of cleavages are observed with the unmodified peptides. This is especially noted in Figure 3.7a and c, which have the greater reduction of overall cleavages. These two observations may suggest that additional sites of lone pairs of electrons, such as
those found on the –NO or –NO₂ groups of the PTMs, in fact compete for the initial metastable atom interaction.

Figure 3.7. Venn diagrams comparing the number of fragmentation ion types produced after He MAD of the modified and unmodified peptides: (a) [DRVYIHPF+2H]2+, (b) [VFDARDCRSAQ+2H]2+, and (c) [EMFTYICNHK+2H]2+. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)

One overall trend observed for the three peptides in Figures 3.3-3.5 is that MAD of the non-modified peptides produced a higher percentage of N–Cₐ backbone cleavages compared to the modified (nitrated and S-nitrosylated combined) peptides. The modified peptides typically produced higher percentages of Cₐ–C and C–N bond cleavages. A comprehensive analysis of a larger pool of peptides with varying types of modifications will need to be assessed before confirming trends in fragmentation behavior in MAD.
Table 3.2.

Comparison of the Type and Charge of the Fragmentation Ions Produced After He MAD of the Modified and Unmodified Peptides: (a) [DRVYIHPF+2H]^{2+}, (b) [VFDARDnCRSAQ+2H]^{2+}, and (c) [EMFTYInCNHIK+2H]^{2+}. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)

<table>
<thead>
<tr>
<th>a)</th>
<th>Product ions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Nitrated</td>
<td>( b_1, c_1, a_1, b_2, c_2, a_2, b_3, c_3, a_3, b_4, c_4, a_4, b_5, c_5, a_5, b_6, c_6, a_6, b_7, c_7, a_7, b_8, c_8, a_8, b_9, c_9, a_9, b_{10}, c_{10}, a_{10}, b_{11} )</td>
</tr>
<tr>
<td>Nitrated</td>
<td>( b_1, c_1, a_1, b_2, c_2, a_2, b_3, c_3, a_3, b_4, c_4, a_4, b_5, c_5, a_5, b_6, c_6, a_6, b_7, c_7, a_7, b_8, c_8, a_8, b_9, c_9, a_9, b_{10}, c_{10}, a_{10}, b_{11} )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b)</th>
<th>Product ions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Nitrosylated</td>
<td>( b_1, c_1, a_1, b_2, c_2, a_2, b_3, c_3, a_3, b_4, c_4, a_4, b_5, c_5, a_5, b_6, c_6, a_6, b_7, c_7, a_7, b_8, c_8, a_8, b_9, c_9, a_9, b_{10}, c_{10}, a_{10}, b_{11} )</td>
</tr>
<tr>
<td>Nitrosylated</td>
<td>( b_1, c_1, a_1, b_2, c_2, a_2, b_3, c_3, a_3, b_4, c_4, a_4, b_5, c_5, a_5, b_6, c_6, a_6, b_7, c_7, a_7, b_8, c_8, a_8, b_9, c_9, a_9, b_{10}, c_{10}, a_{10}, b_{11} )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>c)</th>
<th>Product ions observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Nitrosylated</td>
<td>( a_2, b_2, c_2, a_3, b_3, c_3, a_4, b_4, c_4, a_5, b_5, c_5, a_6, b_6, c_6, a_7, b_7, c_7, a_8, b_8, c_8, a_9, b_9, c_9, a_{10}, b_{10}, c_{10}, a_{11} )</td>
</tr>
<tr>
<td>Nitrosylated</td>
<td>( a_2, b_2, c_2, a_3, b_3, c_3, a_4, b_4, c_4, a_5, b_5, c_5, a_6, b_6, c_6, a_7, b_7, c_7, a_8, b_8, c_8, a_9, b_9, c_9, a_{10}, b_{10}, c_{10}, a_{11} )</td>
</tr>
</tbody>
</table>

3.3.4 Effect of Charge State on Peptide Backbone Cleavage

The amount and type of backbone cleavages observed through MAD of the 1+, 2+, and 3+ charge states of the modified peptides, DRVnYIHPF, GPLEnYGFAKGPLAK, and EMFTYInCNHIK, and the 2+ and 3+ charge state of VFDARDnCRSAQ are compared in Figure 3.8 and Table 3.3. The most striking observation is the vast difference in product ion types observed from the different precursor ion charge states. When comparing the three different charge states examined
for a given peptide, only 13%, 6%, and 16% (Figure 3.6 a, b, and c, respectively) of the total product ions observed are common to all charge states. This leads us to infer that the types of fragment ions produced by MAD are highly dependent on the charge state. There are at least three reasons for the differences in fragmentation types observed for MAD of the same peptide in different charge states. 1) A difference in mechanism; e.g. electron capture versus Penning ionization, 2) a difference in degree; e.g. electron capture of the 3+ precursor is more exothermic than electron capture of the 2+ precursor, or 3) a difference in the conformation of the peptide prior to interaction with the metastable atoms.

There is no observable trend between the precursor ion charge state and the product ion types observed in Figure 3.8. For example, MAD of the 3+ species of angiotensin II and the 1+ species of EMFTYInCNHIK produced the fewest fragment ion types of the 3+ charge states observed for the specific peptide (Figure 3.8a and 3.8b, respectively). However it was the 2+ precursor species that produced the fewest fragment ion types (Figure 3.8c).

![Venn diagrams comparing the number of fragment ion types produced by He MAD of the 1+, 2+, and 3+ charge states of the modified peptides: (a) DRVnYIHPF, (b) EMFTYInCNHIK, and (c) GPLEnYGFAKGPLAK.](image-url)
Table 3.3.

Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD of the Singly-, Doubly-, and Triply-Charged Modified Peptides: (a) DRVnYIHPF, (b) EMFTYInCNHIK, and (c) GPLEnYGFAKGPLAK. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)

<table>
<thead>
<tr>
<th>a) Precursor charge state</th>
<th>b) Precursor charge state</th>
<th>c) Precursor charge state</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M+H]^+</td>
<td>[M+2H]^+</td>
<td>[M+3H]^+</td>
</tr>
<tr>
<td>b3 _1^</td>
<td>b3 _1^</td>
<td>a3 _1^</td>
</tr>
<tr>
<td>b3 _1^</td>
<td>c3 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>c3 _1^</td>
<td>c3 _1^</td>
<td>a3 _1^</td>
</tr>
<tr>
<td>a4 _1^</td>
<td>a4 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>b4 _1^</td>
<td>b4 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>c4 _1^</td>
<td>c4 _1^</td>
<td>a3 _1^</td>
</tr>
<tr>
<td>a5 _1^, _2^</td>
<td>a5 _1^, _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>b3 _1^, _2^</td>
<td>b3 _1^, _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>a7 _2^</td>
<td>a7 _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>b7 _2^</td>
<td>b7 _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>c7 _1^, _2^</td>
<td>c7 _1^, _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>y1 _1^</td>
<td>y1 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>y3 _1^</td>
<td>y3 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>z3 _1^</td>
<td>z3 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>w4 _1^</td>
<td>w4 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>y4 _1^</td>
<td>y4 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>z4 _1^</td>
<td>z4 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>x5 _1^</td>
<td>x5 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>y5 _1^</td>
<td>y5 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>x6 _1^</td>
<td>x6 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>y6 _1^</td>
<td>y6 _1^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>x7 _2^</td>
<td>x7 _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>z7 _2^</td>
<td>z7 _2^</td>
<td>b3 _1^</td>
</tr>
<tr>
<td>b2 _1^</td>
<td>b2 _1^</td>
<td>a3 _1^</td>
</tr>
<tr>
<td>b2 _1^</td>
<td>c3 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>c3 _1^</td>
<td>c3 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>a5 _1^</td>
<td>a5 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>b4 _1^</td>
<td>b4 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>c4 _1^</td>
<td>c4 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>a5 _1^, _2^</td>
<td>a5 _1^, _2^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>b3 _1^, _2^</td>
<td>b3 _1^, _2^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>a7 _2^</td>
<td>a7 _2^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>b7 _2^</td>
<td>b7 _2^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>c7 _1^, _2^</td>
<td>c7 _1^, _2^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>y1 _1^</td>
<td>y1 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>y3 _1^</td>
<td>y3 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>z3 _1^</td>
<td>z3 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>w4 _1^</td>
<td>w4 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>y4 _1^</td>
<td>y4 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>z4 _1^</td>
<td>z4 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>x5 _1^</td>
<td>x5 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>y5 _1^</td>
<td>y5 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>x6 _1^</td>
<td>x6 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>y6 _1^</td>
<td>y6 _1^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>x7 _2^</td>
<td>x7 _2^</td>
<td>b2 _1^</td>
</tr>
<tr>
<td>z7 _2^</td>
<td>z7 _2^</td>
<td>b2 _1^</td>
</tr>
</tbody>
</table>
types for the peptide GPLEnYGFAKGPLAK (Figure 3.8c). For the three peptides studied, the 3+ precursor ions tend to produce more c-/z-type ions, whereas the 1+ precursors tend to produce more a-/x-type ions. The 2+ precursor ions typically produce more b-/y-type ions. These trends have not been validated against a larger database of precursor ions.

3.3.5 S-Nitrosylated and Nitrated Peptide Anions

Figure 3.9 shows the He MAD spectra of three 2- peptides; two contain an S-nitrosylated modification (Figure 3.9a and b) and one contains a nitrated modification (Figure 3.9c). In all cases, the most abundant dissociation channels are neutral losses from the charge-reduced species including H$_2$O (18 Da), CO$_2$ (44 Da), and either NO or NO$_2$ (30 or 46 Da, respectively). All the neutral losses observed from the charge-reduced species for the three peptides are listed in Table 3.4 and are indicated with a dot (•) within the spectra in Figure 3.9. Water and carbon dioxide groups are common neutral losses observed in other electron-induced dissociation methods on peptide anions, such as electron detachment dissociation (EDD) [50, 51] and negative electron transfer dissociation (nETD) [54, 147]. When dissociating protonated ions with MAD, the neutral loss of a hydrogen radical from the charge-reduced species is usually more abundant than the charge-reduced product ion. Deprotonated peptides also show signs of significant hydrogen radical loss, although not quite as abundant as when fragmenting cationic precursor ions. When comparing the $^{13}$C envelope to the expected envelope (Figure 3.9 a-c), neutral losses of hydrogen radicals are still apparent. Figure 3.9a and b show the He MAD spectra of two S-nitrosylated peptides, both containing a modification
Figure 3.9. He MAD spectra of doubly-deprotonated S-nitrosylated: (a) EMFTYInCNHIK, (b) VFDARDnCRSAQ, and (c) GPLEnYGFAKGPLAK. The small inserts compare the actual and expected (vertical lines) isotopic distribution. The precursor is indicated by an arrow, neutral losses from the charge-reduced species are indicated by a dot and the neutral loss of modification is indicated by an asterisk. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)
Table 3.4.

List of the Neutral Losses Observed from the Charge-Reduced Species, \([\text{M-2H}]^+\), After He MAD of the 2-Peptide Ions. (Reproduced with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 221-232.)

<table>
<thead>
<tr>
<th>[M-2H]² peptide ion</th>
<th>Neutral loss (Da)</th>
<th>Proposed loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMFTYInCNHIK</td>
<td>-30</td>
<td>-NO</td>
</tr>
<tr>
<td></td>
<td>-45</td>
<td>-CO₂, -H</td>
</tr>
<tr>
<td></td>
<td>-62</td>
<td>-CO₂, -H₂O</td>
</tr>
<tr>
<td></td>
<td>-75</td>
<td>-NO, -CO₂, -H</td>
</tr>
<tr>
<td></td>
<td>-92</td>
<td>-NO, -CO₂, -H₂O</td>
</tr>
<tr>
<td></td>
<td>-107</td>
<td>-SNO, -CO₂, -H</td>
</tr>
<tr>
<td>VFDARDnCRSAQ</td>
<td>-18</td>
<td>-H₂O</td>
</tr>
<tr>
<td></td>
<td>-30</td>
<td>-NO</td>
</tr>
<tr>
<td></td>
<td>-45</td>
<td>-CO₂, -H</td>
</tr>
<tr>
<td></td>
<td>-49</td>
<td>-NO, -H₂O, -H</td>
</tr>
<tr>
<td></td>
<td>-62</td>
<td>-CO₂, -H₂O</td>
</tr>
<tr>
<td></td>
<td>-75</td>
<td>-NO, -CO₂, -H</td>
</tr>
<tr>
<td></td>
<td>-92</td>
<td>-NO, -CO₂, -H₂O</td>
</tr>
<tr>
<td></td>
<td>-107</td>
<td>-SNO, -CO₂, -H</td>
</tr>
<tr>
<td>GPLEnYGFAKGPLAK</td>
<td>-18</td>
<td>-H₂O</td>
</tr>
<tr>
<td></td>
<td>-36</td>
<td>-H₂O, -H₂O</td>
</tr>
<tr>
<td></td>
<td>-45</td>
<td>-CO₂, -H</td>
</tr>
<tr>
<td></td>
<td>-62</td>
<td>-CO₂, -H₂O</td>
</tr>
<tr>
<td></td>
<td>-90</td>
<td>-CO₂, -NO₂</td>
</tr>
<tr>
<td></td>
<td>-108</td>
<td>-CO₂, -NO₂, -H₂O</td>
</tr>
</tbody>
</table>

Note: The neutral losses are indicated with a dot in the corresponding spectra.

on the 7th residue. A variety of ion types are observed for each peptide, with a majority of a-/x-type ions for the peptide in Figure 3.9a and a majority of c- and y-type ions in Figure 3.9b. In both cases, 70% of information required for sequencing is obtained through the production of ten (Figure 3.9a) and thirteen (Figure 3.9b) distinct fragment ions, including a modification retention percentage of 50% and 43%, respectively. In these two cases, the modification site can be unambiguously identified.

Figure 3.9c shows the MAD spectrum observed for the 2- precursors of the nitrated peptide GPLEnYGFAKGPLAK. Similar to the peptide in Figure 3.9a, a-, b-, and x-type ions are most common. Because of the more stable nature of the nitration
modification for the peptide in 3.9c, all fragment ions retain the modification and enable PTM site determination. Furthermore, no observable trend can be noted based on ion types produced, in contrast to EDD and nETD, which preferentially produce a-/x-type ions. That the fragmentation of EMFTYInCNHIK and VFDARDnCRSAQ appear to be directly adjacent to the highly electronegative nitrosylated cysteine residues supports the proposed mechanism of energy transfer as Penning ionization [58, 68, 106]. The site of preferential fragmentation seems to correlate to the PTM site rather than to the sites of deprotonation.

3.4 Conclusion

Extensive backbone cleavage of both nitrated and S-nitrosylated peptides were obtained after exposure to a high kinetic energy beam of He metastable atoms. MAD is less sensitive than ECD and ETD to the presence of nitrotyrosine, which can act as an electron predator and inhibit fragmentation in the latter two techniques. Complete peptide sequencing and PTM location is obtained for the majority of the peptides studied regardless of charge state or ion polarity. Amino acid side-chain cleavages enable Xle differentiation in several cases. Peptides containing nitrosylated cysteine residues prove to be highly labile, thereby making isolation prior to tandem analysis more difficult in general. However, we demonstrate that by combining multiple spectra of different charge states, accurate PTM site location can be achieved using MAD. Because of the unique fragmentation pathways offered through metastable atom chemistry, MAD is a complementary alternative to collisional or other electron-induced fragmentation methods.
CHAPTER 4: METASTABLE ATOM-ACTIVATED DISSOCIATION MASS SPECTROMETRY OF PHOSPHORYLATED AND SULFONATED PEPTIDES IN NEGATIVE ION MODE.


4.1 Introduction

Post-translational modifications (PTMs) are chemical alterations of peptides and proteins following translation that usually provide a specific function such as signaling and activation. The need to unambiguously identify the type of PTM and the exact site of modification is critical to understand the biological function and consequence. Phosphorylation and sulfonation are two examples of modifications that occur on carbohydrates and lipids in addition to proteins and peptides. One of the more common and well-studied modification is phosphorylation: an addition of \(-\text{HPO}_3\) to tyrosine, threonine, and serine amino acid side chains. Phosphorylation detection is an integral part of proteomics because it is a major player in cell regulating mechanisms and signaling pathways which control a wide variety of biological functions [148, 149]. Tyrosine O-sulfonation is a less prevalent PTM, which occurs by a \(-\text{SO}_3\) transfer from phosphoadenosine phosphosulfate (PAPS) to the side chain of tyrosine residues. O-sulfonation is associated with protein transport and the regulation of protein-protein interactions in the extracellular space [150].
For several decades mass spectrometry (MS) and tandem MS (MS/MS) has been used to identify and locate PTMs. Traditionally, the standard polarity and dissociation method used to analyze PTMs is positive ion mode and CID, respectively. Although there are thousands of articles on PTM analysis in positive ion mode within the past decade, less than 1% of the publications on PTM analysis have been dedicated to the use of negative ion mode. Despite the advances in PTM detection with MS/MS, there are some major drawbacks. Traditional CID preferentially cleaves side chain PTMs rather than the peptide backbone, thereby leading to ambiguous site determination. Even though phosphorylation modifications tend to be among the more stable PTMs, Palumbo and Reid have shown evidence that under positive mode CID conditions, the phosphate groups can migrate to different amino acids and thereby results in ambiguous or unambiguously incorrect PTM site determination [151]. In addition, highly acidic peptides, such as those that contain phospho- and/or sulfo- groups, are difficult to observe in positive ion mode because of their low pKa values and poor ionization efficiency [152, 153]. However, acidic peptides have been shown to ionize more readily in negative ion mode [153, 154]. O-sulfonation, which is highly labile in positive ion mode, has shown to be slightly more stable in negative ion mode [152, 155]. To date, the ability to routinely determine both phosphorylation and sulfonation modifications in anions, especially in highly acidic peptides and proteins, is not a routine mass spectrometry process.

To combat some of these issues, alternative dissociation techniques utilizing electron-radical chemistry such as electron capture and electron transfer dissociation (ECD and ETD) have become the preferred new way to sequence and analyze modified peptides and proteins [30, 31, 132, 133]. These two methods have the ability to retain
labile side chain modifications while achieving extensive N–C\textsubscript{\alpha} bond cleavage of multiply-charged cations. ECD and ETD have enhanced the ability for peptide sequencing and PTM site determination. To date, both methods have been successfully employed to determine phosphorylation [131, 156], glycosylation [134, 135, 136], and sulfonation [132, 133] modifications, among others. On the other hand, it has been recently shown that certain modifications, such as phosphorylation [146] and nitration [138, 139, 140], have hindered ECD and ETD sequence coverage and modification identification. Liu and Håkansson showed that highly acidic peptides with no basic amino acids produced complete loss of the sulfonated modification, thereby rendering site determination impossible by ECD [157]. To achieve improved ionization efficiency and modification retention of highly acidic sulfonated peptides in ECD, charge enrichment through the formation of a divalent metal cation–peptide complex is necessary. In a similar experiment, Medzihradszky et al. investigated the labile nature of sulfonated peptides that contained only one basic amino acid employing both ETD and ECD. Under native ETD and ECD conditions, 15% (in the best case) of the –SO\textsubscript{3} modification retention was observed for the multiply protonated cations [158]. The modification retention improved to 73% (in the best case) when the peptides were complexed with Na\textsuperscript{+}.

Traditionally, ECD and ETD were only applicable to multiply protonated species. Recently, new methods such as electron detachment dissociation (EDD), negative ETD (NETD), and negative-ion mode ECD (niECD) have been explored and applied to peptide anions. EDD, first applied to sulfonated di-anions in 2001 by Zubarev and co-workers, results in C\textsubscript{\alpha}–C peptide backbone cleavage by irradiating peptide anions with
fast electrons (>10 eV), which induces electron detachment from the peptides [50]. Similar to ECD, EDD requires costly FT-ICR instruments and has proven to be an inefficient process with interaction times on the scale of several seconds [52, 53]. Coon et al. first demonstrated NETD or ion/ion reactions of xenon radical cations with polyanions resulting in Cα–C peptide backbone cleavage and neutral losses of CO₂ and HPO₃ from the product ions [54]. More recently, Huzarska et al. utilized a fluoranthene radical (C₁₆H₁₀)⁺⁺ as the NETD reagent cation to induced EDD like fragmentation through the abstraction of an electron from the peptide anion by the reagent cation radical leaving the phosphorylation modification intact [55]. The difference in the degree of modification retainment in NETD is attributed to the difference in the recombination energy of the electron transfer process between the two reagent ions studied; 6.7-8.7 eV for Xe⁺⁺ and 2.5-4.5 eV for the fluoranthene radical. Håkansson et al. unveiled impressive results exploring the ECD version for peptide anions termed negative-ion ECD at the 58th ASMS Conference on Mass Spectrometry and Allied Topics in Salt Lake City. However, little information is available to date [137, 159].

In the last five years, a new type of dissociation method has been introduced in RF ion trap instruments, which uses metastable atoms as the electron source or potential energy source to induce fragmentation. Extensive backbone cleavages in the form of a-, b-, c-, x-, y-, and z-type ions, and PTM retainment, is achieved through the interaction of isolated precursor ions with high or low kinetic energy beam of Noble gas metastable atoms. Our research shows evidence that peptide dissociation occurs through two competing mechanisms, Penning ionization (PI) and charge reduction [68, 160]. The later mechanism is very similar to ECD and ETD. PI occurs when the potential energy of the
metastable atom is greater than the ionization potential of the biomolecular ion and has
been well characterized for neutrals [57, 160]. Metastable atoms are attracted to, and
therefore most reactive with, regions of high electron density such as the lone pairs on
nitrogen atoms or carbonyl oxygen atoms that are found throughout the peptide backbone
and in amino acid side chains. Several papers have been devoted to this new type of
research, characterizing multiply- and singly-charged cations, phosphorylated peptides,
disulfide bond cleavage, isoleucine/leucine differentiation, and proline ring cleavage [58,
67, 68, 69, 106, 160]. However, a small portion of these studies has been dedicated to the
study of the interaction between metastable atoms and peptide anions [58, 68, 160]. To
date, our group has been the only group to explore PTMs on peptide anions using MAD
[160, 161].

In this present study, we investigate the ability of MAD to characterize acidic
phosphorylated and sulfated peptide anions. The majority of fragment ions observed are
a-/x-type ions which correspond to Cα–C peptide backbone cleavages and is consistent
with fragmentation observed in EDD. Additionally, we observed modest neutral losses of
the phosphorylation and the sulfonation modification in comparison to CID. We compare
the non-modified and modified 2- forms of the phosphorylated and sulfonated peptides.
The PTMs did not appear to significantly hinder fragmentation, unlike nitrosylation
[160]. Sequence coverage for the 3- forms of the phosphorylated and sulfonated peptides
was superior to the 2- charge states. Due to the unique chemistry of MAD, we were also
able to acquire MS/MS from the highly acidic 1+ peptides that contain no basic amino
acids. Complete peptide sequencing with unambiguous sulfonation site determination
was also possible in positive ion mode; a unique feature of this technique.
4.2 Experimental

4.2.1 Instrumentation

All of the experiments were performed on a modified Bruker EsquireLC QIT MS (Bruker Daltonics, Bremen, Germany) which contains a 2 mm hole in the top portion of the ring electrode. The metastable atoms are generated by a modified Ion Tech FAB gun (P50 PSU, Teddington, UK), which is centered directly over the ring electrode hole. A deflection electrode positioned and mounted 1 cm from the exit orifice of the FAB gun deflects the majority of the free electrons and ions from entering the trap allowing neutral metastable atoms to enter the top hole of the ring electrode and interact with the isolated precursor ions in the center of the trap. One electrode on the deflection electrode was grounded and the other was biased to +800 V using a high voltage power supply (EL 03R 15L, Glassman High Voltage Inc., High Bridge, NJ). To achieve a pulsed beam of high kinetic energy metastable atoms, the FAB gun anode power supply was replaced with a 10 kV high voltage amplifier (ANT 10B10, Matsusada Precision Inc., Shiga, Japan). To pulse the metastable atom beam on only during the mass spectrometry duty cycle designated for dissociation, a trigger was taken from the Bruker EsquireLC corresponding to the start of ion accumulation and fed to a function/arbitrary waveform generator (33250A, Agilent, Santa Clara, CA). Complete instrumentation modification and pulse operation details has been previously described [68]. In order to decrease background ion signal, the FAB gun was turned on at a constant voltage between 5-6 V to ‘pre-burn’ away residual contaminants for 30 minutes prior to use each day. Although the lack of commercially available instrumentation may hinder the adoption of MAD by
other laboratories, the hardware requirements necessary to achieve MAD are no more
arduous than those required to achieve ETD. The main difference between adding MAD
to a commercial instrument versus adding ETD is the requirement for a straight line-of-
sight path for the metastable atoms to interact with the isolated ion cloud. ETD uses
charge reagent anions, which can be steered using ion optics.

4.2.2 Preparation of Peptides

All of the following peptides were provided by Protea Biosciences Inc.
(Morgantown, WV): the phosphorylated and non-phosphorylated forms of angiotensin II,
cholecystokinin (10-20) and calcitonin (15-29); the sulfated and non-sulfated forms of
cholecystokinin (26-33), leucine-enkephalin, and hirudin. Methanol (HPLC grade),
ammonium hydroxide, and glacial acetic acid were purchased from Sigma-Aldrich (St.
Louis, MO). All peptides were reconstituted in a 1:1 mixture of methanol and water with
either 1% acetic acid or 1% ammonium hydroxide (for the positive and negative ion
mode, respectively) to provide solutions between 20-40 μM of the individual peptides.
Ultrahigh-purity He (Airgas, Parkersburg, WV) was further purified on-line with a noble
gas purifier (HP2, VICI, Houston, TX) before use in the FAB gun.

4.2.3 Method

An electronic syringe pump (BM-1000, Protea Bioscience Inc, Morgantown, WV)
was utilized to directly inject all peptides at a flow rate of 200 μL/hour. Depending on the
modification and precursor charge state, the ion isolation windows varied between m/z 1-
5. Ion accumulation was set to 15-25 ms to reach an ion target of 20,000. Depending on
peptide and precursor charge state, the precursor ion intensity ranged from $8 \times 10^4$ – $4 \times 10^5$ AU. All fragment ion identifications and assignments were manually determined based on predicted fragmentation patterns and were within $m/z \pm 0.8$ of the expected product ions. Peaks intensities needed to meet at least 3 times the signal-to-noise ratio to be assigned. The 2-, 3-, and 1+ precursor ions were identified according to the expected $m/z$ value and the presence of an isotope envelope peak at $m/z +0.5$ and $m/z +0.3$, respectively. Setting a trapping voltage which corresponds to a low mass cutoff value above $m/z$ 200-400 (depending on charge state and ion mode) allowed for the removal of additional low mass background ions, which presumably result from Penning ionization of residual pump oil. In theory, the trapping $q_z$ value could be lowered in MAD to permit the detection of ions well below $\sim 1/3$ the precursor ion mass, which would overcome the low-mass-cut-off (LMCO) problem commonly experienced with CID. However, at present our LMCO is limited by the presence of background ions from residual contaminants, so we cannot yet take full advantage of the potential LMCO benefit of MAD.

4.2.3.1 CID.

All peptides were fragmented using the “SmartFrag” option in the Bruker EsquireLC NT 4.5 software, which applied a sweeping collisional energy starting at 20% and ending at 165% of a set value. The fragmentation time was set to 20 ms in all experiments. The collisional energy was set to 0.5-0.8 V depending on precursor mass and compound stability. CID MS/MS data was acquired in 1 minute intervals, which consisted of approximately 30 scans.
4.2.3.2 MAD.

All peptide precursor ions were exposed to a high kinetic energy beam of He metastable atoms with the FAB anode powered at 6 kV. Depending on the charge state of the precursor ion, the metastable atom beam was pulsed on for 75-150 ms while the isolated precursor ions were ‘fragmented’ at a collision energy of 0 V (i.e. no collisional activation). The pressure was measured with a Granville-Phillips Series 343 Bayard-Alpert vacuum gauge. The pressure in the vacuum chamber, outside the ion trap, was kept at 1.8 mbar (uncorrected) with He leaking out of the trap and an additional 1.0 mbar (uncorrected) of the noble gas from the metastable atom source. MAD MS/MS data was acquired in 2 minute intervals, which consisted of approximately 60 scans.

4.3 Results and Discussion

4.3.1 Phosphorylated Peptide Anions

4.3.1.1 Effect of Phosphorylation Modification on Backbone Cleavage

Phosphorylated peptides had a precursor ion mass increase of 79 Da relative to the unmodified peptides, confirming the addition of a –PO$_3$ group. Peptide sequence inserts in all Figures omit those fragment ions that have undergone a neutral loss of the PTM modification. A lower case ‘p’ indicates the amino acid directly to the right is phosphorylated. Figures 4.1a and 4.1b show the non-phosphorylated and the phosphorylated 2-species of the peptide cholecystokinin (IKNLQSLDPpSH), respectively, which contains two serine residues with the 10$^{th}$ residue phosphorylated (Ser-10). PI causes oxidation or a change of +1 of the charge state of a given target molecule or ion. For cations, PI results in a charge increase, but for anions PI results in a
Figure 4.1. Comparison of He MAD and CID spectra of cholecystokinin and calcitonin in the 2- charge state: (a) MAD of non-phosphorylated IKNLQSLDPSH, (b) MAD of phosphorylated IKNLQSLDpSH, (c) CID of phosphorylated IKNLQSLDpSH, (d) MAD of non-phosphorylated DFNKFHTFPQTAIGV, (e) MAD of phosphorylated DFNKFHpTFPQTAIGV, and (f) CID of phosphorylated DFNKFHpTFPQTAIGV. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)
less negative charge. For this reason, the PI products of 1- charge states are most likely to be overall neutral in charge and not observable. The main features of MAD of the 2-peptides in Figures 4.1a and b are the neutral losses from the oxidized species, [M-2H]\(^-\), corresponding to CO, CO\(_2\), and [CO\(_2\) + H\(_2\)O] for both peptides and HPO\(_3\), and [HPO\(_3\) + H\(_2\)O] (or H\(_3\)PO\(_4\)) for the phosphorylated peptide. Sequence coverage for both peptides is about 80%. MAD of the non-phosphorylated precursor ion (Figure 4.1a) produced twelve distinct fragment ions in total, comprising of eight a-/x-type ions, two c-/z- and two y-type ions. Similarly, MAD of the phosphorylated precursor ion (Figure 4.1b) produced eight a-/x-type ions, three c-/z- and three b-/y-type ions, totaling fourteen distinct fragment ions. The abundant observations of a-/x-type ions is consistent with observation with other types of negative electron transferring dissociation techniques such as EDD [50, 51] and NETD [54, 55]. Only two fragment ions, c\(_7\) and b\(_8\), underwent a neutral loss of the phosphorylation modification. However, both of these ions were also observed with the modification intact. MAD of the doubly-deprotonated cholecystokinin therefore enables unambiguous site determination to the Ser-10 residue. One notable difference between the modified and unmodified forms of the peptide is the relative abundance of the fragment ions x\(_6\) and b\(_8\). These ions are among the most abundant ions in the mass spectrum of the phosphorylated form of cholecystokinin (Figure 4.1b), but are absent or in very low abundance in the non-modified spectrum (Figure 4.1a).

Figures 4.1c and 4.1d show MAD spectra of the non-phosphorylated and the phosphorylated doubly-deprotonated species of the peptide calcitonin (DFNKFHpTFPQTAIGV), respectively, which contains two threonine residues with the 7\(^{th}\) residue phosphorylated (Thr-7). Similar to cholecystokinin, the most dominant
features in Figure 4.1c are the neutral losses of H₂O, CO₂, and [CO₂ + H₂O] from the oxidized species [M-2H]⁺. MAD of the non-phosphorylated calcitonin produced seventeen distinct fragment ions with eleven of those ions comprised of a-/x-type ions. Conversely, when the same peptide is phosphorylated (Figure 4.1d) the number of fragment types slightly decreases and sequence coverage drops from 86% to 72%. MAD of the 2-phosphorylated precursor ion (Figure 4.1d) produced fourteen product ions, with ten a-/x-type ions and three c-/z-type ions. The most abundant peaks in Figure 4.1d correspond to the oxidized species, [M-2H]⁺, and the neutral losses of CO₂ and [HPO₃ + H₂O] (or H₃PO₄) from the oxidized species. Regardless of the slight decrease in sequence coverage, MAD still produces enough information to unambiguously determine the site of modification to the Thr-7 residue. Finally, MAD of the phosphorylated peptides produces very similar results in comparison to the non-phosphorylated peptide spectra such as 1) ample backbone cleavage adjacent to and in the vicinity of the modification and 2) abundant a-/x-type fragment ions showing consistent cleavage mechanism regardless of whether or not the phosphorylation group is present.

4.3.1.2 MAD versus CID.

Figures 4.1b and 4.1c compare the MAD and CID mass spectra of the phosphorylated 2-state of cholecystokinin, respectively. MAD achieves 80% sequence coverage in comparison to only 60% coverage with CID. Using CID, only three fragment ions (Figure 4.1c) retain the modification, thereby obscuring modification site determination. The main dissociation channels in the CID spectrum are the b₁₁ and y₁₁ ions which correspond to neutral losses of H₂O and HPO₃⁻, respectively. Unfortunately, these two fragment ions provide no peptide sequencing information. The MAD and CID
mass spectra of the 2- peptide calcitonin, which contains a modified threonine, are also compared in Figure 4.1e and f, respectively. Sequence coverage was significantly worse for CID compared to MAD. More than half of the fragment ions undergo a neutral loss of the modification in CID, whereas no PTM losses were observed in MAD. The CID spectrum therefore cannot provide the site of the PTM modification. Neutral losses of H$_2$O and HPO$_3^-$ are also dominant in the CID spectrum, but in lower abundance compared to cholecystokinin.

Figures 4.2a and 4.2b compare MAD and CID spectra of the 3- form of cholecystokinin. Both methods provide seven out of ten backbone cleavages. However, more than half of the fragment ions containing the PTM in CID showed a neutral loss of the modification, thereby obscuring modification site determination. In contrast, MAD showed very few PTM neutral losses from the fragment ions. Using CID, neutral losses of HPO$_3$ and [HPO$_3$ + H$_2$O] (or H$_3$PO$_4$) are observed from the precursor ion, and using MAD, the same neutral losses are observed from the oxidized species, [M - 3H]$^{2-}$. However, the neutral losses are more abundant in CID. Neutral losses of H$_2$O and [CO$_2$ + 'OH] from the oxidized species are also observed in the MAD spectrum (Figure 4.2a). The di-radical species, [M - 3H]$^{2-}$, is also observed in MAD, which presumably originates from a second Penning ionization reaction of the [M - 3H]$^{2-}$ primary product ion. The Venn diagram in Figure 4.3 shows that MAD and CID of phosphorylated cholecystokinin have only three fragment ions in common out of a possible nine.

MAD and CID mass spectra of the 3- form of calcitonin are compared in Figures 4.2c and 4.2d. The MAD spectrum in Figure 4.2c allows for complete peptide sequencing and unambiguous site determination. CID only achieves 43% sequence coverage with
Figure 4.2. Comparison of He MAD and CID spectra of different phosphorylated peptides in the 3- charge state: (a) MAD and (b) CID of phosphorylated cholecystokinin (IKNLQSLDPpSH), (c) MAD and (d) CID of phosphorylated calcitonin (DFNKFHpTFPQTAIGV), (e) MAD and (f) CID of phosphorylated angiotensin II (DRVpYIHPF). An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)
four fragment ions showing modification loss. In this case, CID cannot distinguish which of the threonine residue contains the phosphorylation because there are no cleavages between the 7th and 11th residues. In total, MAD produced twenty-four fragment ions and CID produced eight fragment ions. Only four fragments were common to both methods (Figure 4.3). The main dissociation channels in CID are the neutral losses of H₂O, HPO₃, and [HPO₃ + H₂O] (or H₃PO₄) from the precursor ions. In MAD however, CO₂ is the only neutral loss observed from the two oxidized species, [M - 3H]²⁺ and [M - 3H]³⁺.

![Venn diagrams comparing the number of fragmentation ion types produced after He MAD and CID of the phosphorylated peptides: (a) [IKNLQSLDPpSH -3H]³⁺, (b) [DFNKFHpTFPQTAIGV -3H]³⁺, and (c) [DRVpYIHPF -3H]³⁺.](image)

(Figure 4.3: Venn diagrams comparing the number of fragmentation ion types produced after He MAD and CID of the phosphorylated peptides: (a) [IKNLQSLDPpSH -3H]³⁺, (b) [DFNKFHpTFPQTAIGV -3H]³⁺, and (c) [DRVpYIHPF -3H]³⁺. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)

Figures 4.2e and 4.2f show MAD and CID mass spectra of the 3-form of angiotensin II. Again, MAD achieves significantly more sequence coverage in comparison to CID. MAD also retained the phosphorylation modification almost all of the time. Conversely, CID retains the modification only 25% of the time. CID produced only seven distinct fragments whereas MAD produced thirteen. Again, only four fragment ions were common to both methods (Figure 4.3). MAD (Figure 4.2e) produced
neutral losses of CO₂ and [HPO₃ + H₂O] (or H₃PO₄) from the two oxidized product ions, [M - 3H]²⁻ and [M - 3H]⁻, but no neutral loss of the modification from the precursor ion was observed in CID (Figure 4.2f). However, CID of the 3- form of angiotensin II showed a dominant y₄ ion and very little sequence information. The phosphate group was lost in most of the CID fragments.

4.3.1.3 Effect of Charge State on Peptide Backbone Cleavage.

Cleavages produced by MAD of the 2- and 3- forms of phosphorylated cholecystokinin, calcitonin, and angiotensin II are compared as Venn diagrams in Figure 4.4 and in Table 4.1. In general, sequence coverage increases only slightly as charge state increases. This observation is likely due to two reasons: 1) as additional charges are added to the precursor ion, the peptide unravels due to Coulombic repulsion and has a more open structure and 2) the fragmentation energy (exothermicity) is different in Penning ionization of the 3- versus the 2- precursor. In our previous work with positive

---

**Figure 4.4.** Venn diagrams comparing the number of fragment ion types produced after He MAD of the 2- and 3- phosphorylated peptides: (a) cholecystokinin (IKNLQpSLDSPH), (b) calcitonin (DFNKFH₃TFPQT₃AGV), and (c) angiotensin II (DRVpYIH₃PF). (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)
Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD on the \([M-2H]^2\) and \([M-3H]^3\) Precursor Ions of the Following Peptides:

(a) IKNLQpSLDPSH, (b) DFNKFHpTFPQTAIGV, and (c) DRVpYIHPF. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. \textbf{2011}, \textit{22}, 1088-1099.)

<table>
<thead>
<tr>
<th></th>
<th>Precursor charge state</th>
<th></th>
<th>Precursor charge state</th>
<th></th>
<th>Precursor charge state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>([M-2H]^2)</td>
<td></td>
<td>([M-2H]^2)</td>
<td></td>
<td>([M-2H]^2)</td>
</tr>
<tr>
<td>a_7</td>
<td>1</td>
<td></td>
<td>a_7</td>
<td>1</td>
<td>a_7</td>
</tr>
<tr>
<td>b_7</td>
<td>1</td>
<td></td>
<td>a_7</td>
<td>1</td>
<td>a_7</td>
</tr>
<tr>
<td>c_7</td>
<td>1</td>
<td></td>
<td>a_7</td>
<td>1</td>
<td>a_7</td>
</tr>
<tr>
<td>a_8</td>
<td>1</td>
<td>1</td>
<td>a_9</td>
<td>1</td>
<td>a_9</td>
</tr>
<tr>
<td>b_8</td>
<td>1</td>
<td>1</td>
<td>c_9</td>
<td>1</td>
<td>c_9</td>
</tr>
<tr>
<td>a_9</td>
<td>1</td>
<td>1</td>
<td>a_10</td>
<td>1</td>
<td>a_7</td>
</tr>
<tr>
<td>b_9</td>
<td>1</td>
<td></td>
<td>c_10</td>
<td>1</td>
<td>b_7</td>
</tr>
<tr>
<td>a_10</td>
<td>1</td>
<td></td>
<td>a_11</td>
<td>1</td>
<td>z_4</td>
</tr>
<tr>
<td>x_6</td>
<td>1</td>
<td></td>
<td>c_11</td>
<td>2</td>
<td>x_5</td>
</tr>
<tr>
<td>y_6</td>
<td>1</td>
<td></td>
<td>a_12</td>
<td>1</td>
<td>x_6</td>
</tr>
<tr>
<td>x_7</td>
<td>1</td>
<td></td>
<td>a_13</td>
<td>2</td>
<td>y_6</td>
</tr>
<tr>
<td>y_8</td>
<td>1</td>
<td></td>
<td>b_13</td>
<td>2</td>
<td>x_7</td>
</tr>
<tr>
<td>x_9</td>
<td>2</td>
<td></td>
<td>c_13</td>
<td>2</td>
<td>y_7</td>
</tr>
<tr>
<td>z_9</td>
<td>1</td>
<td></td>
<td>a_14</td>
<td>1</td>
<td>z_7</td>
</tr>
<tr>
<td>x_{10}</td>
<td>2</td>
<td></td>
<td>c_14</td>
<td>1</td>
<td>z_7</td>
</tr>
<tr>
<td>z_{10}</td>
<td>1</td>
<td>2</td>
<td>x_6</td>
<td>1</td>
<td>z_7</td>
</tr>
<tr>
<td>x_{10}</td>
<td>1</td>
<td></td>
<td>x_{11}</td>
<td>1</td>
<td>z_7</td>
</tr>
<tr>
<td>x_{12}</td>
<td>1</td>
<td></td>
<td>x_{12}</td>
<td>1</td>
<td>z_7</td>
</tr>
<tr>
<td>y_{12}</td>
<td></td>
<td></td>
<td>y_{12}</td>
<td>2</td>
<td>z_7</td>
</tr>
<tr>
<td>x_{13}</td>
<td>1</td>
<td></td>
<td>x_{13}</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>x_{14}</td>
<td>1</td>
<td></td>
<td>x_{14}</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>y_{14}</td>
<td>1</td>
<td></td>
<td>y_{14}</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>z_{14}</td>
<td>1</td>
<td>2</td>
<td>z_{14}</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
ion mode MAD, it was observed that the types of fragment ions produced by MAD were highly dependent on the charge state of the precursor ion [160]. In contrast to positive ion mode MAD, the types of fragment ions produced in negative ion mode MAD are apparently much less dependent on charge state. Between 1/3 and 1/2 of the total product ions observed in negative ion mode MAD are common to both the 2- and 3- charge states (Figure 4.4a, b, and c).

Figure 4.5 and Table 4.2 compares the amounts and types of fragment ions MAD produces from the 2+ and 2- forms of the phosphorylated peptides. In contrast to negative ion mode MAD, positive ion mode MAD produces more product ion types. Additionally, positive ion mode MAD enabled extensive fragmentation and modification retainment. The phosphorylation modification showed no deleterious effect on positive ion mode MAD backbone cleavage (Figure 4.6) unlike ECD of the 2+ phosphorylated species [146]. Compared to the respective 2+ charge states, MAD of the 2- charge state does not

**Figure 4.5.** Venn diagrams comparing the number of fragment ion types produced after He MAD of the 2+ and 2- phosphorylated peptides: (a) cholecystokinin (IKNLQSLDPpSH), (b) calcitonin (DFNKFHpTFPQTAIGV), and (c) angiotensin II (DRVpYIHPF). (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. *J. Am. Soc. Mass Spectrom.* **2011**, *22*, 1088-1099.)
Table 4.2.

Comparison of the Type and Charge of the Fragmentation Ions Produced by He MAD on the [M+2H]^{2+} and [M-2H]^{2-} Precursor Ions of the Following Peptides: (a) IKNLQSDLPPSH, (b) DFNKFHpTFPQTAIGV, and (c) DRVnYIHPF. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)

<table>
<thead>
<tr>
<th>IKNLQpSLDPSh</th>
<th>DFNKFHpTFPQTAIGV</th>
<th>DRVpYIHPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Precursor charge state</td>
<td>b) Precursor charge state</td>
<td>c) Precursor charge state</td>
</tr>
<tr>
<td>b_{12}</td>
<td>1'^+</td>
<td>a_{13}</td>
</tr>
<tr>
<td>b_{14}</td>
<td>1'</td>
<td>b_{15}</td>
</tr>
<tr>
<td>c_{16}</td>
<td>1'</td>
<td>a_{17}</td>
</tr>
<tr>
<td>a_{18}</td>
<td>2'^</td>
<td>b_{19}</td>
</tr>
<tr>
<td>b_{20}</td>
<td>1'^</td>
<td>c_{21}</td>
</tr>
<tr>
<td>c_{22}</td>
<td>1'^</td>
<td>a_{23}</td>
</tr>
<tr>
<td>a_{24}</td>
<td>1'</td>
<td>b_{25}</td>
</tr>
<tr>
<td>b_{26}</td>
<td>1'</td>
<td>c_{27}</td>
</tr>
<tr>
<td>a_{28}</td>
<td>1'</td>
<td>a_{29}</td>
</tr>
<tr>
<td>b_{30}</td>
<td>1'^, 2'^</td>
<td>b_{31}</td>
</tr>
<tr>
<td>c_{32}</td>
<td>1'^, 2'^</td>
<td>a_{33}</td>
</tr>
<tr>
<td>a_{34}</td>
<td>1'</td>
<td>b_{35}</td>
</tr>
<tr>
<td>b_{36}</td>
<td>1'</td>
<td>c_{37}</td>
</tr>
<tr>
<td>a_{38}</td>
<td>1'</td>
<td>a_{39}</td>
</tr>
<tr>
<td>b_{40}</td>
<td>1'^</td>
<td>b_{41}</td>
</tr>
<tr>
<td>c_{42}</td>
<td>1'^</td>
<td>a_{43}</td>
</tr>
<tr>
<td>a_{44}</td>
<td>1'</td>
<td>b_{45}</td>
</tr>
<tr>
<td>b_{46}</td>
<td>1'</td>
<td>c_{47}</td>
</tr>
<tr>
<td>a_{48}</td>
<td>1'</td>
<td>a_{49}</td>
</tr>
<tr>
<td>b_{50}</td>
<td>1'^, 2'^</td>
<td>b_{51}</td>
</tr>
<tr>
<td>c_{52}</td>
<td>1'^, 2'^</td>
<td>a_{53}</td>
</tr>
<tr>
<td>a_{54}</td>
<td>1'</td>
<td>b_{55}</td>
</tr>
<tr>
<td>b_{56}</td>
<td>1'</td>
<td>c_{57}</td>
</tr>
<tr>
<td>a_{58}</td>
<td>1'</td>
<td>a_{59}</td>
</tr>
<tr>
<td>b_{60}</td>
<td>1'^</td>
<td>b_{61}</td>
</tr>
<tr>
<td>c_{62}</td>
<td>1'^</td>
<td>a_{63}</td>
</tr>
<tr>
<td>a_{64}</td>
<td>1'</td>
<td>b_{65}</td>
</tr>
<tr>
<td>b_{66}</td>
<td>1'</td>
<td>c_{67}</td>
</tr>
<tr>
<td>a_{68}</td>
<td>1'</td>
<td>a_{69}</td>
</tr>
<tr>
<td>b_{70}</td>
<td>1'^</td>
<td>b_{71}</td>
</tr>
<tr>
<td>c_{72}</td>
<td>1'^</td>
<td>a_{73}</td>
</tr>
<tr>
<td>a_{74}</td>
<td>1'</td>
<td>b_{75}</td>
</tr>
<tr>
<td>b_{76}</td>
<td>1'</td>
<td>c_{77}</td>
</tr>
<tr>
<td>a_{78}</td>
<td>1'</td>
<td>a_{79}</td>
</tr>
<tr>
<td>b_{80}</td>
<td>1'^</td>
<td>b_{81}</td>
</tr>
<tr>
<td>c_{82}</td>
<td>1'^</td>
<td>a_{83}</td>
</tr>
<tr>
<td>a_{84}</td>
<td>1'</td>
<td>b_{85}</td>
</tr>
<tr>
<td>b_{86}</td>
<td>1'</td>
<td>c_{87}</td>
</tr>
<tr>
<td>a_{88}</td>
<td>1'</td>
<td>a_{89}</td>
</tr>
<tr>
<td>b_{90}</td>
<td>1'^</td>
<td>b_{91}</td>
</tr>
<tr>
<td>c_{92}</td>
<td>1'^</td>
<td>a_{93}</td>
</tr>
<tr>
<td>a_{94}</td>
<td>1'</td>
<td>b_{95}</td>
</tr>
<tr>
<td>b_{96}</td>
<td>1'</td>
<td>c_{97}</td>
</tr>
<tr>
<td>a_{98}</td>
<td>1'</td>
<td>a_{99}</td>
</tr>
<tr>
<td>b_{100}</td>
<td>1'^</td>
<td>b_{101}</td>
</tr>
<tr>
<td>c_{102}</td>
<td>1'^</td>
<td>a_{103}</td>
</tr>
<tr>
<td>a_{104}</td>
<td>1'</td>
<td>b_{105}</td>
</tr>
<tr>
<td>b_{106}</td>
<td>1'</td>
<td>c_{107}</td>
</tr>
<tr>
<td>a_{108}</td>
<td>1'</td>
<td>a_{109}</td>
</tr>
<tr>
<td>b_{110}</td>
<td>1'^</td>
<td>b_{111}</td>
</tr>
<tr>
<td>c_{112}</td>
<td>1'^</td>
<td>a_{113}</td>
</tr>
<tr>
<td>a_{114}</td>
<td>1'</td>
<td>b_{115}</td>
</tr>
<tr>
<td>b_{116}</td>
<td>1'</td>
<td>c_{117}</td>
</tr>
<tr>
<td>a_{118}</td>
<td>1'</td>
<td>a_{119}</td>
</tr>
<tr>
<td>b_{120}</td>
<td>1'^</td>
<td>b_{121}</td>
</tr>
<tr>
<td>c_{122}</td>
<td>1'^</td>
<td>a_{123}</td>
</tr>
<tr>
<td>a_{124}</td>
<td>1'</td>
<td>b_{125}</td>
</tr>
<tr>
<td>b_{126}</td>
<td>1'</td>
<td>c_{127}</td>
</tr>
<tr>
<td>a_{128}</td>
<td>1'</td>
<td>a_{129}</td>
</tr>
<tr>
<td>b_{130}</td>
<td>1'^</td>
<td>b_{131}</td>
</tr>
<tr>
<td>c_{132}</td>
<td>1'^</td>
<td>a_{133}</td>
</tr>
<tr>
<td>a_{134}</td>
<td>1'</td>
<td>b_{135}</td>
</tr>
<tr>
<td>b_{136}</td>
<td>1'</td>
<td>c_{137}</td>
</tr>
<tr>
<td>a_{138}</td>
<td>1'</td>
<td>a_{139}</td>
</tr>
<tr>
<td>b_{140}</td>
<td>1'^</td>
<td>b_{141}</td>
</tr>
<tr>
<td>c_{142}</td>
<td>1'^</td>
<td>a_{143}</td>
</tr>
<tr>
<td>a_{144}</td>
<td>1'</td>
<td>b_{145}</td>
</tr>
<tr>
<td>b_{146}</td>
<td>1'</td>
<td>c_{147}</td>
</tr>
<tr>
<td>a_{148}</td>
<td>1'</td>
<td>a_{149}</td>
</tr>
<tr>
<td>b_{150}</td>
<td>1'^</td>
<td>b_{151}</td>
</tr>
<tr>
<td>c_{152}</td>
<td>1'^</td>
<td>a_{153}</td>
</tr>
<tr>
<td>a_{154}</td>
<td>1'</td>
<td>b_{155}</td>
</tr>
<tr>
<td>b_{156}</td>
<td>1'</td>
<td>c_{157}</td>
</tr>
<tr>
<td>a_{158}</td>
<td>1'</td>
<td>a_{159}</td>
</tr>
<tr>
<td>b_{160}</td>
<td>1'^</td>
<td>b_{161}</td>
</tr>
<tr>
<td>c_{162}</td>
<td>1'^</td>
<td>a_{163}</td>
</tr>
<tr>
<td>a_{164}</td>
<td>1'</td>
<td>b_{165}</td>
</tr>
<tr>
<td>b_{166}</td>
<td>1'</td>
<td>c_{167}</td>
</tr>
<tr>
<td>a_{168}</td>
<td>1'</td>
<td>a_{169}</td>
</tr>
<tr>
<td>b_{170}</td>
<td>1'</td>
<td>c_{171}</td>
</tr>
<tr>
<td>a_{172}</td>
<td>1'</td>
<td>a_{173}</td>
</tr>
<tr>
<td>b_{174}</td>
<td>1'</td>
<td>c_{175}</td>
</tr>
<tr>
<td>a_{176}</td>
<td>1'</td>
<td>a_{177}</td>
</tr>
<tr>
<td>b_{178}</td>
<td>1'</td>
<td>c_{179}</td>
</tr>
<tr>
<td>a_{180}</td>
<td>1'</td>
<td>a_{181}</td>
</tr>
<tr>
<td>b_{182}</td>
<td>1'</td>
<td>c_{183}</td>
</tr>
</tbody>
</table>

(*Reproduced in part, 1088-1099.)*
provide very many unique ions for two of the three peptides (cholecystokinin and angiotensin II) but shows very unique fragments for the 2-form calcitonin. In general MAD of phosphorylated peptide anions is less dependent on charge state than positive mode and offers simpler mass spectra. Positive ion MAD generates more fragment ion types, but more complicated spectra. Each polarity has pros and cons and provides complementary fragment types.

**Figure 4.6.** The fragment ions produced by He MAD on the following doubly-protonated phosphorylated peptides: (a) cholecystokinin, (b) calcitonin, and (c) angiotensin II. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)

### 4.3.2 Sulfonated Peptide Anions

The precursor ion masses of the various sulfonated peptides showed an increase of 79 Da relative to the non-modified peptide in Figures 4.7b and d, confirming the addition of an –SO₃ group. A lower case ‘s’ indicates the amino acid directly to the right is modified. Figures 4.7a and b are the mass spectra of the 2-form of cholecystokinin, DsYMGWMDF, which corresponds to the non-sulfonated species and the sulfonated species dissociated with MAD. Again, the fragment ions that underwent a neutral loss of the modification are omitted in the peptide inserts in each of the figures. The non-sulfonated 2-precursor ions (Figure 4.7a) produced 100% sequence coverage with five a-
/x-type ions, four c-/z-type ions, and only one b-type ion. The most abundant dissociation
channels are C–N and N–C\(_{\alpha}\) backbone cleavages between the methionine and aspartic
acid residues, which correspond to the fragment ions b\(_6\) and c\(_6\).

MAD dissociation of the sulfonated precursor ions also produced five a-/x-type
ions, three b-/y-type ions, and four c-/z-type ions as seen in Figure 4.7b. When comparing
the degree and type of fragment ions observed between the non-sulfonated and the
sulfonated precursor ions with MAD (Figures 4.7a and 4.7b) a small decrease (two ions)
in sequence coverage is observed. The fragmentation behavior is similar to that observed
by Zubarev and co-workers using EDD of the 2-form of sulfonated carulein
(pEQDsYTGWMDF), a very similar peptide to cholecystokinin [50]. Using EDD,
Budnik et al. observed mainly a-, c-, and x-type ions [24]. However they observed more
neutral losses of CO\(_2\) and HSO\(_3\) from the fragment ions than we observed here with
MAD. Observation of additional backbone cleavage (Figure 4.7b) between the
tryptophan and methionine, especially between the C\(_{\alpha}\)–C bonds (a\(_5\) ion), and the decrease
in the abundance of the b\(_5\) and c\(_5\) ions suggests that sulfonation alters the preferred
dissociation channels compared to the unmodified peptide.

Despite the differences in fragment ion types, an intense oxidized product ion,
[M-2H]\(^+\), is observed in both Figure 4.7a and b. In addition, the same neutral losses for
the sulfonated and the non-sulfonated peptides are observed from the oxidized product
ion, which correspond to H\(_2\)O, CO\(_2\), [CO\(_2\) + H\(_2\)O], and HSO\(_3\). Both peptides undergo a
neutral loss of the aspartic acid side chain, (\(\cdot\)CHO\(_2\) resulting in the d\(_7\) ion. Additionally,
both peptides undergo neutral losses of the side chains of the methionine residue
(indicated with a † in Figure 4.7a and b) corresponding to a neutral loss of 74 Da
Figure 4.7. Tandem mass spectra comparing He MAD and CID of sulfonated cholecystokinin and hirudin in the 2+ charge state: (a) MAD of non-sulfonated DYMGMDF, (b) MAD of sulfonated DsYMGMDF, (c) CID of sulfonated DsYMGMDF, (d) MAD of non-sulfonated DFEEIPEELQ, (e) MAD of sulfonated DFEEIPEEsYLQ, and (f) CID of sulfonated DFEEIPEEsYLQ. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)
(•C₃H₆S) [109], and the tryptophan residue (indicated with a † in Figure 4.7a and b) corresponding to a neutral loss of 116 Da (•C₈H₆N) from the oxidized product ion [41]. When comparing MAD and CID of the sulfonated species in Figure 4.7b and 4.7c, the sequence coverage is clearly superior using MAD. The sulfonation modification is lost only once when employing MAD, but many of the CID fragments lose the sulfonation group. The most abundant peak in the CID spectrum is the neutral loss of water from the precursor ion.

Figures 4.7d-f are MAD spectra of the 2- form of hirudin (DFEIEPEEsYLQ) corresponding to: a) the non-sulfonated species dissociated with MAD, b) the sulfonated species dissociated with MAD, and c) the sulfonated species dissociated with CID. Even though the sequence coverage increases from 40% to 60% from the non-sulfonated to the sulfonated species, the overall fragmentation efficiency is slightly less for the sulfonated peptide. The sulfonated peptide shows a much smaller charge reduced ion, [M-2H]⁺, and a larger [M-H]⁻ ion from a radical hydrogen loss. In addition, the fragment ions produced from the non-sulfonated species undergo several neutral losses, including •OH, CO₂ and [CO₂ + H₂O] from the oxidized product ion. No water losses from the fragment ions are observed when the peptide is modified, but all the neutral losses besides •OH are observed from the oxidized product ion (Figure 4.7e). When dissociating the sulfonated species with MAD (Figure 4.7e), five of the eight fragment ions are a-/x-type ions. CID of the sulfonated 2-form of hirudin (Figure 4.7f) showed a large degree of neutral losses of water from the precursor and product ions. Additionally, CID achieves only three sequence ions and produces only two fragment ions which retain the modification.
MAD and CID fragmentation of the sulfonated 3- peptides of cholecystokinin and hirudin are compared in Figure 4.8. MAD dissociation of the sulfonated cholecystokinin (Figure 4.8a) produced five a-/x-type ions, three b-type ions, and four c-/z-type ions which achieved 72% sequence coverage and no modification losses, a total of thirteen product ions. Additionally, MAD produced a d$_7$ ion, which results from a side chain cleavage of the aspartic acid residue from the a$_7$ ion. Two oxidized species, [M-3H]$^{2-}$ and [M-3H]$^{3-}$, are observed in Figure 4.8a, presumably resulting from two consecutive Penning ionization reactions. CID (Figure 4.8b) only produced one b ion and one c ion and almost no structural information. When the 2- form of the non-sulfonated cholecystokinin is fragmented with MAD the b$_6$ and c$_6$ ions are very dominant (Figure 4.7a). When sulfonated at the tyrosine residue (Tyr-2) the b$_6$ and c$_6$ ions are dramatically reduced and the a$_5$ ion is more dominant (Figure 4.7b). When the same sulfonated peptide is fragmented in the 3- charge state with MAD (Figure 4.8a) the b$_6$ and c$_6$ ions return as the most abundant fragments. CID of either the 2- or 3- charge state of the sulfonated peptide also produces the b$_6$ and c$_6$ in high abundance, indicating that these two fragments have a relatively low activation energy and are highly favorable. These observations indicate that when cholecystokinin is not sulfonated, the charge has a high propensity for the aspartic acid at the Asp-7 position and promotes backbone cleavage adjacent to this residue. When the peptide is sulfonated at Tyr-2, an additional site of potential deprotonation is created. Because of the highly acidic nature of the modification, the charge has a higher probability of residing on the sulfate group than the aspartic acid residue. The remaining charge is then located on one of the three remaining
Figure 4.8. Tandem mass spectra comparing He MAD and CID of sulfonated cholecystokinin and hirudin in the 3- charge state: (a) MAD of sulfonated DsYMGWMDF, (b) CID of sulfonated DsYMGWMDF, (c) MAD of sulfonated DFEEIPEEsYLQ, and (d) CID of sulfonated DFEEIPEEsYLQ. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts. (Reproduced in part with permission from Cook, S. L.; Jackson, G. P. J. Am. Soc. Mass Spectrom. 2011, 22, 1088-1099.)

acidic sites. The effect of sulfonation is to reduce the probability of locating the charge at the Asp-7 position.

When the peptide is in the 3- charge state there is an additional charge, so the probability of deprotonation at the Asp-7 position increases. The increased probability of charge-directed fragmentation adjacent to the aspartic acid residue for the 3- precursor
explains the increased relative abundance of the $b_6$ and $c_6$ ions relative to the 2- charge state. This suggests that the specific site of deprotonation, such as acidic acids and the sulfate moiety, appears to influence the favorability for site-specific cleavages.

MAD dissociation of the sulfonated 3- hirudin (Figure 4.8c) achieved 90% sequence coverage with no observed loss of the modification. CID of the same 3-precursor provided 30% coverage and only three fragment ions that retained the modification (Figure 4.8d). MAD produced seventeen fragment ions in total with eight a-/x-type ions, four b-/y-type ions, and five c-/z-type ions. Two oxidized species are observed in Figure 4.8c resulting from consecutive Penning ionization reactions. However the most intense peaks within the isotopic distributions, $[M-2H]^{-}$ and $[M-H]^{-}$, which correspond to neutral losses of $H\cdot$ and $2H\cdot$ or $H_2$, respectively, which is a common observation in EDD which is similarly indicated by electron abstraction [51]. CID of the 3- form of hirudin produced six distinct fragment ions, all cleaving the weaker C–N peptide backbone bonds. Dissociation with CID produced abundant of water loss from the precursor and product ions, which is not observed with MAD. With the limited number of peptides studied to date, the 3- charge states seem to offer slightly better sequencing and modification retention than the 2- charge states.

4.3.3 Sulfonated Peptide Cations: 1+ Charge State

Unlike ECD and ETD, MAD does not utilize a charged reagent to initiate electron transferring/abstraction processes. Therefore MAD has the unique ability to dissociate 1+ species and perform analysis of peptides without basic amino acids, such as DsYMGWMDF and sYGGFL (Figure 4.9). The mass spectrum in Figure 4.9a is of 1+
Figure 4.9. He MAD spectra of sulfonated angiotensin II and leucine enkephalin in the 1+ charge state: (a) DsYMGMDF and (b) sYGGFL. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.

cholecystokinin which contains no basic amino acids and a sulfonated tyrosine residue at the Tyr-2 position. Therefore, the only places for protonation are at the N-terminus or along the peptide backbone. The oxidized product ion, $[M+H]^{2+}$, along with three $2^+$ product ions provides evidence for the mechanism of Penning ionization. Extensive backbone cleavage in the form of $C\alpha-C$, $C-N$, and $N-C\alpha$ bond cleavages produced 100% sequence coverage with He MAD. In total, twenty-three distinct fragment ions were observed with nine c-/z-type ions, nine b-/y-type ion, and five a-/x-type ions. The intense peaks at $m/z$ 331 and 359, which correspond to the $a_2$ and $b_2$ fragment ions, suggest that the main dissociation channel is adjacent to the sulfonated tyrosine residue. In addition, neutral losses from the side chain of the methionine residue are observed twice. One such loss is the methionine side chain from the oxidized product ion, corresponding to neutral loss of 74 Da (indicated with a † in Figure 4.9). This loss is similarly seen in negative ion mode MAD. The second neutral loss is observed from the $z_5$ ion, corresponding to a neutral loss of a $\cdot$C$_2$H$_5$S, thereby producing the $w_5$ ion. These neutral losses from the methionine residue are also observed in ECD [109, 110]. Several neutral losses from the precursor ions are observed in the form of $\cdot$OH, 2[$\cdot$OH], and CO$_2$. The majority of fragment ions retained the labile sulfate modification, which allowed for unambiguous site determination. These results are in stark contrast to the complete sulfonate loss that Liu and Håkansson observed when employing ECD on 2+ cholecystokinin, which was attributed to the promotion of a mobile proton on the peptide backbone amide nitrogens [157]. Modification retention improved in ECD when the cholecystokinin was complexed with different divalent metal cations. However 100% sequence coverage was
not achieved and significant neutral losses of NH$_3$, CO$_2$, H$_2$O, CH$_3$CO$_2$H, and H$^+$ were observed.

Figure 4.9b shows a MAD spectrum of 1+ leucine-enkephalin with a sulfonated tyrosine residue at the Tyr-1 position and no basic amino acids. Including those fragment ions that have undergone a neutral loss of the modification, cleavage between each amino acid is achieved. A total of twelve fragment ions are observed with the majority consisting of b-/y-type ions. A small PI peak, [M + H]$^{2+}$, is observed, as well as one 2+ product ion, [z$_5$]$^{2+}$. The y$_2$ product ion also undergoes a neutral loss of 43 Da. This ion allows for the differentiation of leucine over isoleucine because of the different neutral losses of the sides chains: 43 Da for leucine (•CH(CH$_3$)$_2$) and 29 Da for isoleucine (•CH$_2$CH$_3$) [88]. The major dissociation channel for leucine enkephalin appears to be the loss of the sulfonation group from the precursor ion. Only three of the twelve fragment ions experienced a neutral loss of the sulfonation. These fragments allowed for unambiguous PTM site determination. Unlike ECD, MAD does not require additional charge enrichment steps to improve ionization efficiency or to stabilize the modification. MAD of the 1+ charge state of the sulfated cholecystokinin appears superior to the 2- and 3- charge states even though the same method of activation, PI, is responsible in all three cases. Clearly, the fragmentation methods and the fragmentation ions resulting from PI are influenced to some degree by the overall charge and conformation of the precursor ion.
4.4 Conclusion

Phosphorylated and sulfonated peptides are highly important modifications that need routine MS/MS analysis. The majority of today’s analysis techniques rely on positive ion mode polarity mass spectrometry coupled with CID for modification identification. However, these techniques are not always optimal due to the modifications acidic nature and their tendency to be cleaved during collisional activation. Coupling negative ion mode with MAD is a viable alternative to the traditional MS/MS analysis because sulfonated and phosphorylated peptides ionize more readily in the negative mode and MAD retains the more labile modifications. Ample backbone cleavage was observed for the 2- and 3- forms of phosphorylated and sulfonated peptides, allowing for peptide sequencing. Regardless of which amino acid is phosphorylated (serine, threonine, or tyrosine) MAD achieves unambiguous modification site determination even when numerous potential phosphorylation sites exist. In addition, MAD has the unique ability to dissociate 1+ peptides without the need of charge enrichment steps or metal adduct formation. MAD offers a complementary alternative to traditional collisional or other electron mediated dissociation techniques.
5.1 Introduction

Recent advances in mass spectrometry (MS) have given the proteomic field an exceptional new tool to study the effects of post-translational modifications (PTMs). Because of the steady improvements in resolving power, sensitivity, and analysis time, tandem mass spectrometry (MS/MS) has become a preferred method for large scale phosphorylation identification. Protein phosphorylation is known as one of the most frequent PTMs and is attributed to a wide range of biological functions and regulatory mechanisms [149]. Phosphorylations occur most often on tyrosine, serine, and threonine amino acid residues and the latter two are notably more labile. In the brain, hyperphosphorylation of serine and threonine residues of tau proteins in neurofibrillary tangles (NFT) is considered to be one of the major hallmarks of Alzheimer’s disease (AD) [162, 163]. To date, more than thirty serine and threonine phosphorylation sites have been characterized within this region. However, it is still undetermined which phosphorylated sites are the critical initiation site for the progression of tau into the symptomatic hyperphosphorylated version [164]. The ability to obtain unambiguous modification site determination is crucial for routine biomarker detection and for intricate mapping studies, especially when multiple modifications are present in close proximity.
The most common MS/MS activation method employed today is CID. Dissociation occurs through collisions of isolated peptide ions with a neutral bath gas which gives rise to heating and subsequent dissociation. CID provides cleavage of the weaker peptidic bonds such as C–N backbone bonds that lead to the observation of b-/y-type ions. CID also provides internal fragments and neutral losses of water, ammonia, carbon dioxide, and labile PTMs. Unfortunately, the slow heating nature of CID leads to the significant neutral loss of PTMs, especially phosphate (-80 Da) or phosphoric acid (-98 Da) from phosphorylated peptides [112, 165]. Whereas neutral losses can confirm the modification type, such fragments often cannot unambiguously locate the modification site [15, 112, 165, 166]. Palumbo and Reid recently demonstrated that during slow heating CID of lower charge-state phosphopeptides, gas-phase rearrangements of the phosphate moiety prior to fragmentation can lead to ambiguous or incorrect residue assignment [151]. The magnitude of this rearrangement problem is thought to be negligible [167], but the PTM assignments of low charge-state peptides cannot easily be verified by an independent method like ETD and ECD.

The introduction of new types of radical-induced dissociation methods have become the preferred way to sequence and identify PTMs. Electron capture dissociation (ECD), introduced by McLafferty and co-workers, leads to predominantly N–Ca backbone cleavages while retaining the more labile PTMs [31]. Dissociation occurs through the interaction of free electrons with isolated peptide ions within a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Similar to ECD, ETD employs radical anions to transfer electrons to peptide cations to produce ECD-like fragmentation in relatively low cost RF ion trapping instruments [30]. Compared to CID,
ETD and ECD have shown superior analysis of labile PTMs such as phosphorylation [30, 131], glycation/glycosylation [134, 135, 136, 168], and sulfonation [132, 133], among others.

Research has shown that phosphorylation modifications can have a negative effect on the efficiency of dissociating 2+ species with ECD [146]. This phenomenon is attributed to the phosphorylation moiety forming a noncovalent salt bridge with a basic amino acid residue which is typically not cleaved in ECD. Additionally, ETD studies have shown that fragmentation can be charge-state dependent with a decrease in fragmentation efficiency of the 2+ species leading to few or no backbone cleavages [48, 49]. Both of these issues can pose a problem with peptides that don’t contain multiple (>2) sites of protonation. The ineffectiveness of ETD and ECD for 2+ peptides is a problem given that a large portion of tryptically digested peptides are doubly-charged [169]. McLuckey and co-workers have shown that raising the bath gas temperature during ETD can increase the sequence coverage of 2+ species [49]. Another technique to enhance the effectiveness of ETD of 2+ peptides is to apply supplemental activation in the form of collisional energy to the charge-reduced product ion to increase fragmentation efficiency, a technique known as ETcaD [92, 93, 170].

The complementary nature of the fragment ion types produced by CID and ETD has led many to combine the methods into either a consecutive alternating mode or as an MS$^3$ mode [47, 171, 172]. By utilizing information from both methods, the overall sequence coverage can be increased to more than 90% [171]. Good et al. illustrated that CID produces superior peptide identification capabilities for 2+ species whereas ETD outperforms CID when precursor charge ($z$) is greater than 2 [92]. CID MS/MS spectra of
higher charge states are difficult to identify or score because of the multiple charge-states of the fragment ions. Automated de novo sequencing algorithms built on fusing CID and ETD spectra can make the identification more reliable [105, 173]. Similar combinations have been utilized for CID and ECD also increasing peptide identifications compared to CID alone [174].

Recently, another type of radical-induced dissociation has been introduced that uses metastable atoms as the electron vehicle, or energy source, for dissociating peptide ions [58, 67, 68, 69, 106, 160]. We refer to the technique as metastable atom-activated dissociation (MAD) [68]. Extensive sequence coverage and backbone cleavages with a-, b-, c-, x-, y-, and z-type ions, while retaining PTMs, are observed through the interaction of a high kinetic energy beam of helium metastable atoms with isolated peptide ions. Several groups have provided evidence that dissociation occurs through two competing mechanisms, charge reduction and Penning ionization (PI) [58, 68, 69, 106]. The charge reduction mechanism is analogous to the mechanism proposed for ECD and ETD and occurs through an electron transfer from the metastable atom to a site of protonation on the peptide ion [34, 40, 175]. The second mechanism, PI, occurs through the detachment of an electron, usually from an n or π orbital, from the peptide ion by the metastable atom. PI generates a radical peptide ion, a neutral atom, and a free electron [58, 68, 106]. PI has been well characterized for neutral molecules and occurs when the potential energy of the metastable atom is greater than the ionization potential of the neutral [57, 176]. Metastable atoms are known to be attracted by dipoles and regions of high electron density such as the lone pairs of electrons on carbonyl oxygen atoms and amide nitrogen atoms [62, 63, 65, 66]. PI leads to direct oxidative radical generation unlike the reductive
radical generation observed in ETD and ECD. To date, MAD has been applied to multiply-charged cations and anions [58, 68, 106], 1+ cations [67, 68, 69], disulfide bridges [58], leucine and isoleucine differentiation [68, 69], proline ring cleavage [68], phosphopeptide cations [58, 67, 68], phosphopeptide anions [177], sulfoproteins [177], nitrated/nitrosylated peptides [160], and lipids [161].

In this work, we investigate and compare the types of fragment ions produced, the sequence coverage, and the ability to unambiguously identify the location of PTMs present by CID, ETD, and MAD for the 2+ and 3+ charge states of several different tau phosphopeptides. The tau peptides studied contain several potential sites of modification in close proximity, thereby increasing the need to achieve high sequence coverage. On average, MAD achieved 83% and 91% percent sequence coverage for the 2+ and 3+ charge states, respectively, which was 15%-20% better coverage than either CID or ETD. MAD unambiguously identified more phosphorylation site locations than CID and ETD.

5.2 Experimental

5.2.1 Preparation of Peptides

Phosphopeptides were synthesized on a Syro 2000 multiple peptide synthesizer (MultiSyn Tech GmbH) according to the procedure detailed by Singer et al. [163]. Subsequent RP-HPLC purification and sequence confirmation by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed (MALDI-TOF-MS, 4700 proteomics analyzer, Applied Biosystems GmbH, Darmstadt, Germany). All peptides were reconstituted to a 1:1 ratio of methanol (Sigma-Aldrich, HPLC grade, St.
Louis, Mo) and water with 1% acetic acid (Sigma-Aldrich). Peptide concentrations were 5 μM for CID and ETD experiments and between 10-20 μM for MAD experiments.

5.2.2 Instrumentation and Method

CID and ETD experiments were performed on a Thermo LTQ Orbitrap XL ETD equipped with a nano-ESI source (Thermo Fisher Scientific GmbH, Bremen, Germany). MAD experiments were performed on a modified Bruker EsquireLC (Bruker Daltonics, Bremen, Germany) with an Ion Tech FAB gun (P50 PSU, Teddington, UK) as the metastable atom source. The FAB gun was positioned approximately 2 cm above the ring electrode. The ring electrode contained a 2 mm hole to allow metastable atoms to enter the trap where they came in contact with isolated precursor ions. The deflection electrodes consisted of two 4-40 stainless steel nuts at the end of two flat-ended screws positioned 1 cm below the exit orifice of the FAB gun to prevent ions and free electrons from entering the trap. The metastable atom beam was pulsed on during the part of the MS duty cycle reserved for collisional activation. Detailed instrumentation modification and metastable atom beam pulse production has been described previously [68].

All fragment ion identifications and assignments were manually determined based on predicted fragmentation patterns and were within ±0.8 m/z of the expected product ions. Peaks were only assigned if the intensities were at least 3 times the signal-to-noise ratio. The 2+ and 3+ ions were identified according to the expected m/z value and the presence of an isotope envelope peak at m/z +0.5 and m/z +0.3, respectively.
5.2.2.1 **CID and ETD**

The 2+ and 3+ charge-state of the phosphopeptides were generated by nano-ESI and directly injected at a flow rate of 0.4-0.6 mL/hr, except for the 3+ charge-state of the P1a and P1b peptides, which were generated in static flow mode. An isolation window of m/z 1 was used to isolate all precursor ions and the low mass trapping voltage (LMCO) was dependent on the precursor mass. The collisional activation energy was set to 35 mV for all CID experiments, whereas the ETD activation time varied between 100-250 ms depending on precursor charge-state and peptide. The CID MS/MS spectra were acquired in intervals between 25-50 s consisting of 7-12 averaged scans. ETD MS/MS spectra consisted of 5-9 scans and were acquired between 25-50 s intervals.

5.2.2.2 **MAD**

The 2+ and 3+ charge-states of the phosphopeptides were generated by ESI at a flow rate of 0.2 mL/hr. The isolation window was varied between m/z 1.0-2.0 based on the lability of the peptide and charge-state. A low mass trapping voltage was set to 200 m/z to minimize background ions from the PI of residue pump oils. The vacuum pressure in the MS chamber outside the trap with He bath gas leaking out of the trap was 1.8 mbar. An additional 1.0 mbar of pressure from the FAB gun was added to produce the He metastable atom beam. The FAB gun anode was powered at 6 keV and the metastable atom beam was activated for 100-250 ms, depending on the charge-state and fragmentation behavior of each peptide. The MAD MS/MS spectra were acquired in 2 min intervals which consisted of 60-70 scans with 5 averages per scans.
5.3 Results and Discussion

The aim of this study is to compare MAD based MS/MS fragmentation with CID and ETD for their ability to identify and localize phosphorylation modifications. Details of the peptides used in this study are listed in Table 5.1. Five different peptide sequences are studied, which represent eleven different analogues, with one or two known phosphorylation sites on either a threonine or serine residue. The peptides contain between 4-8 potential sites of phosphorylation, which makes unambiguous PTM localization a challenge. Additionally, the peptides contain between 2-5 proline residues, which are known to hinder both CID and ETD backbone cleavage. Throughout the text, peptides will be referred to by their given number (P1-P5). Phosphopeptides had a precursor ion mass increase of 80 Da relative to the unmodified peptides, confirming the addition of –HPO$_3$ group. Peptide sequence inserts in all Figures omit those fragment ions that have undergone a neutral loss of the PTM modification. It is important to note that there is a positive correlation between the abundance of isolated precursor ions available for MAD or ETD and the quality of the resulting tandem mass spectra. In the Bruker EsquireLC, we are severely limited by space charge effects in the 3D ion trap and are only able to reach a maximum of $10^5$ – $10^6$ AU signal intensity for the isolated precursor ions. The LTQ Orbitrap is able to store many more precursor ions in the 2D ion trap and thus has a significant starting advantage. In addition, the high resolution of the orbitrap enables greater confidence in assigning product ions than the Bruker 3D ion trap. We acknowledge that many of the peptides in this study provided superior ETD spectra when the 4+ precursor ions of the tau peptides were used in the LTQ instrument. However, the
benefit of high charge states is well known for ETD [92] and is beyond the scope of the current article.

5.3.1 Effect of Precursor Charge State

To study the effects of charge state, the percent sequence coverage was compared for each peptide via CID, ETD, and MAD. Percent sequence coverage is defined as

\[
\text{Percent Sequence Coverage} = \frac{\# \text{ of observed backbone cleavages}}{\# \text{ of backbone bonds}} \times 100\%
\]

where all fragment ion-types cleaved between the same amino acids are counted only once (N-terminal cleavage to the amino acid proline are included in the calculation). For this reason, efficiencies cannot exceed 100% and redundant sequences are not accounted for. Table 5.1 and Figure 5.1 compares the fragment ions generated from CID, ETD, and MAD of the 2+ (left column) and 3+ (right column) charge states of the di-phosphorylated peptide SRpTPpSLPTPPTREP (P1a). For the 2+ precursor, the percent sequence coverage achieved for CID, ETD, and MAD was 50%, 36%, and 86%, respectively. For the 3+ precursor, the sequence converge increases for CID and ETD to 64% and 71%, respectively, whereas the coverage for MAD remains at 86%. Combining the ETD 2+ and 3+ charge state information increases the sequencing efficiency to 79%. Combining the MAD 2+ and 3+ provides 93% coverage. No additional information is obtained by combining the CID information. When comparing the charge states of peptide P1b, CID generated 57% sequence coverage for both charge states whereas ETD generated 7% and 79% for the 2+ and 3+ species, respectively. On the other hand, MAD achieved 100% for the 2+ charge state and 86% for the 3+ charge state.
Table 5.1 and Venn diagrams in Figure 5.2 illustrate the number of fragment ion types generated for each of the three dissociation methods for the 2+ and 3+ species of the di-phosphorylated peptide P2b and P3a. CID of P2b and P3a produced similar product ion numbers regardless of charge state. ETD and MAD both produced more product ion types for the 3+ precursor ion compared to the 2+ precursor, producing 14 and 12 more product ions for ETD and MAD, respectively. When comparing the average percent coverage for each dissociation method for the eleven isoforms, MAD of the 3+ precursors was on average 8% better than for the 2+ precursors. On average, the amount of sequence information generated with CID was roughly the same for either charge state at ~70% sequence coverage. ETD and MAD both provided 8-10% better coverage with

Table 5.1
A Comparison of the Percent Sequence Coverage for CID, ETD, and MAD of Each Tau Phosphopeptide.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>% sequence coverage</th>
<th>CID</th>
<th>ETD</th>
<th>MAD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2+</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>P1a</td>
<td>SRpTPpSLPTPPTREPK</td>
<td>50</td>
<td>64</td>
<td>36</td>
</tr>
<tr>
<td>P1b</td>
<td>SRTpSLPTPPTREPK</td>
<td>57</td>
<td>57</td>
<td>7</td>
</tr>
<tr>
<td>P2a</td>
<td>VAVVRTPPKSPSpSAK</td>
<td>86</td>
<td>93</td>
<td>65</td>
</tr>
<tr>
<td>P2b</td>
<td>VAVVRpTPKpSPSSAK</td>
<td>71</td>
<td>71</td>
<td>65</td>
</tr>
<tr>
<td>P2c</td>
<td>VAVVRTPPKSpSSAK</td>
<td>78</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>P2d</td>
<td>VAVVRTPPKPSPSSAK</td>
<td>93</td>
<td>86</td>
<td>78</td>
</tr>
<tr>
<td>P3a</td>
<td>RSGYSpSPGSPGpTPGSR</td>
<td>82</td>
<td>71</td>
<td>59</td>
</tr>
<tr>
<td>P3b</td>
<td>RSGYSSPGpSPGpTPGSR</td>
<td>47</td>
<td>65</td>
<td>47</td>
</tr>
<tr>
<td>P3c</td>
<td>RSGYSSPGpSPGTPGSR</td>
<td>41</td>
<td>41</td>
<td>71</td>
</tr>
<tr>
<td>P4</td>
<td>TPSLLpTPPPTREPK</td>
<td>67</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>P5</td>
<td>TDHGAEIVYKSPVVSGDTpSPR</td>
<td>85</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>67</td>
<td>71</td>
<td>62</td>
</tr>
</tbody>
</table>

Note: Bold and underline numbers indicate a modification site could not be unambiguously identified.
Figure 5.1. Comparison of the dissociation of the di-phosphorylated Tau peptide P1a SRpTPpSLPTPPTREPK (Tau210-224) generated of the 2+ charge state through (a) CID, (b) ETD, (c) MAD, and of the 3+ charge state through (d) CID, (e) ETD, and (f) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (°) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
Figure 5.2. Venn diagrams comparing the number of product ions types produced for CID, ETD, and MAD of both the 2+ and 3+ charge states of the di-phosphorylated Tau peptides P2b VAVVRpTPPKpSPSSAK (Tau226-240) and P3a RSGYSpSPGSPGpTPGSRSR (Tau194-210).
the 3+ precursor compared to the 2+ precursor. Compared to MAD, CID and ETD achieved 15-20% less sequence coverage for either charge state.

McLuckey and co-workers proposed several reasons for the ETD fragmentation behavior of the 2+ versus 3+ species [48]. One reason for better efficiency with 3+ precursors is that the electron transfer process is simply more exothermic. In charge reduction mode, MAD is equally affected by this phenomenon. However, MAD still achieves ~20% better sequence coverage than ETD for the same charge states. The benefit of MAD over ETD could well reside in the presence of a second fragmentation mechanism such as PI. As with ETD, the electron transfer mechanism in MAD probably leads to the ~8% sequence improvement between the 2+ and 3+ charge states in the phosphopeptides studied. The 20% increase of sequence coverage between ETD and MAD might be attributable to an additional mechanism such as PI, which occurs through electron detachment, not electron transfer. The reaction exothermicity of the electron detachment process is the difference between the potential energy of the metastable atom (~20 eV for He\textsuperscript{M}) and the ionization energy of biomolecular ion. The exothermicity of electron detachment of polypeptides using EDD is approximately 5 eV [50]. Because the ionization potential of the polypeptides is probably in the region of 11-16 eV [178], we estimate the reaction exothermicity of PI using helium to be in the region of 6-10 eV.

5.3.2 Fragmentation Behavior of Phosphorylated Tau Peptides

The major dissociation channels consistently observed in CID are the neutral losses of 98 Da from the precursor and product ions and are indicative of a phosphoric acid loss (−H\textsubscript{3}PO\textsubscript{4}) or the combination of a phosphate group (−HPO\textsubscript{3}) and water.
Additionally, several neutral losses of 80 Da are observed from product ions which correspond to the loss of the phosphate group. In ETD and MAD the main product is usually the charge-reduced species without fragmentation. ETD typically generated two intense charge-reduced species, the \([M+3H]^{2+}\) and \([M+3H]^{3+}\) ions. Compared to ETD, MAD spectra show less intense charge-reduced species. In ETD, many neutral losses are observed from the two charge-reduced species. These neutral losses include \(-\text{H}^+\), \(-\text{OH}\), \(-\text{CO}\), \(-[\text{OH} + \text{NH}_3]\), \(-[\text{CO}_2 + \text{H}]\), and \(-[\text{H}_3\text{PO}_4 / \text{HPO}_3 + \text{H}_2\text{O}]\) (Table 5.2). In contrast to ETD, MAD shows only three neutral losses from the charge-reduced \([M+3H]^{2+}\) product ion: \(-\text{H}^+\), \(-\text{OH}\) and \(-[\text{H}_3\text{PO}_4 / \text{HPO}_3 + \text{H}_2\text{O}]\) (Table 5.2). Furthermore, ETD was unique in producing neutral losses of \(-[\text{CH}_5\text{N}_3]\) and \(-[\text{C}_4\text{HN}_{10}]\), which correspond to neutral losses from the side chain of arginine from peptides P1a and P3b [32].

Figure 5.1 shows CID, ETD, and MAD spectra of peptide P1a, SRpTPpSLPTpTPREPK, which is di-phosphorylated at positions Thr-3 and Ser-5. CID generated backbone fragments either side of modification, thereby providing modification location. Figure 5.1c and d shows ETD of the 2+ and 3+ charge state of P1a, respectively. The lack of dissociation between the first three residues of the 2+ charge state results in ambiguous identification of the modification between the serine and threonine amino acids at the Ser-1 and Thr-3 positions. Similar to CID, ETD produced no cleavages between the Thr-8 and Thr-11 residues for the 2+ charge state of P1a and the 2+ charge states of P1b. MAD produced ample dissociation between all the potential sites of phosphorylation and therefore allowed for unambiguous site determination.
The 3+ charge state of two isoforms of peptide P2 are shown in Figure 5.3. P2a is mono-phosphorylated at position Ser-13 and P2b is di-phosphorylated at positions Thr-6 and Ser-10. All three dissociation methods produced large numbers of product ion types. All three methods, with the exception of CID of the 3+ form of P2a, allow unambiguous PTM localization for both charge states for all four isoforms studied. CID produced sequence coverages of 93% and 71% for P2a and P2b, respectively, whereas ETD was
Figure 5.3. Comparison of the dissociation of the 3+ charge state of the mono-phosphorylated Tau peptide P2a VAVVRTPKSPSAPSAK (Tau226-240) through (a) CID, (b) ETD, (c) MAD, and of the di-phosphorylated Tau peptide P2b VAVVRpTPPKpSPSSAK (Tau226-240) through (d) CID, (e) ETD, (f) and MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (‡) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
able to generate 93% for both peptides. MAD induced cleavage between each backbone bond resulting in 100% sequence coverage for both P2a and P2b and produced up to 25 product ions that retained the modification. Informative spectra were observed for all three methods for P4, TPSLPpTPPTREPK, as illustrated in Figure 5.4. MAD achieved the highest percent sequence coverage at 92%, whereas CID and ETD both produced 67% coverage for the 3+ charge state. However all three methods were able to cleave between the 4 potential sites of phosphorylation and were able to unambiguously identify the location of the modification to the Thr-6 residue.

Figure 5.5 shows the dissociation of the peptide P3b which contains two phosphorylations at positions Ser-9 and Thr-12. MAD of P3b achieves 94% sequence coverage and cleaved between all amino acids with the exception of the Pro-7 and Gly-8 residues. MAD produced cleavage between all potential sites of a phosphorylation modification and allowed for unambiguous PTM localization. ETD and CID both produced 65% sequence coverage, about 30% less than MAD. CID induced fragmentation on either side of the two modifications, which permitted successful PTM site determination. In Figure 5.5b, no observed cleavage between the phosphorylated Thr-12 and the Ser-15 was produced using ETD and thereby results in ambiguous site determination. Similar results are also observed for ETD of the 2+ charge state of P3b, with no cleavage between Thr-12 and Ser-17 residues.

As seen in Figure 5.6, CID and MAD of P5 achieved fairly high sequence coverage at 85 and 90%, respectively. The peptide P5 contains one modification at the Ser-19 position. CID almost produced a complete b-and y-ion series, except for the last several backbone bonds near the C-terminus. Within this section, a crucial cleavage
Figure 5.4. Comparison of He MAD and CID of phosphorylated cholecystokinin in the 3- charge state. An arrow in each plot indicates the precursor. An asterisk (*) indicates fragments having lost the modification. Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
Figure 5.5. Comparison of the dissociation of the 3+ charge state of the di-phosphorylated Tau peptide P3b RSGYSSPGpSPGpTPGSRSR (Tau194-210) through (a) CID, (b) ETD, (c) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (♦) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
Figure 5.6. Comparison of the dissociation of the 3+ charge state of the mono-phosphorylated Tau peptide P5 TDHGAEVYKSPVVSVDTPpSP (Tau386-406) through (a) CID, (b) ETD, (c) MAD. The precursor is indicated by an arrow. Neutral losses are indicated by the following: water (†), phosphorylation modification corresponding to 98 Da (○) and to 80 Da (*). Fragments that don’t retain the modification are omitted from the peptides sequence inserts.
between Thr-18 and Ser-19 is missed which results in CID's inability to unambiguously determine the location of the modification. Cleavage in ETD focuses around residues 6-10 of the peptide leaving little to no fragments over the remaining portions of the peptide. Only 30% sequence coverage of P5 is achieved using ETD. MAD generates cleavage between all potential sites of phosphorylation, including the x_3, y_3, and z_3 ions corresponding to the cleavage between the Thr-18 and Ser-19. MAD therefore achieves unambiguous PTM localization.

Figure 5.7 summarizes the frequency of occurrence of all the product ion types generated from this study of the tau phosphopeptides. CID produces mainly b-/y-type ions with few a-, b-, and z-type ions. ETD produced mainly c-/z-type ions, and more specifically in the form of c•- and z’-type ions. In ETD, y-type ions are more common than a- or b-type ions, but all three are observed in much lower intensities than the c-/z-type ions. ETD is also known to be hindered by proline residues and was unable to produce c-/z-ions on the N-terminal side of proline. In several cases ETD produced a- and y-type ions. For example, in Figure 5.3c, the peptide P2a produced the y_8 and y_9 ions. These observations of a-, b-, and y-type ions in the ETD spectrum are in agreement with several other ETD/ECD studies [33, 179, 180]. MAD produces similar frequencies for all product ion types with the exception of x-type ions which were less common. For multiply-charged cations, the main difference between ETD and MAD is that MAD can induce dissociation through PI. In theory, PI can directly generate radicals anywhere along the peptide backbone where there is a polar group or lone pair of electrons, such as carbonyl oxygen atoms or amide nitrogen atoms, so the fragmentation pattern is expected to be less dependent on sites of protonation [68].
Figure 5.7. Charts plotting the total frequency of occurrence of the product ion types for CID, ETD, and MAD during this study.

Venn diagrams in Figure 5.2 and Figure 5.8 illustrate the complementary nature of CID, ETD, and MAD. CID and ETD typically shares 1-7 (<10%) of the fragment ion types and are therefore more complementary. MAD generates abundant b-, c-, y-, and z-type ions and therefore has more in common with both CID and ETD. In general, MAD produces in one spectrum what CID and ETD must produce in two spectra. MAD therefore provides spectra that might be highly beneficial for data-mining [105, 173].
Figure 5.8. Venn diagrams comparing the number of product ions types produced for CID, ETD, and MAD of 3+ charge states of the three Tau peptides (a) p2a VAVVRTPPKSPpSSAK (Tau226-240), (b) P3b RGYSSPgpSPgTPGRSR (Tau194-210), and (c) P5 TDHGAEIVYKSPVSGDTpSPR (Tau386-406).

5.4 Conclusion

On average, CID produced percent sequence coverage’s of ~70% for the 2+ and 3+ charge states and failed to unambiguously identify the modification site ~20% of the time. Additionally, abundant neutral losses of the phosphorylation modification and water were observed which significantly complicated the spectra. ETD generated a variety of a-, b-, c-, y-, and z-type ions, but the c-/z-ions observed were in greatest abundance. The sequence coverage varied between 7% and 93% and fails to unambiguously identify the location of the PTM ~30% of the time. MAD generated the most backbone cleavages, between 83% and 91% sequence coverage for the 2+ and 3+ charge states, respectively, and only failed to unambiguously identify the modification site on one occasion (~5% of the time).

CID and ETD are routine methods for sequencing peptides and analyzing phosphorylation modifications. However, our data has shown that MAD offers an
alternative method to complement CID and ETD. MAD produces higher sequence coverage than either CID or ETD, and can unambiguously locate the site of multiple modifications.
CHAPTER 6: METASTABLE ATOM-ACTIVATED DISSOCIATION MASS SPECTROMETRY OF SINGLY-CHARGED SPHINGOMYELIN AND PHOSPHATIDYLCHOLINE (16:0-18:1)

6.1 Introduction

Tandem mass spectrometry (MS/MS) is an integral tool used to identify, quantify, and determine structural information of a variety of different biological molecules, including lipids. The study of lipids and how their function affects the surrounding biological system is known as lipidomics [181]. Identification, quantification, and structural characterization can aid in the understanding of the biological function and integral role of a specific lipid. Lipids are well known to be important players in cell signaling pathways and aid in protein-protein interactions [181]. Sphingomyelins (SM) are metabolic precursors of ceramides which are integral compounds utilized in skin protection as well as compounds in nervous tissues and plasma membranes [182]. Phosphatidylcholine (PC) also serve as components of plasma membranes in addition to serving as secondary messengers to metabolism [183].

Several different MS/MS techniques have been employed to determine both primary structural characterization of lipids and to determine the exact site of unsaturation or double bond placement within the fatty acid chain. Turk and co-workers have spent more than a decade investigating lipids through multi-stage collision induced dissociation (CID) MS for structural characterization by creating alkali adducts of the lipid species [182, 184, 185]. Additionally, Hsu and Turk were able to determine the lipid structure, fatty acid substituents, polar head group, and site of unsaturation using CID
MS/MS techniques [185]. Ozone-induced dissociation (OzID) has become a promising method to aid in the localization of the unsaturated bonds, however to achieve complete structural characterization the use of CID in combination with OzID is necessary [186]. Electron capture dissociation (ECD) [31, 33, 35] and electron transfer dissociation (ETD) [30, 48, 133] has become the preferred methods to obtain structural characterization of peptides and proteins. This is contributed to the extensive sequence coverage and its ability to retain post-translational modifications, which is uncharacteristic of CID. McLuckey et al. explored the ability of ETD to characterize a doubly sodiated glycerophosphocholine lipid [183]. They utilized ETD in combination with CID to obtain limited structural information as well as the location of unsaturation.

Recently a novel dissociation method has been introduced which induces fragmentation through radical ion chemistry similar in nature to ETD and ECD through the interaction of metastable atoms with isolated ions. We refer to the technique as metastable atom-activated dissociation (MAD) [68, 160, 177, 183]. Extensive backbone cleavage is observed while retaining labile PTMs when dissociating peptides. The observed dissociation is contributed to two competing mechanisms, charge reduction and Penning ionization (PI) [68, 160, 177]. MAD is applicable to singly- and multiply-charged cations as well as multiply-charged anions, therefore it is of interest to explore the versatility of MAD and observed a variety of ions dissociation behavior when exposed to high kinetic metastable atoms.

In this work we explore the dissociation behavior of the 1+ charge state of protonated and sodiated SM and PC comparing MAD to CID. Extensive fragmentation and complete structural information is obtained for both the protonated and sodiated
forms of SM and PC are generated through MAD. The sodiated species tend to consistently produce the neutral loss of the trimethylamine species from the head group showing possible conformational sensitivity to MAD. The major CID fragmentation pathway show preferential cleavage within the polar head group and produces very little other structural characterization information.

6.2 Experimental

All experiments were performed on a modified Bruker Esquire LC QIT MS (Bruker Daltonics, Bremen, Germany) which contained a 2 mm hole in the top portion of the ring electrode. A modified Ion Tech FAB gun (P50 PSU, Teddington, UK) was used to generate a beam of high kinetic metastable atoms which was placed directly above the hole in the ring electrode of the MS to allow metastable atom to interact with the isolated ions within the ion trap. A full description of the MS modifications, FAB gun modifications, and the metastable atom pulse production has been illustrated in a previous manuscript [68].

Lipids were reconstituted to a 9:1 ratio of methanol (Sigma-Aldrich, HPLC grade, St. Louis, Mo) and water with 1% acetic acid (Sigma-Aldrich). Concentrations were 40 μM for CID and MAD experiments. The 1+ charge state of sphingomyelin and phosphatidylcholine (16:0-18:1) were generated by ESI at a flow rate of 0.4 mL/hr. The isolation window varied between \( m/z \) 1.5-4.0. A low mass trapping voltage was set to 200 \( m/z \) to minimize background ions from the PI of residue pump oils. The collisional activation energy was set to 35 mV for all CID experiments. The FAB gun anode was powered at 6 KeV and the metastable atom beam was activated for 75-125 ms. All
fragment ion identifications and assignments were manually determined based on predicted fragmentation patterns and were within ±0.8 m/z of the expected product ions. Peaks were only assigned if the intensities were at least 3 times the signal-to-noise ratio.

6.3 Results and Discussion

Extensive fragmentation within the glycerol backbone is achieved of the protonated form of SM through MAD as illustrated in Figure 6.1. PI at the carbonyl oxygen atom located between the 18:0 fatty acid chain and the nitrogen atom leads to the 491.2, 239.1, and 225.0 m/z peaks. These correspond to the production of radical fatty acid chain fragments. Additionally, the polar head group (–(PO₄H(CH₂)₂N(CH₃)₃)) is characterized by the peak 547.7 m/z which occurs through PI at the –PO₄ group. The fragments ions of 284.3 and 447.4 m/z in addition to the other cleavages adjacent to the nitrogen atom are due to PI occurring directly at lone pair of electrons on the nitrogen atom. Metastable atoms are known to be attracted to lone pairs of electrons, especially those on amides nitrogen and carbonyl oxygen atoms [62, 65, 66]. A small Penning ionized species, [M+2H]²⁺, is observed. CID of protonated SM produced little structural information (Figure 6.1) which is consistent with literature [182]. The most dominant CID fragmentation pathway is the neutral loss of water (18 Da) from the precursor ion observed at 713.7 m/z. The small peaks observed at 669.6 and 654.6 m/z correspond to the neutral loss of the three methyl groups attached to the trimethylamine and the trimethylamine, respectively. The polar head group is identified through the two fragments, 545.6 and 530.6 m/z.
Figure 6.1. Comparison of He MAD and CID of the [M+H]+ charged species of sphingomyelin. The precursor is indicated by an arrow.
Similar fragment ions are observed for the sodiated SM in comparison to the protonated SM when dissociated by MAD (Figure 6.2). The only negative effect the sodium had was the lack of observed PI at the amide nitrogen atom. However cleavage producing a neutral loss of the trimethylamine group from the head group as well as cleavage within the \(-\text{PO}_4\) group are new fragment patterns observed. On several occasions the fragment ions underwent a neutral loss of the sodium, such as from the precursor ion, the 570.7, and 513.4 \(m/z\) ions. The sodiated form of SM produced two fragment ions through CID providing structural characterization of the head group only (Figure 6.2). The loss of \(-\text{N(CH}_3)_3\) (59 Da) from the polar head group was the most dominant fragmentation channel observed [187, 188]. An abundant peak identified as the loss of the entire head group (184 Da) is also observed at \(m/z\) 572.2.

Figure 6.3 illustrates the MAD fragmentation of the protonated PC species. The \(m/z\) 576.6 ion corresponds to the loss of the head group. The 18:1 fatty acid chain is characterized by the fragment ions at \(m/z\) 236.0 and 524.4, which correspond to PI at the carbonyl oxygen atom and the radical migration toward the chain. In addition, the ions at \(m/z\) 264.2 and 496.3 correspond to the cleavage of the lipid chain and radical migration toward the head group. Systematic fragmentation is observed within both the 18:1 and 16:0 fatty acid chains which indicate that PI could occur at the carbonyl oxygen atoms and then propagates down the chains—a process known as charge remote fragmentation in electron-ionized species [189]. Isolation and MS\(^3\) of the 264.2 and 239.2 ions were unavailable due to low relative intensity. CID of the protonated form of PC produced ample fragmentation to achieve structural characterization even though the fragmentation pattern is very different in comparison to MAD (Figure 6.3). The major structural peaks
Figure 6.2. Comparison of He MAD and CID of the [M+Na]^+ charged species of sphingomyelin. The precursor is indicated by an arrow.
Figure 6.3. Comparison of He MAD and CID of the [M+H]$^+$ charged phosphatidylcholine (16:0/18:1) species. The precursor is indicated by an arrow.
correspond to $m/z$ 577.7, which is the neutral loss of the polar group, $m/z$ 478.3 ([M-RCH=C=O]$^+$) and 496.3 ([M-RCH$_2$COOH]$^+$), which are the neutral losses of the 16:0 fatty acid chain, and $m/z$ 504.4 ([M-RCH=C=O]$^+$) and 522.5 ([M-RCH$_2$COOH]$^+$), which are the neutral losses of the 18:1 fatty acid chain [188]. All peaks produced through CID were of very low relative intensity. Therefore, the isolation and a multi-stage MS/MS experiment to determine the position of any unsaturated bonds is unavailable.

The amount and intensity of MAD fragmentation observed is decreased when PC is sodiated (Figure 6.4) versus when it is protonated. Although the fragment ions necessary to obtain structural characterization are present in the sodiated MAD spectrum, complete structural characterization of the 16:0 chain was not obtained or was below the signal to noise ratio. Several fragment ions correspond to the neutral loss of the trimethylamine (723.6 $m/z$), the trimethylamine and the 16:0 fatty acid chain (513.4 $m/z$), and the trimethylamine and the 18:1 chain (441.3 $m/z$). Several fragment ions corresponding to the possible propagation of the radical down the 16:0 and 18:1 fatty acid chains are illustrated in Figure 6.4. This propagation of fragmentation is of notable importance and could potentially produce information about location and degree of unsaturation within the fatty acid chains. Contrary to the protonated PC CID spectrum, the sodiated PC produced very little structural fragmentation ions through CID (Figure 6.4). The presence of the sodium ion hinders the dissociation within the glycerol backbone leading to no fragmentation near the fatty acid chains. The two fragment pathways observed correspond to the neutral loss of the trimethylamine in the head group (723.9 $m/z$) and of the complete polar head group –PO$_4$(CH$_2$)$_2$N(CH$_3$)$_3$ (599.9 $m/z$).
Figure 6.4. Comparison of He MAD and CID of the [M+Na]$^+$ charged phosphatidylcholine (16:0/18:1) species. The precursor is indicated by an arrow and the neutral loss of the sodium is indicated by an asterisk.
Conformational sensitivity to MAD fragmentation is suggested when we compare the sodiated and the protonated forms of the lipids. PI can only occur at HOMO sites that are accessible to interactions with metastable atoms. When the lipid is protonated the proton bound to the phosphate group or the charge on the quaternary amine group can be stabilized through interactions with the carbonyl oxygen atoms of the fatty acid chains as illustrated in Figure 6.5. When the amine group is stabilized, which is ~1.7 kcal/mol more favorable (Figure 6.5a) as opposed to when the proton is stabilized (Figure 6.5b), the lone pair on the phosphate oxygen closest to the ammonium group (indicated with a circle) is therefore protected from ionization. The favored conformer is consistent with the absence of fragmentation between the phosphate and ammonium group. When the

![Figure 6.5. 3-D model of protonated phosphatidylcholine. The ester-trimethylammonium interaction is 1.7 kcal/mol over the (b) ester-phosphate interaction. Calculations performed by Dr. E. Masson using the B3LYP density functional method and 6-31 G (d) basis set (fatty acid chains were shortened for the calculations).](a) ![Figure 6.5. 3-D model of protonated phosphatidylcholine. The ester-trimethylammonium interaction is 1.7 kcal/mol over the (b) ester-phosphate interaction. Calculations performed by Dr. E. Masson using the B3LYP density functional method and 6-31 G (d) basis set (fatty acid chains were shortened for the calculations).](b)
lipid is sodiated, either the sodium or the ammonium group can be stabilized by the carbonyl groups as illustrated in Figure 6.6. The sodium-ester interaction (Figure 6.6b) is 11 kcal/mol more favorable in comparison to the proton-ester interaction (Figure 6.6a). In the favored configuration, the lone pair on the phosphate oxygen closest to the ammonium group is exposed and is a possible site of ionization. This explains the presence of the fragment ions related to the trimethylamine losses as illustrated is Scheme 6.1.

Figure 6.6. 3-D model of sodiated phosphatidylcholine. The ester-\(\text{Na}^+\)-phosphate interaction (a) is 11 kcal/mol over the (b) ester-trimethylammonium interaction. Calculations performed by Dr. E. Masson using the B3LYP density functional method and 6-31 G (d) basis set (fatty acid chains were shortened for the calculations).
Scheme 6.1. Possible outcome from Penning ionization (PI) on the oxygen atom directly connected to the phosphate atom and two carbon atoms away from the trimethylamine portion of the head group of both the sodiated PC and SM lipids. Dissociation generates cleavage between the nitrogen and carbon atoms.

6.4 Conclusion

Reactions between lipid molecules and high kinetic helium metastable atoms generate amble product ions and a robust method for lipid structural determination. Preferential cleavage is observed at ester linkages for the protonated species with additional trimethylamine neutral losses for the sodiated species with MAD. Additionally, MAD offers the unique ability to induce radical ion chemistry of singly-charged species through the mechanism of Penning ionization unlike ETC and ECD.
CHAPTER 7: CONCLUSIONS AND FUTURE WORK

Protein characterization through MS/MS techniques such as CID, ECD, and ETD, has become a highly used tool in proteomics due to its high sensitivity and specificity. However, research has shown that each method have certain drawbacks. CID typically produces incomplete sequence information and preferentially cleaves labile PTMs and other low energy bonds. ECD is unable to fragment singly-charged cations or negatively-charged anions and must be employed on expensive FT-ICR instruments. Similar to ECD, ETD is most efficacious on multiply-charged cations with fragmentation efficiency depending on charge. As well with ETD, larger bioions tend to undergo charge reduction reactions instead of fragmentation. Therefore employing a combination of these MS/MS methods provides a more robust peptide validation technique. MAD has been demonstrated to efficiently fragment 3+, 2+, 1+, 1-, and 2- charged peptides with high sequence coverage while retaining labile PTMs. Additionally, the fragmentation produced through MAD is indicative of the fragmentation observed through both CID and the radical induced fragmentation techniques of ECD and ETD alleviating the need for multiple fragmentation techniques.

The work presented herein demonstrates proof of principle of our novel dissociation technique, MAD. In the future, systematic fragmentation studies of a variety of different peptides should be conducted to determine the preferred fragmentation pathways of MAD. A large pool of peptides which only differ by one amino acid (i.e. AAALAAA, AAAKAAA, AAAMAAA) needs to be synthesized and analyzed in both polarities in a varieties of charge-states to determine if MAD has a propensity to favor a
specific fragmentation pathway. To probe the fragmentation mechanism further, synthetic peptides with basic residues (K and R) and acidic residues (D, E, N and Q) at different positions should be investigated to determine if the location of charge will have an affect on the fragmentation site. Additionally, a study which analyzes larger peptides with charge states greater that 3+ needs to be conducted to determine MADs ability to fragment peptides of high charge states. Then studies would extend the analysis to top down experiments on proteins.

To date, MAD has been applied to peptides cation/anions, phosphorylated, sulfated, nitrated, nitrosylated, and glycosylated bioions as well as singly-charged lipids. Due to the unique radical induced fragmentation process of MAD, charge reduction and Penning ionization, it is applicable to a wide variety of applications. Chapter 5 discusses our preliminary results from MAD dissociation of singly-charged lipids and illustrates the extensive structural information generated. A more thorough analysis of lipids is still needed to assess the ability of MAD to determine the degree of unsaturation within the lipids fatty acid chains. For this study a group of SP and PC lipids with different amounts and locations of double bonds must be analyzed to determine MADs ability to locate the position and degree of the double bond(s). Additionally, negatively-charged lipids should be investigated.

MAD could also be extended to the study of DNA and carbohydrates. DNA and carbohydrates both ionize readily in negative ion mode; therefore the fragmentation mechanisms of ECD and ETD are not suited to these applications. MADs mechanism of Penning ionization allows ionization of negatively charged ions in a similar mechanism to EDD and currently MAD demonstrates efficient fragmentation and PTMs location for
phosphorylated and sulfonated bioions. Future studies ought to start with small glycans and determine MADs ability to produce cross-ring and glycosidic cleavages for structure information and glycan linkage. A group of synthetic saccharide chains should be generated to record MADs preferential fragmentation pathways. Studies must scale to larger glycans and carbohydrates with multiple branches and assess MADs ability to determine branch linkage and structure. In addition to glycan and carbohydrates, I would also like to see MAD extended to the study of DNA. Proof of principle studies could start with small single stranded DNA sequences to determine MADs ability and compare the fragmentation produced to other well-known fragmentation mechanisms, such as CID. Other factors which influence the fragmentation behavior could also be explored such as nucleotide residue, length of DNA, and single-stand versus double-stand.
REFERENCES


ASMS Conference on Mass Spectrometry and Allied Topics; Salt Lake City, UT, May, 2010.


