A Dissertation by

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The following faculty members have examined the final copy of this dissertation for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy with a major in Chemistry.

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DEDICATION

To my mother Kathleen, father Robert, stepfather Skip, stepmother Donna, and Drs. Ramon and Pearla Hizon, the most supportive and influential people in my life.
I can live with doubt and uncertainty. I think it’s much more interesting to live not knowing than to have answers which might be wrong.

--Richard P. Feynman 1981
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Novel fragmentation pathways of various model peptides were generated by collision-induced dissociation (CID) mass spectrometry. For the series AXAG where X=βA, γAbu, εCap, or 4AMBz, the CID of the \([b_3-1+\text{Cat}]^+\) where the residue to the C-terminal side of X is changed and labeled with \(^{15}\text{N}\) and \(^{13}\text{C}\) leads us to believe that the dissociation pathway most likely leads to a metal-cationized nitrile that is most prominent relative to the \([a_3-1+\text{Cat}]^+\) species in the order \(\beta\text{A} < \gamma\text{Abu} < \varepsilon\text{Cap} < 4\text{AMBz}\). The product ion is also most prominent with \(\text{Li}^+\) and \(\text{Na}^+\) metal-cationized peptides and to a small extent for \(\text{Ag}^+\).

Extensive isotope labeling was also used to investigate using CID and multiple-stage tandem mass spectrometry the elimination of \(\text{H}_2\text{O}\) from lithium-cationized model tripeptide methyl esters. The loss of \(\text{H}_2\text{O}\) is initiated by a nucleophilic attack from the N-terminal side upon an amide carbonyl carbon atom forming a five-membered ring intermediate and successive 1,2-elimination of \(\text{H}_2\text{O}\). The same isotope-type experiments were used to investigate the \(b_3^+\) to \(a_3^+\) dissociation reaction for the model peptide tetraglycine (GGGG). Isotope labeling indicated the reaction pathway involves the elimination of \(\text{CO}\) and \(\text{NH}_3\), most notably the \(\text{NH}_3\) is composed of two amide hydrogen atoms with the third coming from an \(\alpha\)-carbon position. The loss of \(\text{NH}_3\) also involves the loss of the nitrogen atom of the \(b_3^+\) oxazolinone ring. Density functional studies were performed to account for the unexpected scrambling of \(\alpha\)-carbon hydrogens as well as the loss of \(\text{CO}\) and \(\text{NH}_3\) through what is indicated to be a multistep reaction cascade involving various ion-molecule complexes.

Placement of the alternative amino acids \(\beta\text{A}, \gamma\text{Abu}, \varepsilon\text{Cap}, \text{or 4AMBz}\) in varied positions of model peptides, most notably AAXG, AXAG and XAAG, we were able to directly affect the propensity to form specific \(b_n^+\)- and \(y_n^+\)-type product ions for protonated peptides. Substitution of the aforementioned residues at the varied positions within the model peptides confirmed our hypothesis that by forcing the formation of larger ring intermediates, certain ion formations would no longer be favorable. The proton transfer and intramolecular nucleophilic attack required to form the respective \(b_n^+\)- and \(y_n^+\)-type product ions was hindered as indicated by decreased ion intensity or lack of any presence of the desired ions. For the series AXAG and XAAG, the abundance of b-type ions indicates that either the protonated molecular ions are initially protonated at amide oxygen atoms or involve larger, whole peptide cyclic...
intermediates as was studied with the model peptide sequence FGGFL. $^{15}$NF labeling within FGGFL in
the final study was used to validate the mechanism proposed in the previous a$_3^+$ study of tetruglycine for
the formation of the a$_4^+$ ion from a$_4^*$. A new rearrangement pathway is also presented for the a$_4^*$-CO
which transfers the C-terminal residue to the N-terminus.
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1.1 Introduction & literature review

Understanding peptide fragmentation patterns and the correlations to fragmentation mechanisms is the primary goal for this research study. Studies prior to those included in this text built a fundamental foundation in the areas of amino acid/peptide gas-phase chemistry. One of the first mass spectrometric studies performed on amino acids was undertaken by Harrison utilizing chemical ionization as the ionization source.\[1\] As larger peptides and eventually proteins\[2, 3\] became of interest to mass spectrometrists, the establishment of a standard peptide fragmentation notation, that at the time was for FAB (fast atom bombardment), rather than ESI (electrospray) ionization, was set as a beginning milestone in peptide fragmentation chemistry.\[4\] The sequencing of peptides with metal cations such as Na\(^+\), K\(^+\), Li\(^+\) and Ag\(^+\) and mapping of their fragmentation pathways showed that there wasn’t a simple single or even handful of rules that explained the gas-phase dissociation patterns that were observed. The study of how these metals were bound to the peptide residues began.\[5, 6\] These coordination studies with Li\(^+\), Na\(^+\), K\(^+\) and others began to raise questions as to why certain peptides generate specific b- and y-type ions.\[7, 8\] Studies of multiply-charged protonated peptides began to develop as electrospray ionization became more common bringing about some of the first studies on tryptic peptides digested from larger proteins.\[9, 10\] Tandem mass spectrometry was having a significant impact on the progression of the interpretation of mass spectra of peptides and CID sequence specific ions.\[11, 12\] The observation was made, most notably for protonated peptides that there must be a correlation between the site of protonation within a peptide and the dissociation product ions observed in the spectra.\[13-15\] This is where the “mobile proton” model was introduced.\[16\] Since the evolution of the mobile proton model, there have been a great many studies designed to obtain structure and mechanisms for the generation of sequence specific ions including the more recent pathways in competition (PIC) model.\[17-21\]

The advent of computational chemistry and molecular modeling of gas-phase structures brought about a new aspect to mass spectrometric analysis of peptides and the structures of their respective ions.
Mass spectrometry by its very nature generates ions in the gas-phase, devoid of any solvent effects that most chemists have to deal with in the solution-phase. Studying gas-phase fragmentation mechanisms with ab initio (in the earlier days) and density functional theory (DFT) such as the methods available in the Gaussian genre of programs requires minimal molecule to molecule interaction to obtain a fairly accurate representation of the most plausible structures.[22-29]

There still remained a great many questions to be answered for both protonated and metal-cationized peptides. For instance, what is the correlation between the coordination chemistry of Ag⁺ and H₂O adducts that are observed in the spectra?[30, 31] Can the amino acid/peptide fragmentation mechanisms be developed from isotope labeled neutral losses or ions for these systems?[32] Furthermore, experiments involving functional group ring substitution, as well as N-terminal acetylation and C-terminal esterification generate fragment ions via tandem mass spectrometry that differentiate fragmentation pathways from unmodified [M+Cat]⁺ that are dependent upon resonance stabilized, nucleophilic and electrophilic fragmentation effects as well as acidic hydrogen’s from either the N- or C-terminus respectively.[31, 33] The use of the Ag⁺ cation at various MSⁿ stages has also been shown to assist in identification of sequence specific ions such as the [bₙ₊₁+Ag]⁺, [bₙ₋₁+Ag]⁺, [aₙ₋₁+Ag]⁺ and [yₙ₋₁+Ag]⁺ in electrospray ionization tandem mass spectrometric studies for both larger peptides such as leucine enkephalin and the smaller single amino acids such as phenylalanine.[30-37] Varying substituent size or bulkiness for α-amino acids w/ respect to the C-terminal side of the cleavage site has been shown to promote the [bₙ₊₁+Ag]⁺ over the [bₙ₋₁+Ag]⁺ when the amino acid to the N-terminal side of cleavage is glycine indicating that R-group size influences the fragmentation patterns for specific sequences.[34] Rearrangement reactions involving γ-hydrogen’s for ethyl, propyl and butyl esters placed on the C-terminus generates a γ-hydrogen transfer to the C-terminal carbonyl group along with the elimination of an alkene via a McLafferty-type rearrangement which was verified by use of deuterium labeling on the terminal methyl group of the ethyl ester.[35] Suppression and elimination of cyclization reactions which produce the [b₃₊₁+Cat]⁺ for the peptide sequence AcFGGX by introduction of amino acids where X=β-alanine (βA), γ-aminobutyric (γAbu) and ε-amino caproic (Cap) acids or X=p- or m-aminobenzoic acids for the elimination of the cyclization process altogether was observed for the Ag⁺, Li⁺ and Na⁺ cations indicating a direct effect on cyclization by the forced formation of larger ring sizes or the inability to form
Intramolecular proton scrambling was also probed via *in situ* isotope labeling with deuterium replacing all amide exchangeable hydrogen's indicating hydrogen scrambling occurs amongst adjacent exchangeable sites during CID when forming the \([b_2+17+Li]^+\).

As has been shown above, ESI-MS utilizing a quadrupole ion trap is a diverse instrumental method to study gas-phase ions as well as their chemistry not by just observing fragment ions at the detector, but by also directly affecting the activation time from longer durations to no activation times for studying adducts. Activation energies also play a role in ion relative abundance and can be compared across series of molecules as long as vibrational degrees of freedom are taken into account and machine tune parameters are kept constant within the experiment. Multiple-stage tandem mass spectrometry combined with collisional activation or collision-induced dissociation has generated reproducible fragment ion patterns and continues to do so as shown in the following chapters in this dissertation to assist in solving the original problem of possessing a better understanding of gas-phase peptide fragmentation patterns. The following studies are a continuation of this pursuit. The objectives of these further studies is to design and perform CID studies on metal and protonated model peptides that contain specific amino acid residues and isotope labels at strategic positions within each peptide to deduce fragmentation mechanisms for varied sequences of peptides as well as observe positional effects on ion formation. For as much time and resources are available, validate proposed mechanisms and explain observed rearrangement/positional effects within the studied model peptides via DFT studies.

The isotope labeling and larger aliphatic backbone amino acid substitution experiments previously studied \([32, 36]\) were continued for the peptide series AXAG where X=βA, γAbu, Cap as well as the incorporation of 4-aminomethylbenzoic acid (4AMBz) to elucidate a new fragmentation pathway for the CID of \([b_2-1+Cat]^+\) \([38]\) in chapter two. Extensive \(^2\text{H}, \ ^{13}\text{C}\) and \(^{15}\text{N}\) isotope labeling along with collision-induced dissociation (CID) and multiple-stage tandem mass spectrometry is used to investigate the elimination of \(\text{H}_2\text{O}\) from a series of model metal-cationized tripeptide methyl esters in chapter 3.\([39]\) Up until this point there has been much discussion of metal-cationized fragmentation pathways and chapter four shows that the mechanism for the \(a_3^*\) ion, observed from protonated GGGG, can be mapped by utilizing \(^{15}\text{N}, \ ^2\text{H}, \) and \(^{13}\text{C}\) isotope labeling combined with density functional theoretical studies.\([40]\) The study of the influence of alternative amino acids on the formation of various ions was also studied at
positions X in XAAG, AXAG, and AAXG for protonated tetrapeptides [41] in chapter five to show the affect of alternative amino acids X where X=βA, γAbu, Cap and 4AMBz on the formation of various fragmentation ions. For instance, to test a hypothesis that the b$_3^+$ ion is formed through a cyclization reaction across the X in AAXG, by placing an alternative amino acid in the X position, a larger ring (6, 7, or 9-member rings from βA, γAbu, Cap, respectively) would have to be formed which should either reduce ion intensities compared to the template (normal AAAG) or make cyclization unfavorable entirely (as with 4AMBz). In addition to the previous metal-cationized leucine enkephaline study, [33] the model system FGGFL (rather than YGGFL) was subjected to extensive $^{15}$N labeling and successive fragmentation studies to better understand sequence scrambling within the ion trap. The ions generated as well as their mechanisms of formation were modeled with DFT methods and are discussed in chapter six.[42] The closing chapter, chapter seven, is a discussion of the general conclusions and further studies.
CHAPTER 2

NOVEL FRAGMENTATION PATHWAY FOR CID OF (b_n–1+Cat)^+ IONS FROM MODEL, METAL-CATIONIZED PEPTIDES.

2.1 Introduction

In an attempt to enhance the understanding of how cation and sequence influence peptide fragmentation, we recently investigated the dissociation of model N-acetylated tetrapeptides with the general sequence AcFGGX that featured C-termini designed to allow transfer of -OH required to generate the [b_3+17Cat]^+ product ion, but not necessarily as the most favored pathway.[1] The amino acid placed at the position “X” either required a larger cyclic intermediate than the five-membered rings presumably formed with α-amino acids (β-alanine, γ-aminobutyric acid, and ε-amino-n-caproic acid to generate 6-, 7-, or 9-membered rings, respectively) or prohibited cyclization because of the inclusion of a rigid ring (para- and meta-aminobenzoic acid). For Ag^+, Li^+ and Na^+ cationized AcFGGX, formation of [b_3+17+Cat]^+ was suppressed when the amino acids requiring the adoption of larger ring intermediates were used, while amino acids that prohibit cyclization eliminated the reaction pathway completely, an observation in accord with proposed mechanisms for the formation of this important sequence ion.[2-8]

During subsequent experiments involving peptides containing similar “alternative” amino acids, we observed an unusual fragmentation pathway when the [b_3–1+Li]^+ product ion derived from the synthetic peptide A(βA)AG was subjected to collision-induced dissociation (CID). The pathway resulted in a neutral loss one mass unit (u) greater than the residue mass of the amino acid that composed the presumed oxazolinone ring[9-15] of the [b_3–1+Li]^+ species, and thus could not be attributed to the formation of the [b_2–1+Li]^+ ion. We describe here experiments involving a group of tetrapeptides of general sequence AXAG, where X = β-alanine, γ-aminobutyric acid, ε-amino-n-caproic acid, and 4-aminomethylbenzoic acid, and analogous peptides with ^15N and ^13C labels in specific positions that were designed to probe the unusual reaction pathway. This specific set of experiments, focused on the fragmentation of the [b_n–1+Cat]^+ ions (as opposed to their formation), showed that the reaction pathway most likely generates a metal-cationized nitrile product.
2.2 Experimental

All peptides used in this study were generated by conventional solid-phase synthesis methods[16] using 9-fluorenylmethoxycarbonyl (FMOC)-glycine loaded Wang resin (Sigma Chemical, St. Louis MO) and a custom-built, multiple-reaction vessel peptide synthesis apparatus. Glycine-$^{15}\text{N}$, glycine-$^{1,13}\text{C}$, γ-aminobutyric acid ($\gamma\text{Abu}$), and FMOC-chloride were purchased from Sigma Chemical and used to generate FMOC-glycine $^{15}\text{N}$ (G-$^{15}\text{N}$), -glycine-$^{1,13}\text{C}$ (G-$^{1,13}\text{C}$) and $\gamma\text{Abu}$ for incorporation into the model peptides. FMOC protected glycine, alanine, valine, β-alanine ($\beta\text{A}$), ε-amino-n-caproic acid ($\varepsilon\text{Cap}$), and 4-aminomethylbenzoic acid (4Ambz) were purchased from Sigma and used as received. Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies.

Metal nitrate salts (Li$^+$, Na$^+$ and Ag$^+$) were purchased from Aldrich Chemical (St. Louis, MO) and used as received. Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (vol:vol) mixture of HPLC grade MeOH (Aldrich Chemical) and deionized H$_2$O to produce final concentrations of $10^{-5}$-10$^{-4}$ M. Equimolar metal nitrate solutions were prepared in deionized H$_2$O.

ESI mass spectra were collected using a ThermoFinnigan LCQ-Deca ion-trap mass spectrometer (San Jose, CA). Mixtures (1:1 by volume) of metal nitrate and peptide, prepared by mixing 0.25 mL of the respective stock solutions, were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 3-5 $\mu$L/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octupole voltage offsets, etc.) were optimized for maximum (M+Cat)$^+$ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N$_2$ sheath gas flow at 25 units (arbitrary for the Finnigan systems, corresponding to approximately 0.375 L/min) and the capillary (desolvation) temperature at 200°C. The ion trap analyzer was operated at a pressure of $\sim1.5 \times 10^{-5}$ Torr. Helium gas, admitted directly into the ion trap, was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The multiple-stage CID studies were performed as follows. The alkali metal-cationized peptides were isolated for the initial CID stage (MS/MS) using an isolation width of 1.5 mass to charge (m/z) units. The Ag$^+$ cationized peptides were isolated using a width of 6 m/z units, with the isolation mass centered between the $^{107}\text{Ag}$ and $^{109}\text{Ag}$ isotopic peaks. The activation amplitude, which defines the amplitude of the
RF energy applied to the end cap electrodes in the CID experiment, was set between 20% and 35% (chosen empirically, representing approximately 0.66 → 1.54 V, 0-p, laboratory frame) and the activation Q (as labeled by ThermoFinnigan, used to adjust the qz value for the precursor ion) was set at 0.30. The activation time employed at each CID stage was 30 ms.

2.3 Results and Discussion

Figure 2.1 shows the CID (MS/MS stage) spectra generated from Li⁺ cationized AAAG (Figure 2.1a) and A(βA)AG (Figure 2.1b). For both peptides, CID generated the [b₃+17+Li]⁺, [b₃-1+Li]⁺, and [a₃-1+Li]⁺ ions at m/z 238, 220, and 192, respectively. Peaks at m/z 277 and 260, corresponding to the elimination of H₂O (18 mass units, u) and the combination of H₂O and NH₃ (35 u) were generated by CID of AAAG but not of the analogue containing βA. Recent investigations in our laboratory suggest that the reaction to eliminate H₂O and combination of H₂O and NH₃ are initiated by nucleophilic attack by the N-terminal amide carbonyl oxygen atom upon the carbon atom of the adjacent amide group. The absence of the two reaction products for the peptide containing βA adjacent to the N-terminus is consistent with the cyclization and intra-molecular attack involving a kinetically and entropically less favored 6-member ring and with our earlier study of the influence of amino acids on the generation of (bₙ+17+Cat)⁺ products.[1]

Figure 2.1. CID (MS/MS) spectra of Li⁺ cationized tetrapeptides: (a) AAAG; (b) A(βA)AG.
The \([b_3-1+Li]^+\) ion generated from AAAG and \(A(\beta A)AG\) is presumably a lithium-cationized oxazolinone.[11] Often, the most prominent dissociation pathway for \((b_n)^+/[b_{n-1}+Cat]^+\) species is the elimination of CO to produce the \([a_{n-1}+Cat]^+\) ion (Scheme 2.1).[9, 10, 14, 17, 18] Figure 2.2 shows the MS\(^3\) spectra for the dissociation of the \([b_3-1+Li]^+\) species (m/z 220) derived from Li\(^+\) cationized AAAG (Figure 2.2a) and \(A(\beta A)AG\) (Figure 2.2b). Fragmentation pathways for CID of \([b_3-1+Li]^+\) derived from AAAG included formation of \([a_3-1+Li]^+\), m/z 192, by loss of CO and \([b_2-1+Li]^+\), m/z 149, by elimination of 71 u. Fragmentation pathways observed for CID of the \([b_3-1+Li]^+\) species derived from \(A(\beta A)AG\) included formation of \([a_3-1+Li]^+\) and the elimination of 72 u to generate a product ion at m/z 148.

\[
\begin{align*}
&\text{H}_2\text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{O} \hspace{1cm} \text{CO} \\
&\downarrow \hspace{1cm} \text{Li}^+ \\
&\text{H}_2\text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{O} \hspace{1cm} \text{CO} \\
&\downarrow \hspace{1cm} \text{CO} \\
&\text{H}_2\text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{O} \hspace{1cm} \text{CO} \\
&\downarrow \hspace{1cm} \text{Li}^+ \\
&\text{H}_2\text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{N} \hspace{1cm} \text{O} \hspace{1cm} \text{CO} \\
\end{align*}
\]

Scheme 2.1. Suggested mechanism for formation of \([a_3-1+Cat]^+\) from \([b_3-1+Cat]^+\) from Li\(^+\) cationized AAAG.
Figure 2.2. Product ion spectra from CID of [b_{3-1}+Li]^+ derived from dissociation (MS^2 stage) of (a) AAAG and (b) A(βA)AG. Asterisks mark H_2O adducts to product ions. Values in parenthesis represent neutral losses.

The 72 u eliminated from the [b_{3-1}+Cat]^+ product derived from A(βA)AG is 1 u greater than the residue mass of the alanine residue adjacent to the C-terminus of the original peptide and suggested an alternative reaction pathway for decomposition of the Li^+ cationized oxazolinone. Figure 2.3 compares the spectra generated following the CID of [b_{3-1}+Li]^+ initially generated from A(βA)AG (Figure 2.4a), A(γAbu)AG (Figure 2.4b), A(εCap)AG (Figure 2.4c), and A(4AMBz)AG (Figure 2.4d). For this series of peptides, the tendency to eliminate 72 u from the [b_{3-1}+Li]^+ species increased substantially, relative to the more conventional elimination of CO (to furnish the a-type ion), as the size of the amino acid adjacent to the N-terminus increases and was highest for the amino acid containing the aromatic amino acid.
Figure 2.3. Comparison of the product ion spectra generated from CID (MS$^3$ stage) from [b$_{31}$-1+Li]$^+$ sequence ions derived from the dissociation (MS$^2$ stage) of (a) A(βA)AG, (b) A(γAbu)AG, (c) A(εCap)AG, and (d) A(4AMBz)AG. Asterisks mark \( \text{H}_2\text{O} \) adducts to product ions. Values in parenthesis represent neutral losses.
Figure 2.4. Comparison of the product ion spectra generated from CID (MS³ stage) from \([b_3-1+Li]^+\) sequence ions derived from the dissociation (MS² stage) of (a) \(A(\varepsilon\text{Cap})VG\), (b) \(A(\varepsilon\text{Cap})GG\), (c) \(A(\varepsilon\text{Cap})(G-1-13C)G\), and (d) \(A(\varepsilon\text{Cap})(G-15N)G\). Values in parenthesis represent neutral losses.

To determine whether or not the fragmentation reaction leading to the loss of 72 u involved elimination of the amino acid residue that composed the oxazolinone ring (i.e., the residue originally adjacent to the C-terminus), the \([b_3-1+Li]^+\) species derived from Li⁺ cationized \(A(\varepsilon\text{Cap})VG\), \(A(\varepsilon\text{Cap})GG\), \(A(\varepsilon\text{Cap})(G-1-13C)G\), and \(A(\varepsilon\text{Cap})(G-15N)G\) were subjected to CID. As shown in Figure 2.4a and b, the neutral losses to form the peak at \(m/z\) 148 shifted to 100 for the peptide containing V and to 58 for the peptides containing G adjacent to the C-terminus. The elimination of 100 and 58 is 1 u greater than the residue masses of V and G, respectively, confirming the elimination of a portion of the presumed oxazolinone ring. The neutral losses observed shifted to 29 (\(^{13}\text{CO}\)), 45, and 59 when the peptide with G-1\(^{13}\text{C}\) was investigated (Figure 2.4c). For the peptide containing the G-15N (Figure 2.4d) the dominant product ion shifted by 1 u to \(m/z\) 149, indicating retention of the isotope label.
A dissociation reaction in which the net neutral loss was 1 u greater than the residue mass of the amino acid that composed the oxazolinone ring, along with the retention of the $^{15}$N label by the product ion, suggested the formation of a nitrile as depicted in Scheme 2.2a. While the composition and structure of the neutral species is not revealed by the CID experiment, it is depicted in Scheme 2.2a as an $\alpha$-lactone resulting from a direct ring-opening reaction. Formation of $\alpha$-lactones has been proposed to explain certain dissociation products for Ag$^+$-cationized phenylalanine [19, 20]. An alternative reaction pathway might involve another intramolecular nucleophilic attack upon the oxazolinone by the carbonyl group to the N-terminal side of the ring. However, we reason that such cyclization reactions might be less favored for the larger amino acids (i.e., because of kinetic and entropic factors associated with the larger cyclic intermediates, as demonstrated in our earlier study [1] such as $\gamma$Abu and $\varepsilon$Cap, and impossible for 4AMBz because of the rigid aromatic ring).

Scheme 2.2. Formation of (a) nitrile from CID of [b$_3$-1+Li]$^+$ derived from A(ɛCap)AG and (b) 2H-azirine product from CID of [b$_2$-1+Li]$^+$ derived from A(ɛCap)GG.

In the spectra shown in Figure 2.4b and c, the fragmentation pathway involving the elimination of 44 and 45 u is attributed to the loss of CO$_2$ and $^{13}$CO$_2$, respectively, from the oxazolinone ring. Here the
identity of the product ion is less clear, but may be a substituted 2H-azirine as shown in Scheme 2.2b. The elimination of 44 u from the [b₃₋₁+Cat]⁺ species was only observed for the peptides AXGG, suggesting that the relative stability of neutral α-lactones, particularly with respect to the 2H-azirine structure, may play a role in the tendency to produce the nitrile product. 2H-azirines are more stable than the anti-aromatic 1H-azirines, and even the unsubstituted version of the former has been isolated at low temperatures [21], but not the unsubstituted α-lactone.

Based on the observations described above, it appears that the [b₃₋₁+Li]⁺ ion from the AXAG series is capable of fragmenting by several competing pathways: (1) loss of the residue mass of α-alanine to form the [b₂₋₁+Cat]⁺ species, (2) elimination of CO to produce [a₂₋₁+Li]⁺, (3) ejection of an α-lactone to generate a lithiated nitrile, and (4) elimination of CO₂ to leave a lithium cationized 2H-azirine. Formation of the [b₂₋₁+Li]⁺ and [a₂₋₁+Li]⁺ correspond to well-documented reaction pathways as recorded here for Li⁺ cationized AAAG. The absence of the nitrile and azirine products in the CID spectrum of [b₃₋₁+Li]⁺ derived from AAAG suggests a special stabilization of the [a₃₋₁+Li]⁺ product; stabilization that may be lacking in the same product derived from the peptides with the amino acids such as βΑ, γAbu, and εCap at position X. To account for the differences in the competition between imine-type, [aₙ₋₁+Cat]⁺, and nitrile or azirine products, we suggest that the [a₃₋₁+Li]⁺ species derived from AAAG may be stabilized by the pseudo 5-membered hydrogen-bonded structure, a, shown in Figure 2.5. Moreover, the traditional imine-like structure may be in equilibrium with the entropically favored 5-membered ring structure, a', resulting from a Michael-type addition. In structure a', both nitrogen atoms of the oxazolidinone ring can participate in amide-type resonance, a feature lacking in structure a because the lone pair of the imine nitrogen would occupy an sp² orbital to accommodate conjugation with the carbonyl group. Even this conjugation in a of the imine-carbonyl type is less resonance stabilized than the traditional olefin-carbonyl type because of the electronegativity of the nitrogen atom, thus promoting formation of a'. The presence of the imine nitrogen, on the other hand, would facilitate the initial Michael addition (species b in Figure 2.5). In the case of structure a, potentially formed from AXAG (with X representing amino acids other than α-alanine), not only would intra-molecular hydrogen bonding become progressively more difficult as the pseudo-ring enlarges from 5 to 6, 7 or 9 members (as for X = βΑ, γAbu, or εCap) or becomes impossible with 4AMBz, but formation of structure a' would be kinetically less favorable. Reduced
probability for formation of structure \textbf{a}' might then allow pathways leading to the nitrile or azirine to become more competitive. Another factor may be progressively increased stability of the nitrile product in proceeding from \( A \) to \( \beta A, \ \gamma \text{Abu}, \) and to \( \varepsilon \text{Cap} \). This is reminiscent of the increase in proton affinity with chain length of a homologous series of nitriles reported by Cao and Holmes [22], and the trends in gas-phase acidity of alcohols first reported by Brauman and Blair [23]. In part the influence of an alkyl group on the acidity of gas-phase alcohols has been attributed to increasing polarizability with chain length and partial delocalization of charge. In addition to the inability of 4AMBz to compete with formation of the oxazolidinone, \( \textbf{a}' \), in 2.5 on account of its rigid structure, conjugation of the nitrile with the aromatic ring would certainly favor its formation in comparison with the other aliphatic nitriles.

![Figure 2.5. Potential structures for [\textbf{a}_3-1+\text{Cat}]^+ species derived from Li$^+$ cationized AAAG.](image)

We also examined the influence of the cation on the tendency to generate the putative nitrile product. Figure 2.6 shows the spectra generated from the CID of \( \text{A}(\varepsilon \text{Cap})\text{AG} \) when cationized with proton (Figure 2.6a), Li$^+$ (Figure 2.6b), Na$^+$ (Figure 2.6c), and Ag$^+$ (Figure 2.6d). CID of the \( \text{(b}_3\text{)}^+ \) ion, derived from the protonated version of the peptide, caused the elimination of 89 u but not the loss of 28 u to form \( \text{(a}_3\text{)}^+ \) or 72 u to generate the nitrile product. The mechanism behind the elimination of 89 u from the protonated peptide is yet to be resolved. The loss of 72 u to make the nitrile product was the dominant pathway for the CID of both the Li$^+$ and Na$^+$ cationized versions of the species, but only minor for the CID of the Ag$^+$ cationized analog.
Figure 2.6. Comparison of the product ion spectra generated from CID (MS$^3$ stage) from [b$_3$-1+Li]$^+$ sequence ions derived from protonated and metal-cationized A(ε CAP)AG: (a) protonated, (b) Li$^+$ cationized, (c) Na$^+$ cationized, and (d) Ag$^+$ cationized. Values in parenthesis represent neutral losses.

2.4 Conclusions

In summary, we have identified a novel reaction pathway for the CID of [b$_3$-1+Cat]$^+$ product ions. The pathway is not observed when the amino acid adjacent to the ring structure of the oxazolinone is an α-amino acid, but increases in prominence when β- or γ-amino acids are substituted in the same position. For tetrapeptides in which the “alternative” amino acids are incorporated, this reaction pathway is not observed when the residues are positioned either at the N-terminus or adjacent to the C-terminus (spectra not shown). The dissociation pathway is also observed for the CID of (b$_4$-1+Cat)$^+$ derived from model pentapeptides with sequence AAXAG (with X=βA and ε Cap, spectra not shown), suggesting that the reaction is not unique to the fragmentation of [b$_3$-1+Cat]$^+$. By examining the respective neutral losses from a series of peptides, and the retention of a specific $^{15}$N label by the product ion, we conclude that the
reaction most likely leads to the formation of a metal-cationized nitrile. The reaction pathway is most prominent for CID of the Li⁺ and Na⁺ cationized versions of [b3-1+Cat]⁺ species. The reason for the pronounced “metal effect” is not clear and requires a significant computational investigation. Preliminary ab initio modeling of likely precursor and transition state structures, including possible important hydrogen-bonded conformations, is currently underway.
LIST OF REFERENCES


3.1 Introduction

In a previous study of the dissociation patterns of metal-cationized tetrapeptides with the sequence Acetyl-FGGX-OH, the C-terminal sequence position X contained an $\alpha$, $\beta$ or $\gamma$ amino acid, or an aminobenzoic acid, so that we could test the importance of a putative five-membered ring intermediate in the production of the $(b_3+17+\text{Cat})^+$ sequence ion.[1] The intermediate is proposed to arise through an intramolecular nucleophilic attack by the carbonyl oxygen atom of the C-terminal carboxyl group upon the carbonyl group of the adjacent peptide residue.[2-6] Collapse of the cyclic intermediate would result in a net transfer of an oxygen and hydrogen atom to the amino acid adjacent to the C-terminus, concurrent with loss of the residue mass of the C-terminal amino acid. When this pathway was rendered difficult (by demanding a larger cyclic intermediate such as six-, seven- or nine-membered) or impossible (by prohibiting cyclization altogether through incorporation of a rigid aromatic ring) by placing the 'alternative' amino acids at position X, the dominant pathway observed for the Ag$^+$-cationized version of the peptide was production of $[b_3-1+\text{Ag}]^+$, which is thought to be generated by nucleophilic attack on the same carbonyl group but from the opposite direction (attack by the carbonyl group of an adjacent amide on the N-terminus side).[7-14] The dominant product for the collision-induced dissociation (CID) of the Li$^+$- and Na$^+$-cationized versions, however, was instead $[\text{M-H}_2\text{O}+\text{Cat}]^+$. The Ag$^+$-cationized peptides also produced the corresponding $[\text{M-H}_2\text{O}]^+$ ion, but at lower relative abundance.

Elimination of H$_2$O from the carboxyl group of protonated glycine-glycine was investigated by Lifshitz and coworkers, who found the formation of a protonated oxazolinone structure to be kinetically favored over an alternative diketopiperazine conformation.[15] Loss of H$_2$O from the protonated dipeptide through a process involving the C-terminal acid -OH group would lead to the $b_2$ ion. Multiple-stage dissociation experiments showed that the $[\text{M-H}_2\text{O}+\text{Cat}]^+$ ions derived from AcFGGX-OH, whether the cation was Li$^+$, Na$^+$ or Ag$^+$, did not eliminate CO (the main fragmentation pathway for conventional, oxazolinone-type $b_n$-type ions[8, 11]), and thus the ion cannot be considered the $[b_n-1+\text{Cat}]^+$ species generated via elimination of H$_2$O from the C-terminus of the peptide. The $[\text{M-H}_2\text{O}+\text{Cat}]^+$ species is also
the dominant product generated from CID of Li⁺- and Na⁺-cationized methyl esters of the peptides. Because the [M-H₂O+Cat]⁺ species are apparently not oxazolinone-type sequence ions, we were obliged to turn our attention away from any role of the C-terminal -OH group in the loss of water, and focus on alternative pathways.

The loss of water from [M+H]⁺ ions derived from glycine-containing peptides such as GG or AcG was studied by Reid et al.[16] who proposed retro-Koch or retro-Ritter type reactions requiring the elimination of the oxygen atom from the C-terminal -OH group or the amide group, respectively. Both reactions involve addition of a proton to the peptide. A study of metal-cationized esters of the type Ac-(G)ₙ−OR, where R=Me, Et, Pr, Bu or t-Bu and n=2-4, in our laboratory[17] also revealed a prominent loss of H₂O, and led us to propose a mechanism (Scheme 3.1) involving cyclization from the N-terminal side of the site of attack to form a 5-hydroxyoxazolinone intermediate, followed by a 1,2-elimination of water. In this mechanism, the oxygen atom of the amide bond of the N-terminal amino acid is eliminated, while those from the carbonyl or ester groups of the C-terminus are retained. In this respect, the mechanism for loss of H₂O involving amide-position oxygen atoms is reminiscent of the one proposed by Reid and coworkers for protonated peptides.[16]

![Scheme 3.1. Mechanism for loss of H₂O (MS² stage) from the ethyl ester of acetylated triglycine, [AcGGGOEt+Li]⁺.](image)

In preliminary studies conducted prior to the experiments reported here, the CID of [AcGGGOMe+Cat]⁺, where Cat⁺ was Li⁺ or Na⁺, was compared with that of an analogous peptide in which all amide hydrogen (H) atoms were exchanged with deuterium (D) by solution-phase H/D exchange. The CID spectrum for the peptide with all exchangeable sites occupied by D showed losses of both D₂O (100% RI) and HOD (99%). Gas-phase H/D exchange with H₂O present in the ion trap mass
spectrometer was ruled out and the loss of HOD clearly suggested the involvement of H atoms at the α-carbon positions in the dissociation reaction.

Following the preliminary experiments, three questions remained to be answered: (1) Which α-carbon positions(s) is/are involved in the loss of HOD? (2) Is the N-terminal acetyl group, used to facilitate the solution-phase synthesis of our model peptides of the type AcFGGXOR (R=H or Me), the primary cause of the pronounced tendency to eliminate water from the alkali-metal-cationized peptides? This particular question was partially answered by the previous investigation [1] of GGAGOMe and GGVGOMe. CID of these esters cationized by Li⁺ or Na⁺ generated [M-H₂O+Cat]⁺ as the dominant peak. However, a second less prominent peak corresponded to [M-35+Cat]⁺. Multiple-stage tandem mass spectrometry experiments suggested that there is a loss of H₂O and NH₃, with the loss of H₂O preceding that of NH₃. (3) What is the mechanism behind the sequential loss of H₂O and NH₃ from peptides lacking the N-terminal acetyl group? To answer these questions, we embarked on the present study involving site-specific isotope (²H, ¹³C and ¹⁵N) labeled model tripeptides and their characterization by multiple-stage tandem ion trap mass spectrometry. A series of peptides of the type X-GGG-Y, where X=Ac, Bz, or H, and Y=OMe, was prepared in which each glycine residue in turn bore either ²H at α-carbon or ¹⁵N or ¹³C at amide positions. The peptides, in Li-cationized form, were subjected to multiple CID stages in a quadrupole ion trap mass spectrometer to elucidate the dissociation pathways. Using the isotope-labeling results, mechanisms to account for the product ions generated are proposed.

3.2 Experimental

All peptides used in this study were generated by conventional solid-phase synthesis methods[18] using 9-fluorenylmethoxycarbonyl (FMOC)-glycine loaded Wang resin (Sigma Chemical Co., St. Louis, MO, USA) and a custom-built, multiple-reaction vessel peptide synthesis apparatus. Glycine-¹⁵N (H₂¹⁵NCH₂COOH, G-¹⁵N), glycine-¹³C (H₂NCH₂¹³COOH, G-¹³C), α-d₂-glycine (H₂NCD₂-COOH, α-d₂-G) and FMOC-chloride were purchased from Sigma Chemicals and used to generate N-terminus FMOC amino acids for incorporation into the model peptides. FMOC-protected glycine (G) and alanine (A) were purchased from Sigma and used as received. Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies. N-terminal acetylation or benzylation was carried out by incubating peptides in acetic or benzoic anhydride (both compounds purchased from Sigma-Aldrich.
and used as received). Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (v/v) mixture of HPLC-grade methanol (MeOH) (Aldrich Chemicals, St. Louis MO, USA) and deionized H₂O to produce final concentrations of 10⁻⁴ to 10⁻⁵ M. The sequence of each peptide, and in particular the position of isotope labels, was confirmed using multiple-stage CID of Li⁺- and Ag⁺-cationized versions. For experiments in which the CID of amino, amide and acid deuterium-labeled peptides was examined, the relevant peptides were incubated in a 50:50 mixture of D₂O and CH₃OD (Aldrich Chemicals) for 2 hours. The peptide solutions in deuterated solvent were then injected into the electrospray ionization (ESI) source without further modification.

ESI mass spectra were collected using a Finnigan LCQ-Deca ion trap mass spectrometer. Peptide solutions were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 5 mL/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octupole voltage offsets, etc.) were optimized for maximum [M+Li]⁺ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N₂ sheath gas flow at 25 units (arbitrary for the Finnigan systems, corresponding to approximately 0.375 L/min), and the capillary (desolvation) temperature at 200°C. Helium was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The CID studies (MS/MS and MSⁿ) were performed as follows. The [M+Li]⁺ ions were isolated for the initial CID stage (MS/MS) using an isolation width of 0.9 to 1.2 mass-to-charge (m/z) units. Product ions selected for subsequent CID (MSⁿ experiments) were isolated using widths of 1.0–1.3 m/z units. The exact width was chosen empirically and reflected the best compromise between high [M+Li]⁺ abundance and the isolation of a single isotopic peak. The (mass) normalized collision energy, which defines the amplitude of the radio-frequency (RF) energy applied to the end-cap electrodes in the CID experiment, was set between 20% and 25%, which corresponds roughly to 0.55–0.68 V with the instrument calibration used in this study. The activation Q (as labeled by ThermoFinnigan, used to adjust the qz value for the precursor ion) was set at 0.30. Subsequent CID stages were performed using similar activation parameter settings. The activation time employed at each CID stage was 30 ms.
3.3 Results and Discussion

CID was investigated using Li⁺-, Na⁺- and Ag⁺-cationized versions of the isotope-labeled peptides. For each cation, similar results were obtained with respect to the types of product ions observed and the retention or elimination of the isotope labels. For the sake of brevity, the results obtained using the Li⁺-cationized versions of the peptides are discussed below as they are representative of the entire group of metal-cationized peptides. Elimination of H₂O from metal-cationized peptides followed by further fragmentation was studied for two series of compounds: AcGGGOMe and GGGOMe. In each case, extensive isotope labeling formed the basis of the mechanisms that are being proposed. In the following discussion, the labeled residue is indicated, with the specific isotope, in parentheses.

3.3.1 Initial loss of H₂O from [AcGGGOMe+Li]⁺

The fragmentation pathways observed for MSⁿ CID study of [AcGGGOMe+Li]⁺ are provided in Figure 3.1, while the actual CID spectra are shown in Figure 3.2. In the CID spectra, H₂O adducts to product ions, formed by gas-phase reactions with adventitious H₂O in the ion trap, are indicated using an asterisk. The adducts were identified as such based on their appearance when individual product ions (from CID) were isolated and stored in the ion trap, without imposed collisional activation, for 30 ms. During the isolation step, all ions except the one chosen for storage in the ion trap are resonantly ejected. The appearance of ions 18 mass units higher than the ion isolated marks the formation of H₂O adducts.

![Figure 3.1. Major product ions generated from CID (MS^2 stage) of Li^+ -cationized AcGGGOMe and (MS^3 stage) AcGGGOMe-H_2O.](image-url)
Figure 3.2. CID spectra generated from Li⁺-cationized AcGGGOMe: (a) CID of [AcGGGOMe+Li]⁺ (MS/MS stage) and (b) CID of [AcGGGOMe-H₂O+Li]⁺ (MS² stage).

In the CID spectrum generated from [AcGGGOMe+Li]⁺ (Figure 3.2(a)), the loss of H₂O was the dominant fragmentation reaction, and subsequent CID of [M-H₂O+Li]⁺ (Figure 3.2(b)) led to further fragmentation via the multiple pathways depicted above. Described below is each individual fragmentation in terms of key observations and the relevant mechanisms. To elucidate which hydrogen and oxygen atoms are eliminated as H₂O in the dissociation reaction, we performed two sets of experiments: (1) When the peptide ions were generated by ESI from solutions in D₂O/MeOD and the presence of Li⁺ the loss of both D₂O and HOD was observed in roughly equal amounts. Loss of HOD indicates that hydrogen atoms from α-carbon positions are involved. (2) To pinpoint the specific α-carbon from which the transfer of H occurs, we examined the CID of peptides bearing deuterium atoms at the α-carbon position of individual G residues. Loss of both H₂O and HOD (100% and 40% relative intensities, respectively) was observed only when α-d₂-G was incorporated into the peptide ester at the N-terminal position within the sequence. Peptide esters for which the α-d₂-G residue was incorporated at, or next to, the C-terminus exhibited only the elimination of H₂O. Therefore, we propose (Scheme 3.2) that the dissociation reaction to eliminate H₂O from Li⁺ cationized Ac(d₂G)GGOMe commences with a
cyclization step that involves nucleophilic attack by the oxygen atom of the N-terminal acetyl group on the carbonyl carbon atom of the adjacent amide group to the C-terminal side, with proton transfer, to produce a five-membered 5-hydroxyoxazoline ring. The scheme shows the hydroxyl group of this heterocyclic ring combining with an exocyclic NH proton of the middle G residue (loss of H,O) or an endocyclic deuterium atom of the α-carbon of the N-terminal G residue (loss of H,D). The greater acidity of the NH proton is the likely reason for the greater intensity of the peak resulting from loss of H,O. The fragment ion is depicted as a lithiated aromatic oxazole.

Scheme 3.2. Proposed pathway depicting the elimination of H,O versus H,D during CID (MS² stage) of [Ac(d₂-G)GGOMe+Li]⁺.

3.3.2 CID (MS³ stage) of [M-H₂O+Li]⁺ from AcGGGOMe

A second loss of water from the lithiated peptide constitutes a prominent pathway (100%) at the MS³ stage. Incorporation of α-d₂-G into the peptide sequence led to the observation that this further loss
of water occurs in the form of both H$_2$O and HOD when the D-labeled residue was positioned at, or adjacent to, the N-terminus of the peptide. The loss of only H$_2$O was observed for the peptide with α-d$_2$-G at the C-terminus. We propose (Scheme 3.3) that for the [M-H$_2$O+Li]$^+$ ion from Ac(d$_2$G)GGOMe, a tautomeric change in the C-terminal amide bond may lead to an enolic hydroxyl group which can undergo competitive 1,2- and 1,3-elimination to afford a ketenimine and aziridine, respectively [loss of HOD (100%) or H$_2$O (30%)]. In both cases, the resulting ion would be the aromatic oxazole of Scheme 3.2, further stabilized by conjugation with a three-membered ring or double bond of the ketenimine. It is well known that the ring bonds of three-membered rings have considerable p-character (so-called sp$^5$ hybridization[19]) and participate in bathochromatic shifts in UV/visible spectra on account of conjugation,[20] or in stabilization of carbocations.[21]

Scheme 3.3. Route showing further elimination of H$_2$O versus HOD resulting from CID (MS$^3$ stage) of the product ions generated at the MS$^2$ stage: (i) [Ac(d$_2$-G)GGOMe-H$_2$O+Li]$^+$ and (ii) [AcG(d$_2$-G)GOMe-H$_2$O+Li]$^+$. 

![Scheme 3.3](image-url)
For CID of AcG(d2G)GOMe, a similar loss of H2O would lead to the aziridine (100%) and a loss of HOD to the ketenimine (35%). Apparently, for both Ac(d2G)GGOMe and AcG(d2G)GOMe, the ion with the greater intensity involves loss of a proton attached to nitrogen compared to a reaction that involves transfer of a proton attached to carbon.

The loss of 28 u after the initial loss of water from [AcGGGOMe+Li]+ constitutes another pathway observed at the MS3 stage. Loss of 28 u can be the result of elimination of CO, N2 or C2H4. In order to ascertain the exact nature of this process, we studied decomposition of [AcGGGOMe+Li]+ for which individual G residues were labeled with 13C at the carbonyl positions. A neutral loss of 29 u was observed only when the middle G residue was labeled with carbonyl 13C. Moreover, when the middle G residue was replaced with A, CID of [M-H2O+Li]+ generated the elimination of 28 u rather than 42 u (propylene).

We propose (Scheme 3.4) that the source of the loss of 28 u is the carbonyl group of the middle G residue to afford a structure akin to a nitrogen ylide, which is converted into a lithiated imine by proton transfer.

![Scheme 3.4. Pathway depicting the loss of CO during CID (MS3 stage) of [Ac(d2-G)GGOMe-H2O+Li]+ generated at the MS2 stage.](image)

The loss of 59 u was the next most prominent mode of fragmentation at the MS3 stage for CID of [M-H2O+Li]+ derived from AcGGGOMe. One logical combination of neutral species that would have a combined mass of 59 u is [H2O+CH3CN]. If the source of the methyl group of acetonitrile is the N-acetyl group, then replacement of the acetyl group by a benzoyl (Bz) group should lead to a corresponding loss
of $[\text{H}_2\text{O}+\text{C}_6\text{H}_5\text{CN}]$ for a combined 121 u. When $[\text{BzGGGOMe}+\text{Li}]^+$ was subjected to CID, loss of 59 u was absent and was replaced by loss of 121 u. Therefore, we suggest that elimination of two successive molecules of water from $[\text{AcGGGOMe}+\text{Li}]^+$ is followed by loss of acetonitrile (Scheme 3.5) to furnish an epoxypyrrolinimine. Rearrangement of the epoxide functional group to a carbonyl group in the presence of a Lewis acid is well known.[22] Such a rearrangement is envisaged to afford a highly conjugated system as a lithium adduct.

In the CID of both $[\text{AcGGGOMe}+\text{H}_2\text{O}+\text{Li}]^+$ and $[\text{BzGGGOMe}+\text{H}_2\text{O}+\text{Li}]^+$, the loss of 60 u was observed. The loss of 60 u from either species suggests that the N-terminal acyl group is not involved in the reaction, and that loss of 59 u and loss of 60 u are not related processes. One possible neutral species possessing a mass of 60 u is methyl formate, with the methyl group likely being part of the C-terminal methyl ester. Hence, when we examined the fragmentation of $[\text{AcGGGOC}\text{D}_3+\text{H}_2\text{O}+\text{Li}]^+$, the neutral loss of 60 u ($\text{HCO}_2\text{CH}_3$) was shifted to a loss of 63 u ($\text{HCO}_2\text{CD}_3$). Also, when CID of the peptides containing G-1-$^{13}\text{C}$ was examined, the loss of 60 u shifted to 61 u for $\text{AcGG}(^{13}\text{CG})\text{OMe}$ whereas the other versions of the $^{13}\text{C}$-labeled peptide esters exhibited exclusive loss of 60 u.
Therefore, we propose (Scheme 3.6) that the reaction pathway to produce the net loss of 60 u involves a 1,2-elimination of hydrogen and the methyloxy carbonyl group from the C-terminal position to furnish a lithiated and highly conjugated imine.

![Scheme 3.6. Pathway depicting the elimination of methyl formate (MS³ stage) from [Ac(d₂-G)GGOMe-H₂O+Li]+.](image)

The loss of 89 u during CID of [M·H₂O+Li]+ likely involves the elimination of glycine methyl ester from the C-terminal end of the peptide ester. In fact, the neutral loss shifted from 89 to 92 u for CID of [AcGGGOCd₂-H₂O+Li]+. Also, CID of [AcGG(d₂-G)OMe-H₂O+Li]+ resulted in a loss of 91 u, whereas the other analogues incorporating α-d₂-G showed only the loss of 89 u. Therefore, it is proposed (Scheme 3.7) that the reaction to produce the loss of 89 u commences with an intramolecular attack by the nitrogen atom (of the middle G residue of the precursor peptide) on its own carbonyl group to yield a hydroxyaziridine ring, which collapses by loss of glycine methyl ester to furnish a lithiated oxazole joined to an aziridinone. The vinyl aziridinone moiety can relieve its strain by ring expansion to a five-membered ring, akin to the well-known vinylcyclopropane to cyclopentene rearrangement,[23] ultimately leading to a lithiated fused pyrrolo[3,2-d]oxazole system.
Scheme 3.7. Fragmentation (MS$^3$ stage) of [AcGGGOMe-H$_2$O+Li]$^+$ by loss of glycine methyl ester.

Based on three key observations, we suggest that the fragmentation pathway leading to the loss of 99 u involves the elimination of isocyanoacetic ester: (1) CID of [AcGGGOCd$_3$-H$_2$O+Li]$^+$ results in a loss of 102 u instead of 99 u, which indicates involvement of the C-terminal methyl ester. (2) When we examined the peptide bearing deuterium atoms on the $\alpha$-carbon atoms, only the AcGG(d$_2$G)OMe exhibited a loss of 101 u, whereas the other deuterium-labeled analogues exhibited neutral losses of only 99 u. (3) Fragmentation of a [M-H$_2$O+Li]$^+$ ion derived from a peptide in which the middle glycine residue was replaced by alanine, i.e. AcGAGOMe-H$_2$O+Li, also resulted in loss of 99 u. Therefore, we suggest (Scheme 3.8) that an initial tautomerization of the C-terminal amide bond is followed by a rearrangement of a carbocation and subsequent loss of the methyl isocyanoacetate, resulting in the formation of a formamidooxazoline coordinated to Li$^+$. CID of the $m/z$ 135 product generated by loss of 99 u from [M-H$_2$O+Li]$^+$ produced a species at $m/z$ 106 exclusively by loss of 29 u. This loss involves elimination of an imine (Scheme 3.9) and is presumed to form an epoxide. This epoxide undergoes a well-known rearrangement of an epoxide to a carbonyl compound,[22] leading to the lithiated N-formylacrylamide ($m/z$ 106).
Scheme 3.8. Pathway showing the loss of methyl isocyanoacetate from $[\text{AcGGGOMe-H}_2\text{O+Li}]^\pm$.

Scheme 3.9. Loss of imine during fragmentation (MS$^4$ stage) of the ion resulting from elimination of methyl isocyanate shown in Scheme 7.

The elimination of 129 u was a minor but persistent pathway in the CID of $[\text{AcGGGOMe-H}_2\text{O+Li}]^\pm$, leading to a lithiated ion at $m/z$ 105. Pertinent observations that provide a clue to the underlying mechanism are: (1) the $m/z$ value of this product ion shifted to 106 for CID of $\text{Ac}^{(13}\text{C-G})\text{GGOMe}$; (2) the $m/z$ value shifted to 107 for CID of $\text{Ac}(d_2\text{G})\text{GGOMe}$; and (3) the $m/z$ of the product ion is also 105 for CID of $[\text{AcGAGOMe-H}_2\text{O+Li}]^\pm$. Incorporation of deuterium in the $\alpha$-position of the glycine residues either at, or adjacent to, the C-terminus affords only the ion of $m/z$ 105.
In other words, the isotope labels on the N-terminal G residue are retained in this process, and suggests that the rest of the peptide is almost completely eliminated in the form of a conjugated imine (Scheme 3.10) to furnish a lithiated oxazole.

Scheme 3.10. Pathway depicting elimination of 129 u (methyl ester of glyoxaloglycine) from [AcGGGOMe-H2O+Li]+.

3.3.3 Initial loss of H2O from [GGGOMe+Li]+

The fragmentation pathways observed for MS^n CID study of [GGGOMe+Li]^+ are provided in Figure 3.3, while the actual CID spectra are shown in Figure 3.4. In the CID spectra, H2O adducts to product ions, formed by gas-phase reactions with adventitious H2O in the ion trap, are indicated using an asterisk. As for the acetyl analogue, a prominent loss of H2O from [M+Li]^+ was observed for GGGOMe. However, as discussed below, the subsequent fragmentation pattern (MS^3) was very different from the acetylated version discussed above.

Figure 3.3. Major product ions generated from CID (MS^2 stage) of Li^+-cationized GGGOMe and (MS^3 stage) GGGOMe-H2O.
In the [AcGGGOMe+Li]⁺ experiments, our proposal was that the initial loss of water is triggered by the oxygen atom of the acetyl group acting as a nucleophile and forming a five-membered ring as an intermediate. In the present case, because an N-terminal acyl group is absent, the nucleophilic atom that leads to a five-membered ring has to be either the nitrogen atom of the free amino group or an oxygen atom of a peptide bond. In order to resolve this question, we examined GGGOMe peptides for which specific G residues bore deuterium atoms in the α-carbon position. It was observed that loss of both H₂O and HOD occurred as parts of the chief fragmentation pathway only when the N-terminal G residue contained the deuterium label (-H₂O, 100%; -HOD, 16%). This dual loss of water was much more selective for the peptide with deuterium-labeled G in the middle sequence position (-H₂O, 100%; -HOD, 6%), whereas the peptide with the deuterium-labeled G residue at the C-terminus underwent exclusive loss of H₂O. Moreover, when [GGGOMe+Li]⁺ with all amino and amide positions labeled with D (by solution-phase H/D exchange) was subjected to CID, the elimination of both H₂O and HOD was observed. These observations indicate involvement of an α-CH₂ group. Schemes 3.11(a) and 3.11(b) depict a mechanism for the initial loss of water from [GGGOMe+Li]⁺ that commences with the oxygen atom of the N-terminal peptide bond acting as a nucleophile. The subsequent transformations are similar.
to those shown in Scheme 3.1 for the acetylated peptide.

Scheme 3.11(a) depicts the loss of H$_2$O from [(d$_2$G)GGOMe+Li]$^+$, whereas Scheme 3.11(b) shows the corresponding loss of HOD. Obviously, before HOD can be lost, an exchange of hydrogen isotopes at the α-position of the N-terminal G residue must occur. Apparently, the presence of the electron-attracting -NH$_3$ facilitates transfer of the adjacent deuterium atom. Scheme 3.11(c) shows how both H$_2$O and HOD can also be eliminated from [G(d$_2$G)GOMe+Li]$^+$, but to different extents.

There is a greater loss of H$_2$O than of HOD, presumably because the former involves elimination of an exocyclic N-H proton whereas the latter requires removal of an endocyclic C-H proton. The greater acidity of NH is probably one reason for this selectivity. In any case, in all of the Schemes 3.11(a)–11(c), the ion resulting from a loss of water is a stable aromatic oxazole coordinated to Li$^+$.

Scheme 3.11. (a) Pathway showing loss of H$_2$O during CID of [G(d$_2$-G)GOMe+Li]$^+$. (b) Pathway for loss of HOD during CID of [G(d$_2$-G)GOMe+Li]$^+$. (c) Mechanism depicting the competitive losses of H$_2$O versus HOD during CID of [G(d$_2$-G)GOMe+Li]$^+$. 

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3.3.4 CID (MS$^3$ stage) of [M-H$_2$O+Li]$^+$ from GGGOMe

Subsequent CID of [GGGOMe-H$_2$O+Li]$^+$ caused the elimination of 17 u. The neutral species lost is very likely NH$_3$, a process not observed at all for the acetylated versions of the peptide esters. Hence, the free terminal amino group likely plays a role in the fragmentation reaction. In order to probe this point, we examined six tripeptide esters: in one set, each G residue in turn was labeled with $^{15}$N and in the other set each G residue in turn was replaced with $\alpha$-d$_2$-G. For the peptide that contained the $^{15}$N label at the amino terminus (the amino group labeled with $^{15}$N), the loss of 17 shifted to 18 u. The elimination of D atoms from the $\alpha$-carbon positions was observed from the peptides in which either the C-terminal or middle G residues contained the D isotope labels, but not from the peptide in which the N-terminal G residue was labeled. Moreover, when all exchangeable hydrogen atoms of GGGOMe were replaced with deuterium by solution-phase H/D exchange, initial loss of HOD was followed by loss of 18, 19 and 20 u (Figure 3.5).

![Diagram](image)

**Figure 3.5.** Major product ions generated from CID (MS$^2$ stage) of Li$^+$-cationized d$_4$GGGOMe.

Loss of NH$_3$ containing just the N-terminal nitrogen atom but varying amounts of hydrogen isotopes from $\alpha$-carbon positions can be explained if one assumes that extensive scrambling of protons and deuterons at middle and C-terminal G positions precedes the elimination of ammonia, very likely facilitated by the basicity of the free amino group. In Scheme 3.12(a), loss of ND$_2$H and ND$_3$ is depicted as proceeding through a six-centered chair-like transition state leading to structures B and C, respectively. The tautomer of both product ions is a highly conjugated oxazole. The preference for elimination of ND$_2$H (~71%) versus ND$_3$ (~12%) may reflect a kinetic isotope effect. No scrambling of protons is needed to afford B and C. However, loss of NH$_2$D requires extensive scrambling of hydrogen
labels between the middle and C-terminal G residues (Scheme 3.12(b)) prior to loss of ammonia. This is no doubt facilitated by the presence of the basic free amino group and acidity of the α-hydrogen atoms in the five-membered ring (a system akin to β-diketones). Apparently, a requirement of scrambling before elimination of ammonia poses a larger energy barrier than direct elimination of ammonia and could explain the loss of NH$_2^D$ to the extent of only 14%.

Scheme 3.12. (a) Pathway showing the loss of either ND$_2^D$ or ND$_3^D$ from [d$_4$GGGOMe-HOD+Li]$^+$. (b) Mechanism for loss of NH$_2^D$ from same precursor ion shown in (a).

The loss of 29 u was the dominant pathway observed for CID of [GGGOMe-H$_2$O+Li]$^+$ (MS$^3$ stage), and very likely involves the loss of CH$_2$-NH as the neutral fragment. Incorporation of the $^{15}$N label in different positions showed that the nitrogen atom lost arises only from the N-terminal amino group. In addition, the presence of deuterium labels on α-carbon atoms indicated that both α-CH$_2$ hydrogen atoms are lost from the N-terminal glycine residue, whereas either of the other two α-carbon positions lose only one hydrogen atom. Also, CID of GGGOMe in which all exchangeable hydrogens had been replaced by D by solution-phase H/D exchange produced an initial loss of HOD, followed by elimination of 29 or 30 u in the ratio of 5 to 22, respectively. Scheme 3.13 depicts the course of these competing eliminations,
starting from \([d_4\text{GGGOMe-HOD+Li}]^+\), the peptide with D atoms at the amino and amide N positions because of solution-phase H/D exchange. The loss of 30 u is depicted as a non-concerted retro-cycloaddition in which the terminal nitrogen atom and both \(\alpha\text{-CH}_2\) hydrogen atoms are eliminated from the N-terminal glycine residue. The less abundant ion derived from loss of 29 u is proposed to involve elimination of HN-CH\(_2\) and again requires prior scrambling of hydrogen isotopes. As shown in Scheme 3.12(b), the terminal -ND\(_2\) group can convert into -NHD, with the origin of the hydrogen atom being the \(\alpha\)-carbon of the middle or C-terminal G residue. In both cases, the resulting ion represents a substituted aromatic oxazole coordinated to Li\(^+\). For CID of \([\text{GGGOMe-H}_2\text{O+Li}]^+\), the observed elimination of 32 u almost certainly involves the loss of methanol. The -OCH\(_3\) portion is likely derived from the C-terminal ester group, but the source of the hydrogen remains to be ascertained. When the \(\alpha\text{-d}_2\text{-G residue}\) occupied the middle or C-terminal sequence positions in GGGOMe, the initial loss of 18 u was followed by loss of 32 u as the major pathway. Smaller amounts of loss of 33 u (10-20% relative abundance) were also observed. However, when the \(\alpha\text{-d}_2\text{-G residue} \) was located on the N-terminal sequence position, initial loss of 18 u was followed solely by loss of 32 u. In \(d_4\text{-GGGOMe (all exchangeable hydrogen atoms replaced by deuterium through solution-phase H/D exchange)}\), an initial loss of D\(_2\)O or HOD was followed by loss of both 32 and 33u. In particular, loss of HOD was followed by loss of CH\(_3\)OH (100%) and loss of CH\(_3\)OD (70%), as indicated by experiments where the \([\text{GGGOMe-H}_2\text{O+Li}]^+\) and \([\text{GGGOMe-HDO+Li}]^+\) species were independently isolated and subjected to CID. The inference is that scrambling of hydrogen isotopes occurs and both 1,2-and 1,3-eliminations are involved (Scheme 3.14). The 1,2-elimination would result in the production of an amino ketene, whereas the 1,3-elimination would afford initially an aziridinone which can rearrange to a doubly aromatic pyrrolo[3,2-\(d\)]oxazole (akin to the vinylcyclopropane to cyclopentene rearrangement[23]). The loss of 32 u as the major pathway from both G(d\(_2\text{-G})\text{GOME}\) and GG(d\(_2\text{-G})\text{OMe}\) requires rapid scrambling of labels and/or preference for the 1,2-elimination versus 1,3-elimination. The same remarks apply to the case of \([d_4\text{-GGGOMe-HOD}]\) (all exchangeable protons replaced by deuterium).
Scheme 3.13. Mechanism depicting the loss of HOD followed by loss of mono- or undeuterated imine during CID of \([d_4GGGOMe+Li]^+\).

Scheme 3.14. Mechanism showing competitive loss of CH$_3$OH versus CH$_3$OD from the precursor ion \([G(d_2-G)GOMe-H_2O+Li]^+\).

The loss of 99 u was also observed for CID of \([GGGOMe-H_2O+Li]^+\). We propose that this fragmentation pathway resembles the one suggested for CID of \([AcGGGOMe-H_2O+Li]^+\), where the neutral fragment lost was depicted to be isocyanoacetic ester. In the present case, loss of 99 u would involve the nitrogen atom only from the C-terminal G residue ($^{15}$N lost only from this position), hydrogen atoms from $\alpha$-carbon positions within the C-terminal G residue (if CD$_2$ is present in this position, mostly one D is eliminated), and the -CH$_3$ group of the methyl ester (-CD$_3$ lost in the deuterated analogue). These observations again require extensive and rapid scrambling of hydrogen isotopes, as depicted in Scheme...
3.4 Conclusions

The present results corroborate our earlier suggestion that loss of $\text{H}_2\text{O}$ from lithiated- and metal-cationized in general, peptides is initiated by a nucleophilic attack from the N-terminal side to form a five-membered ring as an intermediate followed by 1,2-elimination of water. The nucleophilic atom is most likely the oxygen atom of the N-terminal amide group in the fragmentation of $[\text{AcGGGOMe}+\text{Li}]^+$ as well as $[\text{GGGOMe}+\text{Li}]^+$ while the site of attack is the carbonyl carbon atom of the adjacent amide group to the C-terminal side. Multiple-stage CID shows that the subsequent dissociation of the product arising from the elimination of $\text{H}_2\text{O}$ is markedly different in the two types of peptide as a result of the absence and presence of a free amino group. In particular, extensive scrambling of protons in the $\alpha$-positions of GGGOMe is observed as a consequence of intervention of the basic amino group, a feature almost totally absent in AcGGGOMe. These cyclization pathways should operate also in the corresponding peptides having a free carboxyl group. Delineation of involvement of the C-terminal hydroxyl group in loss of water, if at all, requires the use of labeled oxygen, which has not been undertaken to our knowledge.


ISOTOPE LABELING AND THEORETICAL STUDY OF THE FORMATION OF $a_3^*$ IONS FROM PROTONATED TETRAGLYCINE

4.1 Introduction

CID and tandem mass spectrometry [1, 2] are now well established as effective tools for deriving sequence information from peptides and proteins. The various reaction pathways involved in the dissociation of protonated peptides have recently been extensively reviewed [3]. Fragmentation of protonated peptides under low-energy collision conditions is dominated by reactions that involve cleavage of the amide bonds along the peptide backbone [4, 5]. Qualitatively, protonated peptide dissociation can be described using the "mobile proton model," [6-19] for which a main tenet is the intra-molecular migration of protons from the most basic group on a peptide to the site of cleavage. The recently introduced "pathways in competition" (PIC) fragmentation model [3] provides a more general framework, taking into account specific features of individual peptide fragmentation pathways (PFPs) and their interaction. PIC states that fragmentation abundances in the MS/MS spectra of peptides are determined by predissociation, bond-cleavage, and post-cleavage events. The predissociation phase involves proton transfers (mobile proton) and internal rotations necessary to populate fragmenting species. Besides the actual bond-cleavage mechanisms, energetics, and kinetics that determine competition of the PFPs, the post cleavage phase determines the fate of the added proton (e.g., which fragment will appear as charged species) based on the proton affinities (or gas-phase basicities) of the species involved. In the present article we will show that additional transitions such as association and relay type exchange reactions can occur in post-cleavage ion-molecule complexes formed by peptide fragments.

The dominant product ions from the CID of protonated peptides are those of the $b_n$ and $y_n$ series, with the former representing ionized fragments containing the N-terminus and the latter containing the C-terminus of the original peptide. While originally proposed to be acylium ions [20-22] the $b_n$ ions are now generally accepted to have a cyclic oxazolinone structure [23-30]. The dissociation/decomposition of $b_n$ ions has also been the subject of both experimental and theoretical studies [24, 29, 31, 32]. A principal product derived from the dissociation of a $b_n$ ion is the $a_n$ species, which is formed through a ring-opening reaction and the elimination of CO [24, 33]. A second product, labeled $a_n^*$, has been observed in the
metastable ion dissociation [24] and CID of small peptides.[34, 35] Formation of this particular product involves the net loss of 45 mass units (Da), which in earlier reports was proposed to involve elimination of CO and NH₃.

In an investigation of the CID of protonated alanine oligomers, Harrison and Young proposed that a₃⁺ is generated by decomposition of the a₃ ion through the mechanism shown in Scheme 4.1.[34] The pathway suggested involves proton transfer from the imine nitrogen atom to the N-terminal amino group, followed by nucleophilic attack (by the same imine nitrogen atom) upon the α-carbon position of the N-terminal amino acid and subsequent elimination of the amino terminus as NH₃. The structure proposed for the product ion (a₃⁺) is reminiscent of substituted diketopiperazines, which have been implicated in the formation of yₘ-type ions from protonated peptides.[36]

![Scheme 4.1. Pathway for generation of a₃⁺ from b₃⁺ proposed by Harrison and Young.](image)

Glish and coworkers used the relative reactivities of bₙ⁺ ions with dimethylamine in an ion-trap mass spectrometer to demonstrate an apparent unique behavior for b₃⁺ ions, [35] in particular that the formation rate for a dimethylamine adduct was lowest for b₃ ions when compared to b₂ and b₄ ions generated from a range of peptides. The differences in reaction rate were used as evidence that the b₃ ion may be formed by a pathway similar to that shown in Scheme 4.1, and a similar process was proposed by Vachet et al. to describe formation of a₄ and a₅ ions from a series of leucine enkephalin analogues.[37]

In this article, formation of a₃⁺ ions from a model peptide is discussed using experimental and theoretical strategies. The experimental work involved synthesis of tetruglycine (GGGG) with specific ¹³C, ¹⁵N, and ²H labeling of amino acid residues and elucidation of CID pathways using multiple-stage tandem
mass spectrometry. The goal of the isotope labeling studies was to identify the atoms within the b3 ion that are eliminated upon formation of a3+. A comprehensive molecular dynamics and computation/theoretical investigation was then undertaken to provide mechanistic details of the underlying chemistry necessary to produce a3+ along a pathway that accounts for the observations made in the isotope labeling study.

4.2 Experimental

Experimental work was performed at the Department of Chemistry, Wichita State University, Wichita, KS. The molecular dynamics simulations and the quantum chemical calculations were carried out at the German Cancer Research Center at Heidelberg. Both the experimental and theoretical strategies are briefly described below.

4.2.1 Mass Spectrometry

All peptides used in this study were generated by conventional solid-phase synthesis methods [38] using 9-fluorenylmethoxycarbonyl (FMOC)-glycine loaded Wang resin (Sigma Chemical, St. Louis, MO) and a custom-built, multiple-reaction vessel peptide synthesis apparatus. Glycine-15N (H215NCH2COOH, G-15N), glycine-1-13C (H2NCH213COOH, G-1-13C), α-d2-glycine (H2NCD2COOH, α-d2-G), and FMOC-chloride were purchased from Sigma Chemical (St. Louis, MO) and used to generate N-terminus FMOC-protected amino acids for incorporation into the model peptides. FMOC protected glycine (G) was purchased from Sigma and used as received. Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies. The sequence of each peptide, and in particular the position of isotope labels, was confirmed using multiple-stage CID of Na+ and Ag+ cationized versions. The positions of the isotope labels were also used to confirm that the major sequence ions generated by CID of protonated GGGG are b3+ arising by elimination of the C-terminal G residue, and y2+ arising by elimination of two G residues from the N-terminus. Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (vol:vol) mixture of HPLC grade methanol (Aldrich Chemical, St. Louis, MO) and deionized H2O to produce final concentrations of 10⁻⁵ to 10⁻⁴ M.

For experiments in which the CID of amino, amide, and acid deuterium labeled peptides was examined, the relevant peptides were incubated in a 50/50 mixture of D2O and CH3OD (Aldrich Chemical,
St. Louis, MO) for 2 hours. The peptide solutions in deuterated solvent were then injected into the ESI source without further modification. In this case, ESI produced abundant (M+D)+ ions.

ESI mass spectra were collected using a Finnigan LCQ-Deca ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Peptide solutions were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 5 µl/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole, and octupole voltage offsets, etc.) were optimized for maximum (M+H)+ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N2 sheath gas flow at 25 units (arbitrary for the Finnigan systems, corresponding to ~0.375 L/min) and the capillary (desolvation) temperature at 200°C. Helium was used as the bath/buffer gas to improve trapping efficiency and also as the collision gas for CID experiments. The CID studies (MS/MS and MSn) were performed as follows. The (M+H)+ ions were isolated for the initial CID stage (MS/MS) using an isolation width of 0.9 to 1.2 mass to charge (m/z). Product ions selected for subsequent CID (MSn) experiments were isolated using widths of 1.0 to 1.3 m/z. The exact width was chosen empirically and reflected the best compromise between high precursor ion abundance and the isolation of a single isotopic peak. The (mass) normalized collision energy, which defines the amplitude of the R.F. energy applied to the end cap electrodes in the CID experiment, was set between 20% and 25%, which corresponds roughly to 0.55 to 0.68 V with the instrument calibration used in this study. The activation Q (as labeled by ThermoFinnigan, used to adjust the qz value for the precursor ion) was set at 0.30. Subsequent CID stages were performed using similar activation parameter settings. The activation time employed at each CID stage was 30 msec.

4.2.2 Computational Details

The potential energy surface (PES) of the GGGG b3 ion was investigated using the strategy developed recently [3, 28, 29] to deal with protonated peptides. These calculations involved molecular dynamics simulations using the Insight II program (Biosym Technologies, San Diego, CA) to produce starting geometries and quantum chemical calculations at the HF/3-21G, B3LYP/6-31G(d) and finally at the B3LYP/6-31+G(d,p) levels. Transition structures (TS) corresponding to various fragmentation pathways of the GGGG b3 ion were determined at the B3LYP/6-31G(d) and B3LYP/6-31+G(d,p) levels of
theory. In most of the cases the transition structures were checked by using intrinsic reaction path
calculations (IRC) to unambiguously define which minima are connected by the TS investigated. Relative
energies were calculated by using the B3LYP/6-31+G(d,p) total electronic energies and zero-point energy
corrections (ZPE) determined at the B3LYP/6-31G(d) level. For all ab initio calculations the Gaussian 03
[39] program was used.

4.3 Results and Discussion

4.3.1 CID of Native and Isotope Labeled GGGG

Figure 4.1 shows the multiple-stage CID spectra generated from protonated GGGG. CID
(MS/MS stage) of (M+H)+ at m/z 247 (Figure 4.1a) produced prominent [M-H2O+H], b3 and y2 ions at m/z
229, 172, and 133, respectively. Subsequent CID of b3+ (MS3 stage, Figure 4.1b) produced a3+ and b2+ at
m/z 144 and 115, respectively. The peak at m/z 127 in Figure 4.1b is 45 Da lower in mass than the b3+ ion and represents the a3− ion as identified in an earlier study of the CID of oligoalanine peptides.[34]
Isolation and dissociation of a3+ at m/z 144 (MS4 stage, Figure 4.1c) led primarily to the a3− species, with
the b2 ion as a minor product. The multiple-stage CID results suggest a reaction sequence for generation
of a3− that involves the decomposition of b3+ to a3+, and then a3+ to a3−. Identification of a pathway for
direct formation of a3− from b3+, without a3+ as an intermediate, would require double-resonance
experiments, [31] which are not possible with our unmodified LCQ.
Figure 4.1. Multiple-stage CID spectra derived from protonated GGGG: (a) CID (MS/MS) of (M+H)+, (b) CID (MS3) of b3+, (c) CID (MS4) of a3+.

Figure 4.2 shows the product ion spectrum generated by CID (MS3 stage) of the b3 ion at m/z 176, derived initially from D⁺ cationized GGGG in which all amino, amide, and acid groups were labeled with D via solution-phase hydrogen/deuterium exchange before ESI. The a3⁺ ion appeared as three peaks at m/z 128, 129, and 130, corresponding to net neutral losses (from the b3⁺ species) of 46, 47, and 48 Da and reflecting the elimination of 1, 2, and 3 D atoms, respectively. The elimination of up to 3 D atoms supports the proposal that the MSⁿ CID pathway to generate a3⁺ involves the elimination of CO and NH₃, and the isotopic distribution in Figure 4.2 suggests that the favored route is the elimination of CO and ND₂H for the amino, amide, and acid deuterium exchanged peptide. An alternative reaction pathway that involved the loss of CO and OH (also a net loss of 45 Da for unlabeled GGGG) would produce a maximum shift in the H/D exchange experiment of only 46 Da.
Figure 4.2. CID (MS³) of b³⁺ generated by dissociation (MS/MS) of (M+D)⁺ derived from amino, amide, and acid D-exchanged GGGG.

In the spectrum shown in Figure 4.2, the b₂ species appears as discrete isotopic peaks at m/z 118 and 119. The peak at m/z 118 represents the formation of b₂ via the elimination of 58 Da, consistent with the elimination of CO and a D labeled imine (ND≡CH₂). The peak at m/z 119, instead, reflects the loss of 57 Da during formation of the b₂ ion from the b₃ species. This observation suggests that H atoms from an α-carbon position may be transferred during the reactions to produce certain b ions by CID. Similar observations were made following CID of the peptides containing α-d₂-G as described below.

The product ion spectra generated from CID (MS³ of the b₃ ions initially derived from GGGG precursor peptides containing G-1-¹³C or G-¹⁵N are shown in Figure 4.3 and Figure 4.4, respectively). The loss of 45 Da to produce a₃⁺ shifted to 46 Da when the precursor peptide was either GG(G-1-¹³C)G or GG(G-¹⁵N)G (¹³C or ¹⁵N label contained within the amino acid adjacent to the C-terminus) as shown in Figures 4.3a and 4.4a, respectively. No shift in mass of the neutral eliminated was observed when the glycine residue with the isotope label was positioned either adjacent to the N-terminus (Figures 4.3b and 4.4b) or at the N-terminus (Figures 4.3c and 4.4c).
Figure 4.3. CID (MS³) of b₃⁺ species derived for dissociation (MS/MS) of protonated, ¹³C labeled forms of GGGG: (a) GG(¹³C-G)G, (b) G(¹³C-G)GG, (c) (¹³C-G)GGG.
Figure 4.4. CID (MS$^3$) of b$_3^+$ species derived for dissociation (MS/MS) of protonated, $^{15}$N labeled forms of GGGG: (a) GG(15N-G)G, (b) G(15N-G)GG, (c) (15N-G)GGG.

The amino acid adjacent to the C-terminus in the precursor tetrapeptides makes up the oxazolinone ring portion of the b$_3^+$ product ion. For GG(G-1-$^{13}$C)G the neutral loss to generate the a$_3$ ion from b$_3^+$ (Figure 4.3a) shifted to 29 Da. The mass shift of neutral loss is consistent with the opening of the oxazolinone ring and elimination of CO ($^{13}$CO for the peptide with G-1-$^{13}$C adjacent to the C-terminus).

The product ion spectra generated from CID (MS$^3$ stage) of the b$_3$ ions initially derived from GGGG precursors containing $\alpha$-d$_2$-G are shown in Figure 4.5. For CID of b$_3^+$ generated from GG($\alpha$-d$_2$-G)G ($\alpha$-d$_2$ labeled residue positioned adjacent to the C-terminus), a$_3$ was formed through the net loss of 45 Da (Figure 4.5a). However, the neutral loss associated with formation of a$_3$ exhibited a shift to 46 and 47 Da, with a preference for the loss of 46 Da (one $\alpha$-carbon position D-atom eliminated), when the precursor peptide was G($\alpha$-d$_2$-G)GG (labeled residue positioned adjacent to the N-terminus) as shown in Figure 4.5b. The preferred loss of 1 D atom from the labeled G residue is consistent with the isotopic
distribution in the neutral loss from the amino, amide, and acid deuterium-labeled peptides (Figure 4.2), in which the preference was for a loss of two amide or amino D atoms.

![Image of Figure 4.5]

**Figure 4.5.** CID (MS$^3$) of $b_3^+$ species derived for dissociation (MS/MS) of protonated GGGG containing $\alpha$-d$_2$ glycine: (a) GG($\alpha$-d$_2$-G)G, (b) G($\alpha$-d$_2$-G)GG, (c) ($\alpha$-d$_2$-G)GGG.

In Figure 4.5b, the $b_2$ species generated by CID of the $b_3$ ion derived from protonated G($\alpha$-d$_2$-G)GG was also split into discrete isotopic peaks at m/z 116 and 117. The latter represents the elimination of CO and an imine (as a net loss of 57 u) from the oxazolinone ring to generate $b_2^+$. The former, however, reflects the elimination of 58 Da. The loss of 58 Da suggests the exchange of an H atom from the oxazolinone ring (or portions of the ring) for a D atom at the $\alpha$-carbon position of the amino acid adjacent to the N-terminus during the formation of $b_2^+$. The labeling data suggests that the splitting of the $b_2$ ion in Figure 5.5b is induced by exchange that involves $\alpha$-carbon H atoms of the G residue adjacent to the N-terminus. The exchange/transfer of the H and D atoms during formation of $b_2^+$ from the
peptide containing α-d2-G is consistent with the splitting of the b2 ion peak observed following CID of GGGG with D exchanged onto the amino, amide, and acid positions.

**4.3.2 Mechanism of Formation of a3* Ions**

The consensus based on a number of experimental and theoretical studies is that the b_n ions of peptides with aliphatic side chains have a cyclic, oxazolinone structure.[23–31] With this in mind, the 13C and 15N isotope labeling experiments, particularly the results obtained for protonated GG(G-1-13C)G and GG(G-15N)G (substitution with G-1-13C or G-15N adjacent to the C-terminus of GGGG), demonstrate that formation of a3* involves elimination of the carbonyl group (as CO) and the N atom, as part of NH3, from the presumed oxazolinone ring of the b3 ion. It is interesting to note that N-terminal acetylation completely suppressed the formation of a3* (spectra not shown), a result that suggests that the reaction pathway involves an attack by the N-terminal amino group. However, the fact that the mass of the neutral species eliminated during formation of a3* did not change when the precursor peptide was (G-15N)GGG clearly shows that the formation of a3* does not involve elimination of the protonated N-terminal amino group as suggested in Scheme 4.1.

It is important to recognize that the a3 isomer of Scheme 4.1 is a diketopiperazine derivative having two cis amide bonds. The N-terminal amide bonds of GGGG and that of the related b3 and a3 ions are in the trans isomerization state, and formation of the diketopiperazine derivative a3 of Scheme 4.1 requires their trans-cis isomerization. In other words, the attacking imine N can approach the protonated N-terminal amino group only if the N-terminal amide bond is in the cis isomerization state. This means that the two structures depicted in Scheme 4.1 for the a3 ion are two distinct isomers that are connected by transition structures with significant barriers [40] and cannot simply be equated as shown in Scheme 4.1. Gas-phase trans-cis isomerization of amide bonds involves amide N protonated species as described in detail in a recent study of protonated GGG.[40] The major conclusion of the work with GGG is that trans-cis isomerization of amide bonds is energetically possible but kinetically disfavored compared with other pathways like b2-y1.

The major steps of an alternative pathway to formation of a3*, one that explains the isotope labeling results (including those of the scrambling of H atoms from α-carbon positions), are depicted in (Scheme 4.2) and (Scheme 4.3). The corresponding relative energetics are graphically shown in Figure
4.6. (The most important structures occurring on this multistep pathway are presented in the Appendix section “Total Electronic Energies and 3-Dimensional Structure Depictions” along with their total electronic energies found in Table 4.1.) The first step is loss of CO on the \( b_3 \rightarrow a_3 \) PFP from \( b_3 \) (I in Scheme 4.2), the corresponding TS (I-II) is of 31.9 kcal/mol relative energy \((E_{rel})\) (Scheme 4.2). The \( a_3 \) ion (II) formed (Scheme 4.2) is protonated at the C-terminal imine group and the adjacent carbonyl C becomes a likely target of nucleophilic attack by the N-terminal amide oxygen. The corresponding \( a_3 \rightarrow b_2 \) PFP involves a TS (II-III) of 35.1 kcal/mol internal energy and leads to a complex of the oxazole and the imine fragments (III, \( E_{rel} \) at 30.9 kcal/mol). Under low-energy conditions this complex likely has a short but finite lifetime, which may allow reisomerization to form various proton-bound dimers like \( IV \) (\( E_{rel} \) at 25.3 kcal/mol) and \( V \) (\( E_{rel} \) at 25.3 kcal/mol). For the latter, the imine fragment is protonated and the amino group of the oxazolinone fragment is close to the carbon of the imine. The next step in Scheme 4.2 is association of the imine and the oxazolinone fragments via attack by the amino group of the latter on the imine carbon, a process that involves a TS (V-VI) of 30.0 kcal/mol relative energy. After some exothermic proton transfer reactions, species \( VII \) (\( E_{rel} \) at 17.6 kcal/mol) is formed that eliminates ammonia by passing TS VII-VIII of 33.7 kcal/mol relative energy. Note that this multistep mechanism is energetically feasible: the highest energy point is the \( a_3 \rightarrow b_2 \) TS on the pathway at 35.1 kcal/mol relative energy. Furthermore, Scheme 4.2 clearly explains the \(^{15}\)N labeling data presented above and predicts that the nitrogen of the oxazolinone ring of \( b_3 \) is lost upon formation of \( a_3 \).
Figure 4.6. Schematics of the potential energy surface of the $b_3$ ion of protonated GGGG showing relevant minima, TS’s and their relative energetics.
Scheme 4.2. Multistep pathway for generation of $a_3^+$ from $b_3^+$ of protonated GGGG.
Scheme 4.3. Relay-type H/D exchange pathway to explain scrambling of H atoms from $\alpha$-carbon positions of $a_3^*$ ions. Labile H atoms are denoted by D.

While the reaction pathway proposed in Scheme 4.2 accounts for the elimination of isotope labels from specific amide positions in the $^{15}$N and $^{13}$C labeled GGGG peptides, it does not explain why H atoms are transferred and eliminated from specific $\alpha$-carbon positions (namely, those in the amino acid adjacent to the amino terminus) upon formation of $a_3^*$ and $b_2$. It is worth noting here that scrambling of $\alpha$-carbon H atoms is usually not observed upon CID.[11] A plausible mechanism that accounts for the $\alpha$-carbon H scrambling of the $a_3^*$ ion is depicted in Scheme 4.3 where all labile H atoms are replaced by D atoms to reflect the experiment on $D^+$ cationized GGGG in which all amino, amide, and acid groups were labeled with D via solution-phase H/D exchange before ESI.

Species passing the VII-VIII ammonia-loss TS (Figure 4.7) can form various complexes and proton-bound dimers (for example, VIIIa...NH$_3$, Appendix) of $a_3^*$ and NH$_3$ under low-energy CID conditions (the four $a_3^*$ isomers (Villa-d) considered in the text are depicted in Figure 4.8). The lifetime of these species can be long enough to allow proton transfer reactions and reisomerizations to form complex VIIIc...NH$_3$ (Figure 4.7) in which the oxazolinone fragment is protonated at the oxazolinone N and ammonia is bonded by the C-H...ND$_3$ bridge. A relay-type H/D exchange TS (relay HDX TS, Figure 4.7), as invoked for gas-phase H/D exchange reactions, [41, 42] would lead to a complex of another $a_3^*$ isomer (Villld) and NH$_2$. It is worth noting here that complex Villld...NH$_3$ is the global minimum on the PES of $a_3^*$ and NH$_3$ while species Villa...NH$_3$ is only slightly less favored (Appendix section). To explain the observed high H/D scrambling reaction rates of the oxazolinone $\alpha$-carbon H’s, we note that the relay...
HDX TS (37.1 kcal/mol relative energy) is energetically more favored than separated \( a_3^* \) and ammonia (37.9 kcal/mol relative energy, Figure 4.6). Spatial separation of \( \text{VIIId} \) and NHD\(_2\) in \( \text{VIIId}. . .\text{NHD}_2\) (Figure 4.7) leads to a singly H/D exchanged \( a_3^* \) species. Exchange of the other \( \alpha \)-carbon H can occur after rotation of NHD\(_2\) around the O-D...N axis in \( \text{VIIId}. . .\text{NHD}_2\) and reversal of the reaction of Scheme 4.3 and an additional relay step to form finally \( \text{VIIId}. . .\text{NH}_2\text{D} \) where \( \text{VIIId} \) bears two D’s at the \( \alpha \)-C and OH positions.

Figure 4.7. \( a_3^*\)-NH\(_3\) complexes and TS HDX relay TS.
In **VIIId**, the 5-membered ring features a stabilizing aromatic character because of two ring double bonds and a lone pair on the ring oxygen atom, and the former carbonyl O becomes an alcohol group. It is worth noting here that **VIIId** is the energetically most favored \( a_5^+ \) isomer, the relative energies of **VIIa**, **VIIb**, and **VIIId** (Figure 4.8) compared with **VIIc** are 5.6, 2.8, and 7.7 kcal/mol, respectively. Isomers such as species **VIIId** are possible for various \( b \) ions as well and their structure and reactivity will be subject of a forthcoming study.
The DFT results suggest that the scrambling of the $\alpha$-carbon H atoms observed in the experimental study does not occur before the first fragmentation step ($b_3 \rightarrow a_3$ PFP) as usual for labile H atoms of protonated peptides but happens in the post dissociation complexes of the fragments. It is worth noting here that this observation is difficult to explain using the “mobile proton” model, which deals strictly with predissociation events of peptide fragmentation.[3] On the other hand, the recent PIC fragmentation model [3] provides a flexible framework to understand such complex chemistries like formation of $a_3^*$ ions and related scrambling of $\alpha$-carbon H atoms of protonated peptides. Likely a similar mechanism can be invoked to explain $\alpha$-carbon H scrambling of the $b_2$ ion observed in Figure 4.2 and Figure 4.5.

### 4.4 Conclusions

A comparison of the CID of native and amino, amide and acid D-exchanged GGGG demonstrates that the formation of $a_3^*$ from $b_3^+$ involves the elimination of CO and NH$_3$, with the latter neutral species carrying two H atoms originating from amino or amide sites and one H atom from an $\alpha$-carbon position. The use of $^{15}$N labeled glycine residues within the GGGG sequence showed that the reaction to produce $a_3^*$ does not result in the elimination of the N-terminal amino group, as suggested in Scheme 4.1, but instead involves the amide N atom from the presumed oxazolinone ring of the $b_3$ ion, two H atoms from amide positions, and one H atom from the $\alpha$-carbon of the amino acid adjacent to the oxazolinone ring.

A multistep reaction pathway involving elimination of CO from $b_3^+$ to form $a_3^+$ and loss of NH=CH$_2$ from $a_3^+$ to form $b_2^+$ is proposed. The NH=CH$_2$ and $b_2$ products can form various complexes and proton-bound dimers allowing nucleophilic attack of the oxazolinone amine N atom on the imine C atom. The attack leads to another $a_3$ isomer that can easily eliminate an ammonia molecule that includes the former C-terminal imine N atom of $a_3^+$. Scrambling of H atoms from $\alpha$-carbon positions is explained by interaction of ND$_3$ and various $a_3^*$ isomers on energetically feasible relay-type H/D exchange pathways. These results indicate that the MS/MS spectra of protonated peptides can be a result of rather complicated chemistries involving reisomerization, fragment association and exchange reactions. While the complicated chemistry is not readily explained using the “mobile proton” model of peptide fragmentation, the alternative PIC [3] model can easily account for the observed phenomena.
LIST OF REFERENCES


APPENDIX

TOTAL ENERGIES WITH ZPE CORRECTIONS AND 3-DIMENSIONAL STRUCTURE DEPICTIONS

<table>
<thead>
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<th>Species</th>
<th>Total energy</th>
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<th>Total energy</th>
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<tr>
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<td>-511.054874</td>
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<td>VIII d</td>
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</tr>
</tbody>
</table>

Table 4.1. B3LYP/6-31+G(d,p) total ZPE corrected energies (Hartree) of the investigated species.

![Structure depictions](image)
APPENDIX (continued)

VI     VII

VII_VIII     VIIIa...NH₃
5.1 Introduction

Fragmentation of protonated peptides in tandem mass spectrometry (MS/MS) proceeds through reactions that involve cleavage of the amide bonds along the peptide backbone.[1, 2] The dominant product ions from the collision-induced dissociation (CID) of protonated peptides are those of the $b_n^+$ and $y_n^+$ series,[3, 4] with the former representing fragments containing the N-terminus and the latter containing the C-terminus of the original peptide. The formation of $b_n^+$ and $y_n^+$ product ions has been the subject of numerous studies,[5-13] and the consensus is that mechanistic steps include cyclization and intramolecular nucleophilic attack (Scheme 5.1) upon an amide carbonyl carbon atom by the oxygen atom of the carbonyl group of the amide group to the immediate N-terminal side of the cleavage site.
Scheme 5.1. Pathway to $b_3^+$ from protonated AAAG through the oxazolinone mechanism.

The general reaction pathways involved in the dissociation of protonated peptides have been the subject of a recent, comprehensive review.[14] Qualitatively, protonated peptide dissociation can be described using the “mobile proton” model.[15-28] A main tenet of the mobile proton model is that protons undergo intramolecular migration from the most basic group on a peptide to the site of cleavage, where they can weaken the C-N bond and make the carbonyl C atom more susceptible to intermolecular nucleophilic attack. The recently introduced “pathways in competition” fragmentation model [14] provides a more general framework, taking into account specific features of individual peptide fragmentation.
pathways that include pre-dissociation events such as proton transfer and peptide isomerization, structures and transition state energies in the dissociation step, and the thermodynamics associated with the separation of ion-molecule complexes into fragmentation products (so-called post-dissociation steps).

In an attempt to enhance the understanding of how cation and sequence influence peptide fragmentation we recently investigated the dissociation of metal-cationized, model N-acetylated tetrapeptides, with the general sequence AcFGGX, that featured C-termini designed to allow transfer of the -OH required to generate the \([b_{3+17}+\text{Cat}]^+\) product ion, but not necessarily as the most favored pathway.\[29\] The amino acid placed at position X either required a larger cyclic intermediate than the five-membered ring presumably formed with \(\alpha\)-amino acids (\(\beta\)-alanine, \(\gamma\)-aminobutyric acid and \(\varepsilon\)-amino-n-caproic acid to generate six-, seven- or nine-membered rings, respectively) or prohibited cyclization because of the inclusion of a rigid ring (\(\text{para-}\) and \(\text{meta-}\)aminobenzoic acid). For \(\text{Ag}^+\), \(\text{Li}^+\) and \(\text{Na}^+\)-cationized AcFGGX, formation of \([b_{3+17}+\text{Cat}]^+\) was suppressed when the amino acids requiring the adoption of larger ring intermediates were used, while amino acids that prohibit cyclization eliminated the reaction pathway completely; an observation in accord with proposed mechanisms for the formation of this important sequence ion.\[30-36\]

Formation of five-membered cyclic intermediates is also an integral part of the proposed pathway leading to \(b_n^+\) and \(y_n^+\) sequence ions from protonated peptides through the oxazolinone mechanism (Scheme 5.1).\[6, 7, 10-13\] Quantum chemical calculations have provided evidence that charge solvation of the protonated amide N atom in the reactive configuration assists in the approach of the nucleophile (amide carbonyl O atom) to the electrophilic site of attack (adjacent amide carbonyl C atom) and formation of the five-membered ring intermediate.\[9, 11\] The goal of this study was to build upon our earlier study of metal-cationized peptides, and to determine the extent to which changes of the size of the putative cyclic intermediate may influence the tendency to generate sequence ions from a series of model protonated peptides. This investigation involved three series of peptides with sequence \(\text{AAXG}, \text{AXAG}\) and \(\text{XAAG}\), where the sequence position X was occupied by an ‘alternative’ amino acid such as \(\beta\)-alanine, \(\gamma\)-aminobutyric acid, \(\varepsilon\)-aminocaproic acid or 4-aminomethylbenzoic acid. As in our earlier study, these particular amino acids were selected because they either require larger cyclic intermediates in the nucleophilic attack step, or block cyclization altogether.
5.2 Experimental

All peptides used in this study were generated by solid-phase synthesis methods [37] using Wang resin, conventional coupling procedures and FMOC-protected amino acids in a custom-built, multiple reaction vessel peptide synthesis apparatus. FMOC-chloride and D,L-alanine-3,3,3-d₃ [CD₃CH(NH₂)CO₂H, d₃A] were purchased from Sigma Chemical (St. Louis, MO, USA) and used to generate FMOC-d₃A for incorporation into the model peptides. Glycine-loaded Wang resin and FMOC-alanine (A), β-alanine [NH₂CH₂CH₂CO₂H, βA], γ-aminobutyric acid [NH₂CH₂CH₂CH₂CO₂H, γAbu], ε-aminocaproic acid [NH₂CH₂CH₂CH₂CH₂CO₂H, Cap), 4-aminomethylbenzoic acid [NH₂CH₂C₆H₅CO₂H, 4AMBz] and histidine (H) were also purchased from Sigma and used as received. Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies.

Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (v/v) mixture of HPLC-grade methanol and deionized H₂O to produce final concentrations of 10⁻⁵ to 10⁻⁴ M. Electrospray ionization (ESI) mass spectra were collected using a ThermoFinnigan LCQ-Deca ion trap mass spectrometer (San Jose, CA, USA). Peptide stock solutions were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 3-5 µL/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octupole voltage offsets, etc.) were optimized for maximum [M+H]⁺ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N₂ sheath gas flow at 25 units (arbitrary units, corresponding to approximately 0.375 L/min) and the capillary (desolvation) temperature at 200°C. The ion trap analyzer was operated at a pressure of ~1.5 x 10⁻⁵ Torr. Helium gas, admitted directly into the ion trap, was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The CID studies (MS/MS and MSⁿ) were performed as follows. The [M+H]⁺ ions were isolated for the initial CID stage (MS/MS) using an isolation width of 0.9 to 1.2 m/z units. Product ions selected for subsequent CID (MSⁿ experiments) were isolated using widths of 1.0-1.3 m/z units. The (mass) normalized collision energy, which defines the amplitude of the radio frequency (RF) energy applied to the end cap electrodes in the CID experiment, was set between 20% and 25%, which corresponds roughly to 0.55-0.68V with the instrument calibration used in this study. The activation Q (as labeled by
ThermoFinnigan, used to adjust the $q_z$ value for the precursor ion) was set at 0.30. Subsequent CID stages were performed using similar activation parameter settings. The activation time employed at each CID stage was 30 ms.

5.3 Results and Discussion

5.3.1 CID of AAXG

Figure 5.1 shows CID (MS/MS stage) spectra for the [M+H]$^+$ ions of AAAG (Figure 5.1(a)), AA($\beta$A)G (Figure 5.1(b)), AA($\gamma$Abu)G (Figure 5.1(c)), AA(Cap)G (Figure 5.1(d)), and AA(4AMBz)G (Figure 5.1(e)). To generate each spectrum, the normalized collision energy was adjusted such that the signal for the [M+H]$^+$ ion was reduced to ca. 1-5% relative intensity. The AAAG peptide served as a control, and the use of A and G at the amino and carboxy termini, respectively, allowed for unambiguous identification of $b_n^+$ and $y_n^+$ ions in the CID spectrum. For AAAG (Figure 5.1(a)), the prominent sequence ions observed included $b_3^+$ and $b_2^+$ at $m/z$ 214 and 143, respectively, and $y_2^+$ at $m/z$ 147. Also observed were the [M-H$_2$O+H]$^+$ and $a_3^*$ ions at $m/z$ 271 and 169, respectively. Formation of [M-H$_2$O+H]$^+$ may occur through the oxazolinone mechanism, with loss of H$_2$O from the C-terminus, to generate $b_4^+$. Elimination of H$_2$O from the carboxyl group of protonated glycine-glycine was investigated by Lifshitz and coworkers, who found formation of a protonated oxazolinone structure to be kinetically favored over an alternative diketopiperazine conformation.[38] An alternative formation mechanism for [M-H$_2$O+H]$^+$, proposed first by Reid and coworkers[39] and supported by subsequent experiments by our group with metal-cationized peptides,[29, 40] involves a retro-Ritter or retro-Koch type reaction and elimination of an amide O atom. The formation mechanism for the $a_3^*$ ion, which involves the net elimination of 45 Da from the $b_3^+$ ion, is yet to be fully resolved. In a study of the CID of protonated oligoalanine peptides, Harrison and Young[41] proposed a mechanism that involves attack upon the imine portion of the $a_3$ ion by the N-terminal amino group. A more recent study, in which a combination of isotope labeling and quantum chemical calculations was used, provided evidence that the mechanism may instead involve a complex rearrangement of the $a_5^*$ ion and elimination of the imine N atom as part of the NH$_3$ molecule.[42]
Figure 5.1. CID (MS/MS) spectra from protonated peptides in the AAXG series: (a) AAAG, (b) AA(βA)G, (c) AA(γAbu)G, (d) AA(Cap)G, and (e) AA(4AMBz)G.

The focus of this study was the influence of the 'alternative' amino acids on the generation of sequence (bₙ⁺ and/or yₙ⁺) ions rather than non-specific species such as [M-H₂O+H]⁺. At the normalized collision energy used to generate the spectrum shown in Figure 5.1(a), the most abundant product ion from AAAG was b₃⁺ at m/z 214. Preferential formation of b₃⁺ is in accord with theoretical studies by Paizs and Suhai, who have shown that amine N atom protonated structures that make up the reactive
configuration of the amide cleavage step, and the transition state structures on the oxazolinone pathway to $b^+$ and $y^+$ ions, are energetically more favorable when the protonated amide lies near the C-terminus.[12] The calculations of transition state energies performed by Paizs and Suhai qualitatively supported the relative $b_n^+$ and $y_n^+$ ion signal intensities from (experimental) CID studies by Harrison and Young of protonated alanine oligomers.[41]

Subsequent CID of $b_3^+$ derived from protonated AAAG (MS$^3$, spectrum not shown) generated the $a_3^+$ and $a_3'^+$ species, via the elimination of 28 and 45 Da, respectively, but only a minor amount of $b_2^+$ (less than 10% relative intensity). It is therefore likely that the appearance of the prominent $b_2^+$ ion in the CID spectrum of AAAG reflects generation of the species directly from [M+H]$^+$ via the oxazolinone mechanism, as shown in Scheme 5.2. A prior theoretical study suggests that the oxazolinone and peptide fragments such as those shown in Scheme 5.2 initially form a loose complex that can rearrange to a proton-bound dimer.[11] As the dimer dissociates the relative proton affinities of the respective products (oxazolinone and peptide) determine the $b_2^+$ and $y_2^+$ signal intensities. Based on this argument, the relative intensities of the $b_2^+$ and $y_2^+$ species generated from protonated AAAG (Figure 5.1(a)) suggest that the AG product has the higher proton affinity.
Scheme 5.2. Pathway for generation of $b_2^+$ directly from protonated AAAG through the oxazolinone mechanism.

For the AAXG series of peptides, the hypothesis tested was that placement of $\beta$A, $\gamma$Abu or Cap at position X would suppress or inhibit formation of $b_3^+$ because the nucleophilic attack and proton transfer steps would be forced to proceed through larger cyclic intermediates. As is apparent in a comparison of the spectra shown in Figure 5.1, the signal intensity of $b_3^+$ decreased when $\beta$A was substituted for the A residue adjacent to the C-terminus, and is present in Figure 5.1(b) (CID of AA($\beta$A)G) at a relative signal intensity of only ca. 5%. The $b_3^+$ ion was not observed when either $\gamma$Abu or Cap was substituted for A in the same position (Figures 5.1(c) and 5.1(d), CID of AA($\gamma$Abu)G and AA(Cap)G, respectively). The decrease in $b_3^+$ intensity when X= $\beta$A, $\gamma$Abu or Cap is consistent with, at least in part, potential changes to the rates of the putative ring-closure step leading to nucleophilic attack in the oxazolinone mechanism. The larger cyclic rings would be kinetically slower to form than the conventional five-membered rings.
associated with peptides composed entirely of \(\alpha\)-amino acids. In addition, the thermodynamic stability of the rings may also play a role. As noted in an earlier study of the influence of similar amino acids on the tendency to form \([b_{n}+17+\text{Cat}]^+\) ions from metal-cationized peptides, [29] the general trend in intramolecular nucleophilic substitution reported for the ring-closure of diethyl (\(\omega\)-bromoalkyl)malonate anions in solution is \(5>6>3>7>4>8–10\).[43] The trend reflects competition between enthalpy and entropy changes, with entropy playing a major role in the formation of rings larger than six members. Dissection of the energy of activation of ring-closure reactions would show that \(\Delta H\) for formation of three- and four-membered rings is normally higher than that for the corresponding five- and six-membered rings, whereas \(\Delta S\) is least negative for three-membered rings, is comparable for four-, five- and six-membered rings, and then becomes more negative as the ring size increases above seven. The \(\Delta H\) term reflects developing strain, whereas the large negative \(\Delta S\) of large rings indicates the difficulty of achieving the correct orientation for ring closure. The combination of these two factors is most favorable for a five-membered ring.[44]

For 4AMBz, our hypothesis was that placing the residue at position X in AAXG would eliminate cyclization and ring formation necessary to generate \(b_3^+\) through the oxazolinone mechanism because the rigid aromatic ring would separate the nucleophile and electrophilic site of attack. A previous study by our group demonstrated that the rigid ring prevented transfer of the O and H atoms necessary to generate \([b_{n}+17+\text{Cat}]^+\) ions from metal-cationized peptides.[29] However, as shown in Figure 5.1(e), a minor peak (ca. 5% relative signal intensity) was observed at \(m/z\) 276, which corresponds to the loss of 75 Da (C-terminal G residue) from \([\text{M}+\text{H}]^+\) to form \(b_3^+\). The \(b_3^+\) species generated from AA(4AMBz)G may be an acylium-type ion generated through the mechanism proposed in Scheme 5.3, in which the peptide is initially protonated at the C-terminal amide position. The low intensity of the \(b_3^+\) species for AA(4AMBz)G may reflect a relatively low probability of generating the requisite amide-protonated precursor species, and the fact that the reaction would involve direct amide bond cleavage, which is likely to be a higher energy process than a pathway that involves intramolecular nucleophilic attack. Experimental and theoretical studies of the formation of \(b_n\)-type ions suggest that the acylium-type structure is unstable and would rapidly decompose to \(a_n^+\)-type species.[6] For the \(b_3^+\) species derived from AA(4AMBz)G, the
stability of \( b_3^+ \) with an acylium structure might be enhanced because of the proximity of the aromatic ring and the generation of a highly conjugated system.

![Scheme 5.3. Formation of acylium-type \( b_3^+ \) ion from protonated AA(4AMBz)G.](image)

For the AAXG peptides, the decrease in \( b_3^+ \) signal intensity was accompanied by an increase in the relative abundance of the \( y_n \) ions. The \( y_2^+ \) ion (\( m/z \) 147) was the base peak in the CID spectrum of AA(\( \beta \)A)G (Figure 5.1(b) and remained dominant in the spectra generated from AA(\( \gamma \)Abu)G and AA(Cap)G (\( m/z \) 161 and 189 in Figures 5.1(c) and 5.1(d), respectively). The \( y_3^+ \) species was particularly abundant in the CID spectra generated from AA(Cap)G (\( m/z \) 260 in Figure 5.1(d)) and AA(4AMBz)G (\( m/z \) 280 in Figure 5.1(e)). Several theoretical studies have demonstrated that proton transfer steps between initial structures in which the N-terminal amino group is protonated, and the reactive configurations with protonated amide N-atoms, have internal energies well below threshold values for dissociation.[27, 28, 45, 46] Because of the importance of cyclization to the mechanism, it would seem that adoption of configurations that facilitate proton transfer between amide O atoms would be less favorable if forced to proceed through larger rings such as those that would be formed in peptides that contain larger amino acids such as \( \gamma \)Abu and Cap. 4AMBz, because of the rigid aromatic ring, should interrupt proton transfer by blocking the cyclization. This ‘bottleneck’ to proton migration may limit or prevent the transfer of the mobile proton ‘across’ the amino X in AAXG and increase the probability that the N atom of the A–X amide bond will be the next most favored site of protonation. An alternative hypothesis is that fragmentation may occur through precursor \([M+H]^+\) structures that are initially protonated at amide carbonyl O atoms (see below).
Using the hypothesis that the y ions are generated via the pathway depicted in Scheme 5.2, and are a potential ‘bottleneck’ to proton transfer across the residue X, the increased $y_2^+$ signal intensity for AAXG with $X=\beta A$, $\gamma$Abu, Cap and 4AMBz may be rationalized by considering possible changes in proton affinity of the $b_2$ and $y_2$ products. To the best of our knowledge, the proton affinities of $\gamma$Abu, Cap and 4AMBz have not been reported. However, Table 5.1 contains the proton affinities and gas-phase basicities of amine analogs to the amino acids used in this study.[47] The proton affinities of ethyl-, n-propyl- and n-pentylamine are ~13, 18 and 24 kJ/mol higher than that of methylamine. Assuming that the relative differences of proton affinity for the amino acids mirror those for the analogous amines, the data in Table 5.1 supports the hypothesis that higher proton affinities of the ‘alternative’ amino acids should promote increases in $y_2^+$ ion intensities.

Table 1. Proton affinities and gas-phase basicities of amine analogs to the ‘alternative’ amino acids (in kJ/mol)

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<th>Proton affinity (kJ/mol)</th>
<th>Gas-phase basicity (kJ/mol)</th>
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<td>878</td>
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<td>$\text{C}_6\text{H}_5\text{CH}_2\text{NH}_2$</td>
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<td>879.4</td>
</tr>
</tbody>
</table>

Table 5.1. Proton affinities and gas-phase basicities of amine analogs to the ‘alternative’ amino acids (in kJ/mol)

It is interesting to note the fact that the proton affinity of benzylamine, which is approximately 14 kJ/mol higher than that of methylamine, is comparable with that of ethylamine and ca. 4 and 10 kJ/mol lower than n-propyl- and n-pentylamine, respectively. With the assumption again that the relative differences in proton affinity for the amino acids mirror those for the analogous amines, a lower relative proton affinity of 4AMBz when at the N-terminal end of the dipeptide $y_2$ product would explain the prominence of the $b_2^+$ species (ca. 50% relative intensity) following CID of AA(4AMBz)G (and the lack of the same product ion in the CID spectra generated from AA($\gamma$Abu)G and AA(Cap)G): the lower relative proton affinity of the $y_2^+$ product ion derived from 4AMBzG ($y_2^+$) would make transfer of the proton to the N-terminal product, and formation of $b_2^+$, more favorable.

The $y_3$ ion was most prominent in the CID spectra generated from AA(Cap)G and AA(4AMBz)G (Figures 5.1(d) and 5.1(e), respectively). Computational studies by Paizs and Suhai provide evidence
that the \( y_3^+ \) species from the tetrapeptides used here probably arise along an integrated pathway that involves simultaneous cleavage of C-C and C-N bonds, as depicted in Scheme 5.4, to generate \( y_3^+ \), \( a_1^+ \) and CO.\[48\] Such a pathway was found, for protonated glycine-glycine, to be energetically more favorable than one involving formation of an aziridinone product via cyclization and attack by the N-terminal amino group. It is reasonable to assume that the potential bottleneck to proton transfer suggested earlier would increase the probability that the mobile \( H^+ \) remains on the N-terminal side of the X residue in AAXG and thus increase the tendency to generate \( y_3^+ \).

Scheme 5.4. Formation of \( y_3^+ \) from protonated AA(4AMBz)G through integrated cleavage of C-C and C-N bonds.

5.3.2 CID of AXAG

Figure 5.2 shows CID (MS/MS stage) spectra for the [M+H]\(^+\) ions of A(\( \beta \)A)AG (Figure 5.2(a)), A(\( \gamma \)Abu)AG (Figure 5.2(b)), A(Cap)AG (Figure 5.2(c)), and A(4AMBz)AG (Figure 5.2(d)). Based on the oxazolinone mechanism, the position of the larger amino acids should not have a prohibitive effect on the cyclization and nucleophilic attack required to generate \( b_3 \) or \( y_1 \) products. Instead, the larger amino acids are positioned such that they should interfere with formation of the \( b_2^+ \) and \( y_2^+ \) ions. As is apparent in the spectra shown in Figure 5.2, the most abundant product generated by CID of AXAG, regardless of the amino acid at position X, was the \( b_3^+ \) ion at \( m/z \) 214, 228, 256 and 276 for the peptides containing \( \beta \)A,
γAbu, Cap and 4AMBz, respectively. The $b_2^+$ ion was prominent (above 10% relative intensity) only for CID of the control peptide, AAAG (Figure 5.1(a)) and for A($\beta$A)AG ($m/z$ 143, Figure 5.2(a)). One reasonable explanation for the appearance of the $b_2^+$ species in the CID spectrum of A($\beta$A)AG is that, while kinetically less favorable than for the five-membered oxazolinone ring, a six-membered ring would be less strained and thermodynamically more stable once formed.

Figure 5.2. CID (MS/MS) spectra from protonated peptides in the AXAG series: (a) A($\beta$A)AG, (b) A(γAbu)AG, (c) A(Cap)AG, and (d) A(4AMBz)AG.
For AXAG peptides with γAbu, Cap and 4AMBz, the \( b_2^+ \) and \( y_2^+ \) ions either were not observed, or appeared at relative intensities below 2\%, consistent with the prohibitive effect of forcing nucleophilic attack to occur through larger cyclic intermediates, or blocking cyclization altogether. For A(γAbu)AG (Figure 5.2(b)), the only other notable product ion generated was \( a_3^+ \) at \( m/z \) 200. The \( a_3^+ \) species, a characteristic product for the dissociation of the oxazolinone ring, was observed as a major ion following CID of \( b_3^+ \) generated from A(βA)AG, A(γAbu)AG and A(4AMBz)AG (MS\(^3\) stage, Figures 5.3(a), 5.3(b) and 5.3(d), respectively). CID of \( b_3^+ \) generated from A(γAbu)AG also generated a prominent ion through the loss of 89 Da (\( m/z \) 139, Figure 5.3(b)). The loss of 89 Da to produce an ion at \( m/z \) 167 was the dominant pathway for CID of \( b_3^+ \) derived from A(Cap)AG (Figure 5.3(c)): the \( a_3^+ \) species was observed at only ca. 5\% relative intensity. The product ion at \( m/z \) 167 was also prominent in the CID spectrum (MS/MS stage, Figure 5.2(c)) for protonated A(Cap)AG.
Figure 5.3. CID (MS<sup>3</sup> stage) spectra of b<sub>3</sub><sup>*</sup> derived from protonated AXAG: (a) A(bA)AG, (b) (A(gAbu)AG, (c) A(Cap)AG, and (d) A(4AMBz)AG.

The neutral loss of 89 Da corresponds to the free-acid mass of alanine. CID of A(Cap)AG containing a single A residue labeled with deuterium on the α-carbon methyl group (d<sub>3</sub>A) showed that the neutral loss shifted from 89 to 92 Da when the isotopically labeled A residue was positioned at the N-terminus (i.e. (d<sub>3</sub>A)CapAG). No shift in the mass of the eliminated neutral was observed when the deuterium labeled A residue was positioned adjacent to the C-terminal residue (ACap(d<sub>3</sub>A)G).
isotope labeling results demonstrate that the loss of 89 Da shown in Figure 5.2(c) involves the elimination of the N-terminal amino acid. The mechanism for this dissociation pathway is yet to be resolved, and is being investigated in greater detail using a more extensive series of isotope labeled (\(^{15}\)N and \(^{13}\)C) peptides. For the present study, the most important point is that formation of \(b_2^+\) and \(y_2^+\) is suppressed or eliminated with the AXAG set of peptides when \(X\) is \(\gamma\)Abu, Cap or 4AMBz.

A surface-induced dissociation study of small, model peptides provided evidence that peptides lacking amino acids with basic side groups (such as lysine or arginine) are initially protonated at the N-terminal amine.[49] Proton migration from the initial protonation site (whether the N-terminal amine or a basic side group) to the amide bond cleaved in the dissociation reaction is thought to proceed through a series of intramolecular cyclization and transfer steps between adjacent amide O atoms. Generation of prominent \(b_3^+\) ions from AXAG, especially when \(X=\gamma\)Abu, Cap or 4AMBz, is therefore noteworthy because their formation should be inhibited, at least in part, by changes to the rates and energetics associated with the transfer of the mobile proton to the site of nucleophilic attack due to the presence of the larger amino acids or the aromatic ring within the peptide backbone.

With inhibition or suppression of cyclization because of the alternative amino acids, proton transfer may occur instead through a much larger cyclic intermediate involving the entire peptide. In this case, transfer would be from the amino-terminus directly to the C-terminal amide O atom, then to the amide N atom, to facilitate the nucleophilic attack. Larger, ‘whole peptide’ cyclic intermediates have been invoked to explain the formation of certain \(a_n^+\) product ions from protonated peptides such as YGGFL [50] and AAAA.[41] One would expect, however, that achieving the correct conformation by cyclizing the entire peptide to be kinetically and entropically less favorable than the smaller rings assumed to be formed more locally with the \(\gamma\)Abu and Cap residues.

The prominent \(b_3^+\) species observed for AXAG (and for XAAG described below) may instead suggest that for these peptides the fragmentation reactions proceed through initial isomers of \([M+H]^+\) species that are protonated at the C-terminal amide O atom. This would eliminate the requirement for proton transfer across the larger cyclic intermediates or across the aromatic amino acid residue. Burlet et al. [21] invoked a heterogeneous population, with respect to the location of the charge-bearing proton, of
peptide precursor ions to explain features in the dissociation patterns of protonated peptides containing cysteine and cysteic acid.

With the proton at the site of attack, the cyclization and nucleophilic attack steps to generate $b_3^+$ from the AXAG peptides would occur through the favored five-membered ring reactive configuration of the oxazolinone pathway. As noted earlier, calculations suggest that reactive configurations that feature amine N atom protonation are energetically favored, and the transition state structures within the oxazolinone mechanism have lower relative energies, if the site of protonation is near the C-terminus. During the CID reaction, formation of $b_3^+$ from AXAG where $X=\beta A, \gamma Abu$ or Cap may be enhanced by the fact that the products will have large alkyl substituents on the resulting oxazolinone ring. Recently, a similar enhancement in the yield of nitrile products from the decomposition of $[b_{n-1}+\text{Cat}]^+$ from metal-cationized (Li$^+$, Na$^+$ and Ag$^+$) peptides containing $\beta A, \gamma Abu$, Cap was reported, [51] and the stabilization afforded by the large substituents was reminiscent of the increase in proton affinity and gas-phase basicity of alkylamines reported by Cao and Holmes.[52] For $b_3^+$ generated from A(4AMBz)AG, the highly conjugated nature of the oxazolinone would be expected to confer additional stability. Preliminary studies by our group have revealed that the formation of the $[b_{2-1}+\text{Cat}]^+$ (the metal-cationized analog of the $b_2^+$ ion generated from protonated peptides) dominates the multiple-stage CID spectra of Li$^+$-, Na$^+$- and Ag$^+$-cationized A(4AMBz)AG (S. Osburn, M.J. Van Stipdonk, unpublished results), despite the overwhelming preference for generation of rival $[b_{2+17}+\text{Cat}]^+$ sequence ions for Li$^+$-and Na$^+$-cationized peptides in particular.

Another interesting observation is the lack of prominent $y_3$ and $y_2$ ions in the CID spectra generated from the AXAG set of peptides. The $y$ ions were especially prominent in the CID spectrum of AA(4AMBz)G (Figure 5.1(e)). For AXAG, the absence of $y_2^+$ can be explained by the fact that these species are the complementary products to the $b_2^+$ ions generated via the oxazolinone mechanism, and the $b_n^+/y_n^+$ pathway for this group of peptides appears to be inhibited by the larger cyclic intermediates formed because of the presence of the $\beta A, \gamma Abu$, Cap residues at position $X$.

One would expect the inhibition of proton transfer across the larger amino acids or the 4AMBz residue in the AXAG series to enhance the formation of the $y_3$ ion via the integrated pathway described earlier. In this pathway, proton transfer would occur between the N-terminal amino group and the N-
terminal amide group in a process that is kinetically and energetically feasible based on calculations for GG by Paizs and Suhai.[48] However, the \( y_3^+ \) species was not observed in the CID spectra of any AXAG peptide. One plausible explanation for this observation is that fragmentation is generated from precursors in which the initial protonation site is the C-terminal amide group, which would favor the formation of \( b_3^+ \) over other competing pathways.

5.3.3 CID of XAAG

For the XAAG series of peptides, the \( \beta \)A, \( \gamma \)Abu, Cap and 4AMBz residues positioned at the N-terminus were not expected to interfere with generation of \( b_3, b_2 \) and \( y_2 \) ions by inhibiting the nucleophilic attack step important to the oxazolinone mechanism. Instead, the hypothesis tested was that the 'alternative' residues might interfere with proton transfer from the N-terminal amino group, assuming this was the initial protonation site, to more C-terminal amide groups. Figure 5.4 shows CID (MS/MS stage) spectra for the [M+H]+ ions of (\( \beta \)A)AAG (Figure 5.4(a)), (\( \gamma \)Abu)AAG (Figure 5.4(b)), (Cap)AAG (Figure 5.4(c)), and (4AMBz)AAG (Figure 5.4(d)). For (\( \beta \)A)AAG, the \( b_3^+ \) and \( b_2^+ \) species (m/z 214 and 143, respectively) appeared at nearly identical relative intensities. The \( y_2^+ \), \( a_3^+ \) and \( a_3^{*+} \) ions at m/z 147, 186 and 169, respectively, were also observed. The same product ions were observed in the CID spectrum generated from (\( \gamma \)Abu)AAG. However, the \( b_3^+ \) ion (m/z 228, Figure 5.4(b)) was the base peak, and the \( b_2^+ \) ion (m/z 157, Figure 5.4(b)) appeared at a significantly lower relative intensity than the (\( \beta \)A)AAG analog. While the \( b_3 \) and \( b_2 \) ions (m/z 256 and 185, respectively) were prominent in the CID spectrum generated from (Cap)AAG (Figure 5.4(c)), the most abundant product was an ion at m/z 168. The species at m/z 168, and a less abundant ion at m/z 167, were also generated by CID (MS\(^3\) stage, spectrum not shown) of the \( b_2 \) ion at m/z 185 through the loss of 17 and 18 Da, respectively. The mechanism by which either NH\(_3\) or H\(_2\)O is eliminated is yet to be resolved and is currently under more intensive investigation using isotopically labeled peptides. For CapAAG, the MS\(^3\) results clearly suggest that the m/z 167 and 168 product ions present in Figure 5.4(c) are derived from the decomposition of the \( b_2 \) ion, though rigorous exclusion of a pathway to m/z 167 and 168 directly from [M+H]+, without initial generation of \( b_2^{*+} \), requires a double resonance experiment [53] which is not an option on our unmodified LCQ instrument.
Figure 5.4. CID (MS/MS) spectra from protonated peptides in the XAAG series: (a) (βA)AAG, (b) (γAbu)AAG, (c) (Cap)AAG, and (d) (4AMBz)AAG.

For protonated (4Ambz)AAG (Figure 5.4(d)), the $b_2^+$ species was the base peak, and the $b_3^+$, $a_2^+$, $y_2^+$ and $b_1^+$ ions ($m/z$ 276, 177, 147 and 134, respectively) were all observed at less than 20% relative intensity. The dominant $b_2^+$ species generated from protonated (4AMBz)AAG probably reflects the stabilization of the oxazolinone structure afforded by the aromatic ring substituent. The $b_1^+$ species, which is an uncommon ion in the CID spectra of larger peptides, is probably an acylium ion that is also stabilized by the aromatic ring. A possible route for the production of $b_2^+$ directly from [M+H]$^+$ is shown in
Scheme 5.5, with the caveat that without a double resonance experiment, a pathway involving generation of \( b_2^+ \) by decomposition of energetic \( b_3^+ \) cannot be rigorously excluded. A reaction similar to that shown in Scheme 5.5 probably accounts for the pronounced intensity of \( b_3^+ \) from A(4AMBz)AG (Figure 5.2(d)) discussed earlier.

For the XAAG series of peptides, the alternative amino acids were not positioned within the sequence such that they influence formation of \( b^+ \) and \( y^+ \) ions by inhibiting or suppressing the nucleophilic attack necessary for amide bond cleavage, as was the case for the AXAG and AAXG peptides. Instead, the \( \beta \)A, \( \gamma \)Abu, Cap and 4AMBz amino acids may have an effect on the initial transfer of the ‘mobile’ proton from the amino-terminus to the site of cleavage. The spectra in Figure 5.4 clearly show that, despite the presence of the larger or aromatic amino acids, prominent \( b_2^+ \) and \( b_3^+ \) ions are generated from protonated XAAG.
5.3.4 Influence of multiple 4AMBz residues on $b_n$ and $y_n$ ion formation

To explore further the effect of 4AMBz residues in particular on the formation of $b_n^+$ and $y_n^+$ ions, the CID of a series of pentapeptides containing two 4AMBz residues was investigated. Figure 5.5 shows the spectra generated from the CID of protonated (4AMBz)(4AMBz)AAG (Figure 5.5(a)), (4AMBz)(4AMBz)AG (Figure 5.5(b)) and A(4AMBz)(4AMBz)AG (Figure 5.5(c)). For (4AMBz)(4AMBz)AAG, the sequence ions observed were $b_4^+$ and $b_3^+$ at m/z 409 and 338, respectively. The most abundant product ion was $b_3^+$, consistent with the hypothesis that the formation of a highly conjugated substituted oxazolinone structure will enhance the probability that a given product ion is generated. Consistent with the prohibitive effect of 4AMBz of the cyclization/intramolecular nucleophilic attack, neither $b_1^+$ nor $b_2^+$ was observed.
Figure 5.5. CID (MS/MS) spectra from protonated pentapeptides containing two AMBz residues: (a) (4AMBz)(4AMBz)AAG, (b) (4AMBz)A(4AMBz)AG, and (c) A(4AMBz)(4AMBz)AG.

For (4AMBz)A(4AMBz)AG, CID of [M+H]+ generated \(b_4^+\) (m/z 409), \(b_2^+\) (m/z 205), \(y_3^+\) (m/z 280) and \(a_2^+\) (m/z 177). The lack of a \(b_3^+\) ion in the CID spectrum of (4AMBz)A(4AMBz)AG is consistent with the inhibition of cyclization due to the presence of the aromatic amino acid, while both \(b_2^+\) and \(b_4^+\) represent formation of the presumed stable highly conjugated oxazolinones. For A(4AMBz)(4AMBz)AG, the two aromatic residues occupy adjacent sequence positions. As such, they should inhibit formation of \(b_2^+\) and \(b_3^+\) via the oxazolinone mechanism. As shown in Figure 5.5(c), the product ion generated for CID of A(4AMBz)(4AMBz)AG was \(b_4^+\). The appearance of a minor \(b_3^+\) ion may be due to formation of an acylium product, as described earlier for AA(4AMBz)G.
The results obtained using the larger peptides containing two 4AMBz residues support the general observations described above for the experiments involving the tetrapeptides, in that the position of the aromatic residues influences the favored sequence ions generated. More interesting is the fact that despite the potential suppression of intramolecular proton migration because of the presence and position of two aromatic residues, the \( b_4^+ \) species dominates the CID spectra of \((4AMBlz)A(4AMBlz)AG\) and \(A(4AMBlz)(4AMBlz)AG\). We suggest that this observation supports the hypothesis that fragmentation of the peptides containing 4AMBz, and the peptides bearing the larger amino acids in general, may proceed through precursor \([M+H]^+\) ion structures in which the initial protonation site is an amide carbonyl O atom.

The influence of the aromatic amino acid on the intramolecular migration of protons was also investigated using the peptides \(H(4AMBlz)AG\) and \(HAAG\). With these experiments, the hypothesis tested was that the basic side group of H would increase the probability that the initial protonation site would be the N-terminal residue. Histidine was chosen over lysine and arginine because the relatively small methylimidazole group should limit (compared with the larger alkylamine and alkylguanidinium groups of lysine and arginine, respectively) the potential for proton transfer to amide O atoms of the residues closer to the C-terminus by a cyclization step involving the side chain. The CID spectra for protonated HAAG and \(H(4AMBlz)AG\) are shown in Figures 5.6(a) and 5.6(b), respectively. The dominant sequence ions generated from HAAG were \(b_2^+\) and \(b_3^+\) at \(m/z\) 209 and 280, respectively. The more abundant \(b_2^+\) species can be attributed to a side-group nucleophilic attack characteristic of peptides containing H.[18] The \(b_2^+\) ion was not observed in the CID spectrum for \(H(4AMBlz)AG\), consistent with the inhibition of the side-chain nucleophilic attack step because of the presence of the rigid aromatic ring. Despite the potential sequestering of the proton at the H side chain, and the presence of the 4AMBz to block cyclization to transfer protons across the peptide backbone, the dominant product ion generated from CID of \(H(4AMBlz)AG\) was \(b_5^+\). It is difficult to rationalize such prominent production of \(b_5^+\) from this particular peptide without either invoking larger, ‘whole peptide rings’, or fragmentation reactions that are initiated with isomers of the \([M+H]^+\) ion which feature protonated amide O atoms.
5.4 Conclusions

The influence of the presence and position of a single \( \beta \)A, \( \gamma \)Abu, Cap or 4AMBz residue on the fragmentation patterns was examined, and more specifically the tendency to form \( b_n^- \) and \( y_n^- \)-type product ions, from a group of protonated tetrapeptides with general sequence XAAG, AXAG and AAXG (where X refers to the position of amino acid substitution). The hypothesis tested was that the ‘alternative’ amino acids would influence product ion intensities by inhibiting or suppressing either the nucleophilic attack or key proton transfer steps by forcing the adoption of large cyclic intermediates or blocking cyclization altogether. The observations reported here confirm that the ‘alternative’ amino acid significantly alters the product ion intensities compared with those of a control peptide (AAAG). Specific \( b \) ions were reduced in signal intensity or eliminated completely when \( \beta \)A, \( \gamma \)Abu, Cap or 4AMBz residues were positioned such that they directly affected the intramolecular nucleophilic attack step. In addition, differences in the relative proton affinities of the alternative amino acids appear to influence the competition between complementary \( b_n \) and \( y_n \) ions.
For both the AXAG and the XAAG series of peptides, CID generated prominent b ions despite a potential inhibition or suppression of intramolecular proton migration by the presence of the βA, γAbu, Cap or 4AMBz residues. Prominent bₙ₊⁻*-type ions were also observed for larger peptides that contained two 4AMBz residues, and model tetrapeptides designed to both sequester the migratory proton at the N-terminal residue and block proton transfer using a 4AMBz residue. The prominent appearance of b ions from the AXAG and XAAG peptides is noteworthy, and suggests that proton migration may occur through larger, ‘whole’ peptide cyclic intermediates. An alternative explanation is that fragmentation proceeds through isomers of [M+H]⁺ that are initially protonated at amide O atoms.

Although not discussed here, we found that the XAAG and AXAG peptides did not require significantly higher normalized collision energies to generate the prominent bₙ₊⁻ and yₙ₊⁻ ions, as might be expected if the proton transfer or nucleophilic attack steps have higher activation energies for those peptides that contain the larger amino acid residues. It is clear that the present experimental results would benefit from a molecular dynamics and ab initio theoretical study of the energetic costs for proton transfer through the larger cyclic intermediates, and through larger ‘whole peptide’ rings that facilitate transfer to amide groups remote from the initial protonation site. Also of interest would be the determination of the transition state energies and overall energetics of the intramolecular nucleophilic attack steps involving the larger ring structures, and the influence of the aromatic ring in 4AMBz on the stability of oxazolinones.
LIST OF REFERENCES


CHAPTER 6

STRUCTURE AND REACTIVITY OF a_n AND a_n* PEPTIDE FRAGMENTS INVESTIGATED USING ISOTOPE LABELING, TANDEM MASS SPECTROMETRY, AND DENSITY FUNCTIONAL THEORY CALCULATIONS

6.1 Introduction

The primary method used for peptide and protein identification in proteomics is tandem mass spectrometry (MS/MS or MS²).[1, 2] In most MS/MS experiments, protonated peptides are excited collisionally to induce dissociation (collision-induced-dissociation, CID) and the fragment ion spectrum is used to elucidate peptide sequences. The CID spectra of peptides in proteomics studies are commonly assigned by bioinformatics tools that implement sequencing algorithms and peptide fragmentation models. Regrettably, the existing sequencing programs are based on rather limited fragmentation models that poorly approximate the rich dissociation chemistry of protonated peptides.[3] These limitations often lead to erroneous assignment of peptides and proteins, and the resulting uncertainty in the evaluation of the raw MS/MS data is one of the major limiting factors in large-scale protein identification studies.[4, 5] The incorporation of more detailed peptide fragmentation mechanisms and spectral characteristics into these sequencing algorithms would undoubtedly place MS/MS based sequencing on a much more robust basis.

In general, fragmentation of protonated peptides under low-energy collision conditions involves proton-driven reactions in which amide bonds are cleaved along the peptide backbone and b, y, and a ions [6, 7] are formed. The energetics and kinetics of the necessary proton mobilization (mobile proton model[8, 9]) and amide bond cleavage pathways [3, 10-14] have received significant research interest. On the other hand, much less attention has been devoted to the structure and reactivity of the primary fragments formed by backbone cleavages. According to the recent pathways in competition (PIC) fragmentation model, [3] the thermodynamic properties and the reactivity of these fragments play a significant role in the post-cleavage phase of peptide fragmentation and can be used to understand some fragment intensity relationships.

Early studies demonstrated that the C-terminal y fragments are truncated peptides and their structure and reactions can be explained based on the chemistry of their parent peptides.[3, 14-16] On
the other hand, N-terminal fragments ($b$ and $a$ ions) show new C-terminal functionalities that are introduced by the initial amide bond cleavage. Ions of the $b$ series were originally considered to have the charged acylium group.[2] Harrison and coworkers also suggested the oxazolinone structure,[10, 17] which could explain most of the reactions of $b$ ions. The oxazolinone structure has found support from IR spectroscopy and modeling.[18, 19] In a recent paper, Harrison and coworkers [20] proposed that oxazolinone terminated linear $b$ ions can cyclize by nucleophilic attack of the N-terminal amino group on the charged oxazolinone ring. This reaction leads to a macro-cyclic $b$ isomer,[21] the existence of which has recently been demonstrated by Gaskell and coworkers [22] using ion mobility spectroscopy. Cyclic $b$ ion structures can in principle open up at any amide bond ($b$-type scrambling) and this reaction can lead to linear isomers with scrambled primary structures. Further fragmentation of such ions results in non-direct sequence ions [20] in the MS/MS spectra of protonated peptides.

Other alternative ion structures can be derived if His, Arg, Asp, or Lys occupies the C-terminal position in $b$ ions [23–26]. In these cases the side-chain nucleophiles of the preceding amino acids are responsible for cleavage of the amide bond or reisomerization of the primarily formed oxazolinone ring. To complicate matters even further, recent statistical studies by Zubarev and coworkers [27] suggest that $b_2$ ions of doubly protonated tryptic peptides are unusual and might be protonated diketopiperazines. The structure of $b$ fragments is still heavily debated (see contributions from Harrison, Zubarev, and Gaskell in this focus issue) and further studies are needed to answer a number of open questions in this respect.

Comparatively little is known about the structure and reactivity of $a$ and $a^*$ ions. The former are assumed to have imine groups at their C-terminus and can be formed from $b$ ions by loss of CO [10] or directly from protonated peptides.[28] Under low-energy collision conditions, the first reaction is preferred. Harrison and coworkers have studied formation [10] of $a$ ions from $b$ fragments with assumed oxazolinone structures (study of the formation of $a$ fragments from alternative $b$ structures still awaits). Their kinetic energy release measurements indicate CO loss occurs through a high barrier and the product energy level is below that of the transition structure involved. A mechanism that fits these experimental characteristics was proposed by Paizs and coworkers [29] based on quantum chemical calculations.
Vachet et al. discovered [30] that the a → a*(a-NH₃) pathway involves a rearrangement of the former C-terminal residue to the N-terminus. For example, CID of protonated leu-enkephalin (YGGFL) under some circumstances results in the a₄-NH₃-G fragment at m/z 323 that is formed from the a₄ ion by losing ammonia and one of the formerly internal Gly residues. In recent work,[19] Polfer et al. studied the structure of the a₄ of YGGFL using IR spectroscopy and modeling. This study indicated that the linear a₄ isomer is protonated at the N-terminal amino group and the C-terminal imine is in the trans isomerization state. Furthermore, a cyclic isomer formed by nucleophilic attack of the N-terminal amino group on the charged imine carbon exists. Quantum chemical calculations indicated that the cyclic form is energetically more favored than the linear structures. Ion mobility experiments [22, 31] confirmed that a ions have multiple structures. A recent CID and theoretical study [32] from our laboratories indicated that the CID spectra of a₅ fragments of YAGFL-NH₂ can reasonably be understood by assuming an interplay of b-type scrambling [20] of the corresponding b parent population and a → a* type rearrangements.[30]

The present study reports a combined CID, labeling, and computational study of the structure and reactions of a and a* ions. Multiple-stage CID of unlabeled FGGFL, and versions of the peptide bearing ¹⁵N and α-d₂ isotope labels, were used to examine the fragmentation and in particular the apparent rearrangement of sequence of this model peptide. This specific sequence was chosen as a good compromise between reproduction of past CID results for YGGFL and reasonable cost of synthesis of a series of isotope labeled peptides.

6.2 Experimental

Experimental work was performed at the Department of Chemistry, Wichita State University, Wichita, Kansas. The molecular dynamics simulations and the quantum chemical calculations were carried out at the German Cancer Research Center in Heidelberg. Both the experimental and theoretical strategies are briefly described below.

6.2.1 Mass Spectrometry

All labeled and unlabeled versions of FGGFL were generated by conventional solid-phase synthesis methods [33] using 9-fluorenylmethoxycarbonyl (FMOC) glycine loaded Wang resin (Sigma Chemical, St. Louis, MO) and a custom-built, multiple-reaction vessel peptide synthesis apparatus. FMOC-protected glycine (G), phenylalanine (F), glycine-¹⁵N(H₂¹⁵NCH₂COOH, G-¹⁵N), phenylalanine
$^{15}$N(H$_2^{15}$NCH(CH$_2$C$_6$H$_5$)COOH, F$^{-^{15}}$N) and leucine (L)-loaded Wang resin were purchased from Sigma Chemical and used as received. Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies. Peptide sequence, and in particular the position of isotope labels, was confirmed using multiple-stage CID of Na$^+$ and Ag$^+$ cationized versions (which is an effective approach for sequencing from the C-terminus in the gas-phase [34]). Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (vol:vol) mixture of HPLC grade methanol (Aldrich Chemical, St. Louis, MO) and deionized H$_2$O to produce final concentrations of 10$^{-5}$ to 10$^{-4}$ M.

ESI mass spectra were collected using a Finnigan LCQ-Deca ion-trap mass spectrometer (Finnigan, CA, USA). Peptide solutions were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 5 $\mu$L/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octupole voltage offsets, etc.) were optimized for maximum (M+H)$^+$ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N$_2$ sheath gas flow at 25 units (arbitrary for the Finnigan systems, corresponding to ~0.375 L/min) and the capillary (desolvation) temperature at 200°C. Helium was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The CID studies (MS/MS and MS$^n$) were performed as follows. The (M+H)$^+$ ions were isolated for the initial CID stage (MS/MS) using an isolation width of 0.9 to 1.2 mass to charge ($m/z$) units. Product ions selected for subsequent CID (MS$^n$ experiments) were isolated using widths of 1.0 to 1.3 $m/z$ units. The exact width was chosen empirically and reflected the best compromise between high precursor ion abundance and the isolation of a single isotopic peak. The (mass) normalized collision energy, which defines the amplitude of the r.f. energy applied to the end cap electrodes in the CID experiment, was set between 20% and 25%, (which corresponds roughly to 0.55-0.68 V with the instrument calibration used in this study). The activation Q (as labeled by ThermoFinnigan, used to adjust the $q_z$ value for the precursor ion) was set at 0.30. Subsequent CID stages were performed using similar activation parameter settings. The activation time employed at each CID stage was 30 ms.

6.2.2 Computational Details

The potential energy surface (PES) of the FG_GFIm a*-CO fragment (an ion that features the
CHR=N-CHR'-CO-moiety (denoted by XY_) at the N-terminus) derived from the GGFF\textsubscript{oxa} \textit{b}_4 ion was investigated using the strategy developed recently to deal with protonated peptides.[3, 13] These calculations began with molecular dynamics simulations using the Insight II program (Biosym Technologies, San Diego, CA) in conjunction with the AMBER force field,[35] modified in-house to enable the study of oxygen and nitrogen protonated amide bonds and amide bond cleavage transition structures (TS). During the dynamics calculations we used simulated annealing techniques to produce candidate structures for further refinement, applying full geometry optimization using the AMBER force field. These optimized structures were analyzed by a conformer family search program developed in-house. This program groups optimized structures into families for which the most important characteristic torsion angles of the molecule are similar. The most stable species in the families were then fully optimized at the PM3, HF/3-21G, B3LYP/6-31G(d), and finally at the B3LYP/6-31+G(d,p) levels, and the conformer families were regenerated at each level. The Gaussian set of programs [36] was used for all ab initio and DFT calculations.

For the energetically most preferred structures, we performed frequency calculations at the B3LYP/6-31G(d) level of theory to verify that each was a valid minima with no negative frequencies. Transition states were verified to have one negative eigenvalue via frequency calculations. The relative energies were calculated by correcting the B3LYP/6-31+G(d,p) total energies for zero-point vibrational energy (ZPE) contributions determined from the unscaled B3LYP/6-31G(d) frequencies.

6.3 Results and Discussion

6.3.1 CID and Fragmentation Pathways of FGGFL

Figure 6.1 shows the multiple-stage CID spectra generated from protonated FGGFL. CID (MS/MS stage) of (M + H)\textsuperscript{+} at \textit{m/z} 540 (Figure 6.1a) produced \textit{b}_4 at \textit{m/z} 409, and the (M-H\textsubscript{2}O+H)\textsuperscript{+}, \textit{a}_4 and \textit{y}_3 ions at \textit{m/z} 552, 381, and 336, respectively. The dominant product ion generated by subsequent CID of \textit{b}_4 (MS\textsuperscript{3} stage, Figure 6.1b) was the \textit{a}_4 ion at \textit{m/z} 381. Also observed were peaks at \textit{m/z} 364 and 262. The peak at \textit{m/z} 364 is 17 mass units (u) lower than the \textit{a}_4 ion, and corresponds to formation of the \textit{a}_4\textsuperscript{*}(\textit{a}_4-NH\textsubscript{3}) ion.[37] The peak at \textit{m/z} 262 represents the elimination of 147 u, corresponding to the residue mass of F, to form \textit{b}_3. Isolation and dissociation of \textit{a}_4 at \textit{m/z} 381 (MS\textsuperscript{4} stage, Figure 6.1c) led primarily to the \textit{a}_4\textsuperscript{*} peak at \textit{m/z} 364, the \textit{b}_3 ion at \textit{m/z} 262, with additional peaks at \textit{m/z} 307, 234, and 217.
The peak at m/z 307 corresponds to elimination of 57, or G from $a_4^*$. The peaks at m/z 234 and 217 are formed by elimination of 147 u from $a_4$ and $a_4^*$, respectively. Fragmentation of $a_4^*$ at m/z 364 (MS$^5$ stage, Figure 6.1d) led to formation of the peaks at m/z 336 ($a_4^*$-CO), 307 ($a_4^*$-G), 222 and 217 ($a_4^*$-F).

Figure 6.1. Multiple-stage CID spectra derived from protonated FGGFL: (a) CID (MS/MS) of (M+H)$^+$, (b) CID (MS$^3$) of $b_4$, (c) CID (MS$^4$) of $a_4$, (d) CID (MS$^5$) of $a_4^*$.

In general, the product ions generated in the multiple-stage CID of FGGFL are consistent with those reported by Vachet et al.,[30] and subsequently by Barr and Van Stipdonk [34] for YGGFL. Of particular interest were the prominent $a_4$ and $a_4^*$ peaks, $a_4^*$ created by dissociation of $a_4$ and also the subsequent elimination of 57 and 147 u from $a_4^*$. The latter clearly suggests that the $a_4^*$ ion population consists of two isomers, one losing F and the other eliminating G upon CID. Clearly, this observation is in line with our recent proposal [32] on the interaction of $b$-type scrambling and the $a \rightarrow a^*$ rearrangement.
pathway. The original FGGF$_{oxa}$ $b_4$ ion can undergo cyclization and subsequent ring opening [20] to form other linear structures like GGFF$_{oxa}$, GFFG$_{oxa}$, and FFGG$_{oxa}$. CID of the $b_4$ ion population (Figure 6.1b) leads to the $b_4$-$F$ peak, the $b_4$-$G$ fragment at $m/z$ 352 is not observed. This suggests that the $b_4$ isomers GFFG$_{oxa}$ and FFGG$_{oxa}$ are unlikely to be formed in the scrambling process because their subsequent fragmentation would result in formation of the $b_4$-$G$ fragment. This speculation is supported by computational data on the YGGF$_{oxa}$, GGFY$_{oxa}$, GFYG$_{oxa}$, and FYGG$_{oxa}$ linear $b_4$ isomers derived from YGGFL (B. Paizs, unpublished results). These indicate that GGFY$_{oxa}$ and YGGF$_{oxa}$ are energetically more favored than GFYG$_{oxa}$ and FYGG$_{oxa}$, so ring-opening pathways that place G to the C-terminus of the linear isomers appear to be thermodynamically controlled. Consequently, in the following we assume that the $b_4$ population consists of the FGGF$_{oxa}$ and GGFF$_{oxa}$ linear isomers.

Two major mechanisms have been proposed for the $a \rightarrow a^*$ rearrangement pathway. In their original study, Vachet and Glish suggested [30] that the $a^*_4$ ion was formed via a $S_N 2$-type reaction involving attack by the C-terminal imine group on the N-terminal $\alpha$-carbon forming a cyclic $a^*_4$ ion as the N-terminal NH$_3$ is lost (worked out for the FGGF$_{oxa}$ and GGFF$_{oxa}$ isomers in Scheme 6.1). Subsequent opening of the macro-ring leads to a rearranged sequence and further fragmentation can result in loss of the formerly internal residue. The linear $a^*_n$ ions feature the CHR=N-CHR'-CO-moiety (denoted by XY$_-$) at their N- and an oxazolinone group at their C-terminus, respectively. The CHR=N-CHR'-CO-moiety is likely to be more stable than the C-terminal oxazolinone group, therefore dissociation of $a^*_n$ ions is likely to be dominated by gradual degradation at their C-terminus. Such a fragmentation pathway explains loss of the internal G residues from the $a$ ions like FGGF$_{im}$ and YGGF$_{im}$. 
Scheme 6.1. A reaction pattern for the $b_4$ ion of FGGFL that combines $b$-type scrambling of the FGGF$_{oxa}$ structure to form GGFF$_{oxa}$ and the Vachet-Glish mechanism of the $a \rightarrow a^*$ pathway. This reaction pattern is presented for the $b_4$ fragments derived from ($^{15}$N-F)GGFL. The $^{15}$N isotope is explicitly labeled in the chemical structures while the isotope-labeled phenylalanine residues are noted as F (bold and underlined) in the shorthand notations like FGGF$_{oxa}$; $m/z$ values of the various ions are noted in italics.

An alternative mechanism proposed recently by us [38, 39] is shown on Scheme 6.2. (A similar mechanism was proposed by Uggerud and coworkers for elimination of ammonia from glycinamide.[40])

This mechanism involves cleavage of the C-terminal–CO-NH$^+$ bond by nucleophilic attack of the N-terminal adjacent amide oxygen on the carbonyl carbon. This reaction leads to the next lower $b$ ion that is terminated by an oxazolinone ring at its C-terminus (FGG$_{oxa}$ in Scheme 6.2) and the imine of the former C-terminal residue. Under low-energy CID conditions these two fragments form a proton-bound dimer (PBD), which can undergo proton transfer to the imine monomer and subsequent re-association via nucleophilic attack of the N-terminal amine on the protonated imine creating a new N-terminus. This reaction effectively transfers the formerly C-terminal residue to the N-terminus, thus creating new
functionalities at both termini. Loss of ammonia occurs after proton transfer to the new N-terminal amino group leaving behind the CHR=N-CHR'-CO-moiety.

Scheme 6.2. A reaction pattern for the \( b_4 \) ion of FGGFL that combines \( b \)-type scrambling of the FGGF_{oxa} structure to form GGFF_{oxa} and the PBD mechanism of the \( a \rightarrow a^* \) pathway. This reaction pattern is presented for the \( b_4 \) fragments derived from (\(^{15}\)N-F)GGFL. The \(^{15}\)N isotope is explicitly labeled in the chemical structures while the isotope-labeled phenylalanine residues are noted as F (bold and underlined) in the shorthand notations like FGGF_{oxa}; \( m/z \) values of the various ions are noted in *italics*.

The Vachet-Glish (VG) and the PBD mechanisms of the \( a \rightarrow a^* \) rearrangement pathway lead to final products that are formally the same (Schemes 6.1 and 6.2), via expulsion of ammonia. However, the VG and the PBD mechanisms involve elimination of different nitrogen atoms, the N-terminal or the C-terminal nitrogens, respectively. To decipher which mechanism was responsible for the \( a \rightarrow a^* \) rearrangement pathway, we synthesized isotopically-labeled variants of FGGFL, systematically varying the position of \(^{15}\)N along the backbone. With these peptides, the pattern with respect to retention or elimination of the \(^{15}\)N as part of the departing NH\(_3\) molecule could be observed to help elucidate which
mechanism is responsible for the $a \rightarrow a^*$ rearrangement pathway.

The CID spectra (MS$^4$ stage) generated by isolation and collisional activation of $a_4$ of unlabeled FGGFL, ($^{15}$NF)GGFL, F($^{15}$N-G)GFL, FG($^{15}$N-G)FL, and FGG($^{15}$N-F)L, are shown in Figure 6.2. Note that only portions of those spectra, focused on the $m/z$ range that includes the $a_4^*$ ion, collected using the higher resolution, Zoom scan function of the LCQDeca platform, are displayed. For the unlabeled peptide, and F($^{15}$N-G)GFL and FG($^{15}$NG)FL, formation of $a_4^*$ from $a_4$ involved primarily the elimination of $^{14}$NH$_3$. For example, the $a_4^*$ ion appears at $m/z$ 364 when generated from $a_4$($m/z$ 381) derived from (unlabeled) FGGFL. Likewise, the $a_4^*$ species appears at $m/z$ 365 when generated from $a_4$ ($m/z$ 382) derived from either F($^{15}$N-G)GFL or FG($^{15}$N-G)FL. Conversely, the $a_4^*$ species appears as two peaks separated by 1 u when generated from the $a_4$ ion derived from either ($^{15}$N-F)GGFL or FGG($^{15}$N-F)L.
Figure 6.2. CID (MS$^4$ of a$_4$ derived from unlabeled FGGFL, and the group of $^{15}$N labeled version of the peptide: (a) FGGFL, (b) FGG($^{15}$N-F)L, (c)FG($^{15}$N-G)FL, (d) F($^{15}$N-G)GFL, and (e) ($^{15}$N-F)GGFL.

The observation of split a$_4^*$ peaks can be explained in three ways. The first argument would be that formation of a$_4^*$ involves two distinct pathways (the VG and the PBD mechanisms), one that involves elimination of the N-terminal amino group and one that eliminates the N atom of the initially C-terminal amide group. Another feasible explanation is that two isomers (FGGF$\_oxa$ and GGFF$\_oxa$) are formed in b-type scrambling of the parent b$_4$ population and the position of the labeled residue changes due to the scrambling process. Finally, these two effects can in principle interact leading to four different cases: FGGF$\_oxa$ and GGFF$\_oxa$ can both be present in the mass spectrometer and they can fragment further on the VG and/or the PBD pathway.
The apparent generation of two distinct $a_4^*$ fractions from ($^{15}$N-F)GGFL or FGG($^{15}$N-F)L provided an opportunity to examine each case individually. Figure 6.3 shows the CID spectra (MS$^5$ stage) generated by isolation of $a_4^*$ from FGGFL (Figure 6.3a), FGG($^{15}$N-F)L (Figure 6.3b and c), and ($^{15}$N-F)GGFL (Figure 6.3d and e). The two distinct fragmentation patterns observed in Figure 6.3 clearly suggest that different forms of $a_4^*$ (isolated at m/z 364 and 365) exist, and these ions exhibit very different chemical behavior. In the following we will concentrate on the last two panels of Figure 6.3 and analyze the chemistry of the $a_4^*$ ions derived from ($^{15}$N-F)GGFL.

Figure 6.3. CID spectra (MS$^5$ stage) generated by isolation of $a_4^*$ from (unlabeled) FGGFL: (a), ($^{15}$N-F)GGFL (b) and (c), and FGG($^{15}$N-F)L (d) and (e).
Scheme 6.1 displays the reaction pattern derived by combining the \( b \)-type scrambling and VG \( a \rightarrow a^* \) pathways. \( \text{FGGF}_{\text{oxa}} \) (the \( \times \) shorthand notation is used to note an \( ^{15}\text{N} \) labeled residue here) loses CO and then \( ^{15}\text{NH}_3 \) on the VG pathway to form the \( a^*_4 \) ion with the \( \text{FF}_\text{G}_\text{G}_{\text{oxa}} \) composition at \( m/z \) 364. The \( m/z \) 364 fraction of the \( a^*_4 \) ion population (Figure 6.3e) fragments by losing CO to form \( a^*_4\text{-CO} \) and by eliminating F to form \( m/z \) 217 (formation of the ion at \( m/z \) 222 will be discussed separately in section 3.2). The \( a^*_4 \) ion structure of \( \text{FF}_\text{G}_\text{G}_{\text{oxa}} \) as proposed by the VG mechanism would however eliminate G from its C-terminus to form \( \text{FF}_\text{G}_\text{oxa} \) at \( m/z \) 307. No such ion is observed in Figure 6.3e suggesting that the \( a^*_4 \) ion population derived from \( \text{FGGF}_{\text{oxa}} \) is not formed on the VG \( a \rightarrow a^* \) pathway.

The \( \text{GGFF}_{\text{oxa}} \) \( b_4 \) isomer is formed from \( \text{FGGF}_{\text{oxa}} \) by cyclization and subsequent reopening. Note that the labeled phenylalanine residue is relocated to the C-terminus by this chemistry. \( \text{GGFF}_{\text{oxa}} \) loses CO and then \( ^{14}\text{NH}_3 \) on the VG pathway and forms the \( a^*_4 \) ion with the \( \text{FG}_\text{G}_\text{G}_{\text{oxa}} \) composition at \( m/z \) 365. According to Figure 6.3d, the \( m/z \) 365 fraction of the \( a^*_4 \) ion population fragments to form ions at \( m/z \) 337 and 308. On the other hand, \( \text{FG}_\text{G}_\text{G}_{\text{oxa}} \) is expected to fragment by eliminating the C-terminal F to form \( \text{FG}_\text{G}_{\text{oxa}} \) at \( m/z \) 218. No such ion is observed in Figure 6.3d, suggesting that the \( a^*_4 \) ion population derived from \( \text{GGFF}_{\text{oxa}} \) is not formed on the VG \( a \rightarrow a^* \) pathway.

Scheme 6.2 displays the reaction pattern derived by combining the \( b \)-type scrambling and PBD \( a \rightarrow a^* \) pathways. \( \text{FGGF}_{\text{oxa}} \) loses CO and then \( ^{14}\text{NH}_3 \) on the PBD pathway to form the \( a^*_4 \) ion with the \( \text{FF}_\text{G}_\text{G}_{\text{oxa}} \) composition at \( m/z \) 365. The \( m/z \) 365 fraction of the \( a^*_4 \) ion population fragments (Figure 6.3d) by losing CO to form \( a^*_4\text{-CO} \), \( m/z \) 337, and by eliminating G to form \( m/z \) 308. The \( a^*_4 \) ion structure of \( \text{FF}_\text{G}_\text{G}_{\text{oxa}} \) as proposed by the PBD mechanism for \( m/z \) 365 most likely eliminates G from its C-terminus to form \( \text{FF}_\text{G}_{\text{oxa}} \) at \( m/z \) 308. This ion is observed in Figure 6.3d, clearly suggesting that the \( a^*_4 \) ion population derived from \( \text{FGGF}_{\text{oxa}} \) can be formed on the PBD \( a \rightarrow a^* \) pathway. Elimination of the specific G residue shown in Scheme 6.2 to produce the \( m/z \) 308 product was confirmed using peptides with both \( ^{15}\text{N} \) and (glycine) \( \alpha\text{-d}_2 \) isotope labels (spectra not shown). We found that the loss of 57 u shifted to loss of 59 when \((^{15}\text{N-F})(\alpha\text{-d}_2\text{-G})\text{FL}\) was used as the precursor peptide. The loss remained at 57 u when \((^{15}\text{N-F})(\alpha\text{-d}_2\text{-G})\text{GFL}\) was used as the precursor peptide instead. Therefore, use of the doubly-labeled peptides allows us to conclude that the residue eliminated to create the species at 307 or 308 in Figure 6.3d and e, respectively, is the C-terminal most G.
The GGF\textsubscript{oxa} \textit{b}$_4$ isomer is formed from FGGF\textsubscript{oxa} by \textit{b}-type scrambling and the labeled phenylalanine residue gets relocated to the C-terminus in this rearrangement. The GGF\textsubscript{oxa} \textit{b}$_4$ isomer loses CO and then $^{15}$NH$_3$ on the PBD pathway to form the a$_4^*$ ion with the FG\textsubscript{GF} composition at m/z 364. According to Figure 6.3e, the m/z 364 fraction of the a$_4^*$ ion population fragments to form ions at m/z 336 and 217. The FG\textsubscript{GF} ion structure proposed by the PBD mechanism for m/z 364 most likely eliminates F from its C-terminus to form FG\textsubscript{GF} at m/z 217. This ion is observed in Figure 6.3e clearly suggesting that the a$_4^*$ ion population derived from GGF\textsubscript{oxa} can be formed on the PBD a $\to$ a$^*$ pathway.

The preceding analysis of the fragmentation patterns of the isolated m/z 364 and 365 a$_4^*$ populations of (15N-F)GGFL strongly suggests that the a $\to$ a$^*$ reaction involves the PBD mechanism. The plausibility of the PBD mechanism is also supported by similar analysis of the corresponding FGG(15N-F)L data (Schemes 6.1A and 6.2A in the Appendix), which reach the same conclusion, that the a$_4^*$ ions are formed according to the PBD chemistry. It should be noted however that while supporting the PBD mechanism, the FGG(15N-F)L data are unable to definitively exclude the VG mechanism.

It has been previously suggested that a direct pathway involving concerted loss of G and NH$_3$ form the a$_4$ ion (loss of H$_2$N-CH$_2$CO-NH$_2$) could also be the source of the a$_4^*$-G peak (m/z 307) in Figure 6.1. The main argument behind this proposal is that the CID spectra displayed in Figure 6.1c and d show significantly different intensities for the peak that corresponds to the loss of CO from a$_4$. The very low intensity of the peak at m/z 336 in Figure 6.1c could appear to suggest that there exists a separate pathway to elimination of G and NH$_3$ (nominally C$_2$H$_6$NO) directly from a$_4$, without passing through the a$_4^*$-CO species. Identification of such a direct pathway would best be done using a double-resonance experiment with a short timescale, which we are currently not equipped to perform.

However, this phenomenon can be explained by considering the energetics of the a$_n$ $\to$ a$_n^*$ and a$_n^*$ $\to$ a$_n^*$-X (X is the eliminated amino acid residue) fragmentation channels that is sketched for the present case in Figure 6.1A in the Appendix. Our calculations on a$_5$ of YAGFL [32] suggest that the highest TS on the a $\to$ a$^*$ multistep pathway is around 20 kcal/mol and the relative energy of a$_4^*$ is around 10 kcal/mol. The latter has an oxazolinone C-terminus, the fragmentation energetics of which has recently been studied.[41] These studies indicate that the CO-loss and subsequent imine loss TS’s have very similar threshold energies and the final a$_4^*$-G level is just slightly above those of these TS’s (Figure
6.1A in the Appendix). Consequently, the energetics suggest that the less energized part of the 4a population fragments to form 4a* while the high-energy tail is not likely to be observed as 4a*-CO, these species will fragment further to 4a*-G. On the other hand, the low-energy species of the 4a* population are likely to be observed as 4a*-CO, and only the high-energy tail will fragment further to form 4a*-G. These considerations clearly explain why 4a*-CO is not observed in Figure 6.1c while this is the main peak in Figure 6.1d without introducing a new direct a → a*-X mechanism.

6.3.2 Formation of the m/z 222 Fragment of 4a*

Figure 6.3a shows formation of an ion at m/z 222 that appears in panels 6.3b (shifted by 1 u to m/z 223) and 6.3e as well. Our analysis above suggested that 4a* ions are formed on the PBD a → a* pathway and the m/z 364 fraction of the 4a* population of (15N-F)GGFL is FG_GF_oxa formed from the GGFF_oxa b4 isomer. According to Figure 6.3 FG_GF_oxa loses CO (to form FG_GF_im at m/z 336) and F to form FG_G_oxa at m/z 217 and forms the ion at m/z 222. This ion can be assigned as C6H5-CH2-CH=N+=CH-CH2-C6H5, a structure that contains a fixed charge at the central nitrogen. This ion is formed from FG_GF_im where the two phenylalanines occupy the N- and C-terminal positions (Structure I in Figure 6.4). This suggests that formation of the m/z 222 fragment takes place on a complex rearrangement pathway (Scheme 6.3).
Figure 6.4. Various structures on the FG_GF_{im} PES (relative energies are included for clarity).
Scheme 6.3. A rearrangement pathway for the $\alpha_4^\ast$-CO fragments of ($^{15}$N-F)GGFL shown from the FG_GF$_{im}$ structure.

Nucleophilic attack of the FG glycine carbonyl oxygen of FG_GF$_{im}$ on the C-terminal carbonyl carbon initiates formation of a new oxazolinone ring and cleavage of the C-terminal amide bond. The corresponding transition structure (TS) is shown in Figure 6.4 as Structure II. The relative energy ($E_{Rel}$) of this TS compared with the energetically most favored FG_GF$_{im}$ structure is 18.2 kcal mol$^{-1}$. This reaction produces various proton bound dimers of FG$_{Goxa}$ and the phenylalanine imine. These fragments can re-associate in the PDB via a TS (17.1 kcal mol$^{-1}$ relative energy, Figure 6.4, Structure III) to form F$_{F}$FG$_{ox}$ (isomer of FG_GF$_{im}$, Scheme 6.3, the X_Y_ notation is used for RCH=N-R’CH-). F_F-G-G$_{ox}$ can undergo numerous proton transfer reactions (Structures IV, V, VI, at 5.2, 0.0, and 12.2 kcal mol$^{-1}$ $E_{Rel}$, Figure 6.4).
The last of these (Structure VI) fragments to form the ion at m/z at 222 through a TS at 27.0 kcal mol\(^{-1}\) (Structure VII, Figure 6.4). To the best of our knowledge, this is the first report of such an ion. The mechanism of Scheme 6.3 can easily be adapted to explain formation of the m/z 223 ion in Figure 6.3b from the \(a_4^*\) ion of FGG(\(^{15}\)N-F)L where the \(^{15}\)N is incorporated in the F_F ion (SI Scheme 6.4A). The chemistry shown in Scheme 6.3 effectively transfers the former C-terminal amino acid residue to the N-terminus.

### 6.4 Conclusions

We have described here an investigation of the fragmentation of the model peptide FGGFL that was conducted using extensive \(^{15}\)N and \(^2\)H labeling and multiple-stage tandem mass spectrometry under low-energy CID conditions in a quadrupole ion-trap mass spectrometer. Of particular interest in this study was formation and further dissociation of the \(a_4\) and \(a_4^*\) \((a_4\text{-NH}_3)\) fragments, and apparent rearrangement and scrambling of peptide sequence during multiple-stage CID. With respect to the retention or elimination of the \(^{15}\)N labels, the patterns observed indicate that formation of \(a_4^*\) can occur through different structures and distinct pathways. For \((^{15}\)N-F)GGFL and FGG(\(^{15}\)N-F)L, the \(a_4^*\) ion population appears as two distinct peaks separated by 1 mass unit. These peaks could be separated and fragmented individually providing useful information about the chemical structure of these two fractions of the \(a^*\) population. Careful analysis of the observed fragmentation patterns indicate that \(b\)-type scrambling of the parent \(b_4\) population leads to a mixture of FGGF\(_{oxa}\) and GGFF\(_{oxa}\), and the \(a^*\) ions are formed on the PBD \(a \rightarrow a^*\) pathway. It was also shown that \(a^*-\text{CO}\) ions can rearrange as well, on a pathway that effectively transfers the former C-terminal amino acid residue to the N-terminus.
LIST OF REFERENCES


Scheme 6.1A. A reaction pattern for the $b_d$ ion of FGG($^{15}$N-F)L that combines $b$-type scrambling of the FGG$_{oxa}$ structure to form GGFF$_{oxa}$ and the VG mechanism of the $a \rightarrow a^*$ pathway. The $^{15}$N isotope is explicitly labeled in the chemical structures while the isotope-labeled phenylalanine residue is noted as F (bold and underlined) in the shorthand notations like FGG$_{oxa}$ $m/z$ values of various ions are noted in italics.
Scheme 6.2A. A reaction pattern for the $b_4$ ion of FGG($^{15}$N-F)L that combines $b$-type scrambling of the FGG$_{oxa}$ structure to form GGF$_{oxa}$ and the PBD mechanism of the $a \rightarrow a^*$ pathway. The $^{15}$N isotope is explicitly labeled in the chemical structures while the isotope-labeled amino acid residues are note as **X** (**bold** and **underlined**) in the shorthand notations like FGG$_{oxa}$. $m/z$ values of the various ions are noted in **italics**.
Figure 6.1A. Energetics of the $a_n \rightarrow a_n^*$ and $a_n^* \rightarrow a_n^*-X$ fragmentation channels. Note that barriers are approximate as they are extrapolated from calculations on a very similar system.
CHAPTER 7
GENERAL CONCLUSIONS

7.1 General Conclusions

In chapter two we reported a new fragmentation pathway for the CID of \([b_{3-1}+\text{Cat}]^+\) product ions derived from the model peptide AXAG, where \(X = \beta\text{-alanine, } \gamma\text{-aminobutyric acid, } \epsilon\text{-amino-n-caproic acid, or } 4\text{-aminomethylbenzoic acid.}\) By changing the amino acid to the C-terminal side of the amino acid X, and incorporating \(^{15}\text{N}\) and \(^{13}\text{C}\) labeled residues at the same position, we conclude that the dissociation pathway most likely leads to a metal-cationized nitrile. With respect to the various amino acids at position X, the putative nitrile product becomes more prominent, relative to the conventional \((a_3-1+\text{Cat})^+\) species, in the order \(\beta\text{-alanine} < \gamma\text{-aminobutyric acid} < \epsilon\text{-aminocaproic acid} < 4\text{-aminomethylbenzoic acid.}\) The pathway is not observed for peptides with \(\alpha\text{-amino acids at position X.}\) The product ion is observed most prominently during the CID of \(\text{Li}^+\) and \(\text{Na}^+\) cationized peptides, only to a small extent for \(\text{Ag}^+\) cationized peptides, and not at all from protonated analogues.

In our study of \(\text{Li}^+\) cationized tripeptide methyl esters, extensive isotope labeling \((^2\text{H}, ^{13}\text{C} \text{ and } ^{15}\text{N})\), collision-induced dissociation (CID) and multiple-stage tandem mass spectrometry were used to investigate the elimination of \(\text{H}_2\text{O.}\) The present results corroborate our earlier suggestion that loss of water from lithiated peptides is initiated by a nucleophilic attack from the N-terminal side upon an amide carbonyl carbon atom to form a five-membered ring as an intermediate followed by 1,2-elimination of water. We show that the nucleophilic atom is the oxygen atom of the N-terminal amide group in the fragmentation of \([\text{AcGGGOMe} + \text{Li}]^+\) as well as \([\text{GGGOMe} + \text{Li}]^+.\) However, the subsequent fragmentation is markedly different in the two cases as a result of the absence and presence of a free amino group. In particular, extensive scrambling of protons in the \(\alpha\)-positions of GGGOMe is observed, presumably as a consequence of intervention of the basic amino group.

In a continuation of our isotope labeling studies of fragmentation mechanisms, extensive \(^{13}\text{C}, ^{15}\text{N},\) and \(^2\text{H}\) labeling of tetruglycine was used to investigate the \(b_{3}^+ \rightarrow a_3^+\) reaction during low-energy collision-induced dissociation (CID) in a quadrupole ion-trap mass spectrometer. The patterns observed with respect to the retention of the isotope labels demonstrate that the reaction pathway involves the
elimination of CO and NH$_3$. The labels demonstrate that the reaction pathway involves elimination of CO and NH$_3$. The ammonia molecule includes 2 hydrogen atoms from amide or amino positions, and one from an $\alpha$-carbon position. The loss of NH$_3$ does not involve elimination of the N-terminal amino group but, instead, the N atom of the presumed oxazolinone ring in the b$_3^+$ ion. The CO molecule eliminated is the carbonyl group of the same oxazolinone ring, and the $\alpha$-carbon H atom is transferred from the amino acid adjacent to the oxazolinone ring. Quantum chemical calculations indicate a multistep reaction cascade involving CO loss on the b$_3$ $\rightarrow$ a$_3$ pathway and loss of NH=CH$_2$ from the a$_3$ ion to form b$_2$. In the postreaction complex of b$_2$ and NH=CH$_2$, the latter can be attacked by the N-terminal amino group of the former. The product of this attack, an isomerized a$_3$ ion, can eliminate NH$_3$ from its N-terminus to form a$_3^*$. Calculations suggest that the ammonia and a$_3^*$ species can form various ion-molecule complexes, and NH$_3$ can initiate relay-type mobilization of the oxazolinone H atoms from $\alpha$-carbon positions to form a new oxazolinone isomer. This multiple-step reaction scheme clearly explains the isotope labeling results, including unexpected scrambling of H atoms from $\alpha$-carbon positions.

Our study of the effects of the influence of the presence of a single $\beta$-alanine, $\gamma$-aminobutyric acid, $\varepsilon$-aminocaproic acid or 4-aminomethylbenzoic acid residue on the tendency to form b$_n^+$- and y$_n^+$-type product ions was determined using a group of protonated tetrapeptides with general sequence XAAG, AXAG and AAXG (where X refers to the position of amino acid substitution). The hypothesis tested was that the ‘alternative’ amino acids would influence product ion signal intensities by inhibiting or suppressing either the nucleophilic attack or key proton transfer steps by forcing the adoption of large cyclic intermediates or blocking cyclization altogether. We found that specific b ions are diminished or eliminated completely when $\beta$A, $\gamma$Abu, $\varepsilon$Cap or 4AMBz residues are positioned such that they should interfere with the intramolecular nucleophilic attack step. In addition, differences in the relative proton affinities of the alternative amino acids influence the competition between complementary b$_n$ and y$_n$ ions. For both the AXAG and the XAAG series of peptides, collision-induced dissociation (CID) generated prominent b ions despite potential inhibition or suppression of intramolecular proton migration by the $\beta$A, $\gamma$Abu, $\varepsilon$Cap or 4AMBz residues. The prominent appearance of b ions from the AXAG and XAAG peptide is noteworthy, and suggests either that proton migration occurs through larger, ‘whole’ peptide cyclic
intermediates or that fragmentation proceeds through a population of $[M + H]^+$ isomer that are initially protonated at amide O atoms.

By placing alternative amino acids such as $\beta$A, $\gamma$Abu and Cap at strategic positions (i.e. AAXG to hinder $b_3^*$ formation) many questions have been answered with respect to ring size and ion intensities as well as whether an ion can even be formed depending on basicities of the PBD’s. The alternative amino acid study brought about some interesting questions such as whether sequences containing 4AMBz and histidine could possibly result in macrocyclic ring scrambling.

A study of such macrocyclic ions was undertaken utilizing extensive $^{15}$N labeling where multiple-stage tandem mass spectrometry was used to investigate the fragmentation pathways of the model peptide FGGFL during low-energy collision-induced dissociation within an ion-trap mass spectrometer. Of particular interest was the formation of $a_4$ from $b_4$ and $a_4^*$ ($a_4$-NH$_3$) from $a_4$ ions correspondingly, and apparent rearrangement and scrambling of peptide sequence during CID. It is suggested that the original FGGF$_{oxa}$ b$_4$ structure undergoes b-type scrambling to form GGFF$_{oxa}$. These two isomers fragment further by elimination of CO and $^{14}$NH$_3$ or $^{15}$NH$_3$ to form the corresponding $a_4$ and $a_4^*$ isomers, respectively. For $^{(15N-F)}$GGFL and FGG$^{(15N-F)}$L the $a_4^*$ ion population appears as two distinct peaks separated by 1 mass unit. These two peaks could be separated and fragmented individually in subsequent CID stages to provide a useful tool for the exploration of a potential mechanism along the $a_4 \rightarrow a_4^*$ pathway reported previously in the literature [19, 43]. These mechanisms result in formally the same $a_4^*$ structures but differ in the position of the expelled nitrogen atom. Detailed analysis of the observed fragmentation patterns for the separated light and heavy $a_4^*$ ion fractions of $^{(15N-F)}$GGFL indicates that the mechanism proposed by Cooper et al. is consistent with the experimental findings, while the mechanism proposed by Vachet et al. cannot account for the labeling data. In addition, a new rearrangement pathway is presented for $a_4^*$ -CO ions that effectively transfers the former C-terminal amino acid residue to the N-terminus.

Further DFT studies of the alternative amino acid models XAAG, AXAG, and AAXG are underway and preliminary results indicate that the transition state energies required going from smaller to larger rings sizes to form the observed ions in the spectra increase which is in agreement with the observed trends. The study of d- and l-amino acid substitutions within model peptides has also been performed.
with the results from the experimental aspects still being assembled which will then be published with the finished DFT results to show the effects of functional groups on fragmentation patterns.
REFERENCES
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