Influence of Amino Acid Side Chains on Apparent Selective Opening of Cyclic b_5 Ions

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In this study, the possible influence of acidic, basic, and amide side chains on the opening of a putative macrocyclic *b* ion (b_5^+) intermediate was investigated. Collision induced dissociation (CID) of b_5 ions was studied using a group of hexapeptides in which amino acids with the side chains of interest occupied internal sequence positions. Further experiments were performed with permuted isomers of glutamine (Q) containing peptides to probe for sequence scrambling and whether the specific sequence site of the residues influences opening of the macrocycle. Overall, the trend for (apparent) preferential/selective opening of the cyclic b_5^+, presumably due to the side chain, followed by the loss of the amino acid with active side group is: Q > K > D > N ~ E. (J Am Soc Mass Spectrom 2010, 21, 1028–1036) © 2010 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Within the domain of proteomics, tandem mass spectrometry (MS/MS) and collision-induced dissociation (CID) remain amongst the most important tools used for peptide and protein identification [1, 2]. Sequencing, whether done by comparison to established fragmentation patterns for peptides, or by using bioinformatics approaches that utilize sequencing programs [3], is dependent, in part, on product ion distributions generated by CID. For bioinformatics approaches in particular, prediction of fragmentation patterns often employs rules that are rudimentary and simple. This may lead to invalid assignments of peptide and protein identity [3, 4]. Certainly, a better understanding of fundamental gas-phase peptide fragmentation chemistry and physics would potentially lead to enhanced and more accurate bioinformatics-based MS/MS sequencing.

Using low-energy collision induced dissociation (CID), fragmentation of protonated peptides traditionally involves charge (proton) mediated reactions, with induced cleavage of amide bonds leading to the generation of *b*, *y*, and *a* ions [5, 6]. Development of the mobile proton model [7, 8] of peptide fragmentation, and related amide bond cleavage pathways [9–14], has been focused on the energetics and kinetics of proton mobilization. The more recent pathways in competition (PIC) fragmentation model [14] uses the mobile proton model as a foundation for understanding, but takes into account the structures and reactivity of key reactive configurations and primary fragments as well as transition states and their energies.

There is a great deal of evidence that N-terminal b_n type fragment ions have structures that include, at least in part, C-terminal oxazolone rings [9, 15], and retain much of the primary sequence of the precursor peptide ion. However, more recent experiments [16–19] strongly suggest that a macro-cyclic *b* ion isomer, or intermediate, can arise through cyclization of the linear, oxazolone-terminated *b* ions; this macrocyclic species can then open at different amide bonds to regain a linear, oxazolone terminated structure. One problematic outcome of such a cyclization and reopening process is the associated scrambling of the original primary sequence. This type of pathway is referred to as *b*-type scrambling of peptide fragment ions [16]. Above and beyond the "head to tail" type formation of the macrocycle, there exist several other possible processes that could play a significant role in the scrambling of sequence, the majority of which involve opening of the cyclic *b* ion. For example, peptides that contain acidic, basic, and amide side amino acids feature side chains that can serve as nucleophiles and thus affect bond cleavage. Because of this fact, investigating the fragmentation behavior of these peptides could further our understanding of the reactions that lead to opening of cyclic *b* ions and scrambling of sequence information.

Charge-directed amide bond cleavage mediated by the attack of the nucleophilic groups of amino acid side chains has been well documented and studied in great detail by Paizs and Suhai [14], primarily through use of density functional theory calculations. For peptides with amino acids such as Lys (K) and Arg (R), the basic side chain can influence peptide dissociation by participating in nucleophilic attacks [20–23], providing stabilization through salt bridges [23, 24], or by facilitating proton transfers. Peptides with Asn (N) and Gln (Q) residues, which feature amide side chains, can experience attack by the side-chain amide oxygen on the

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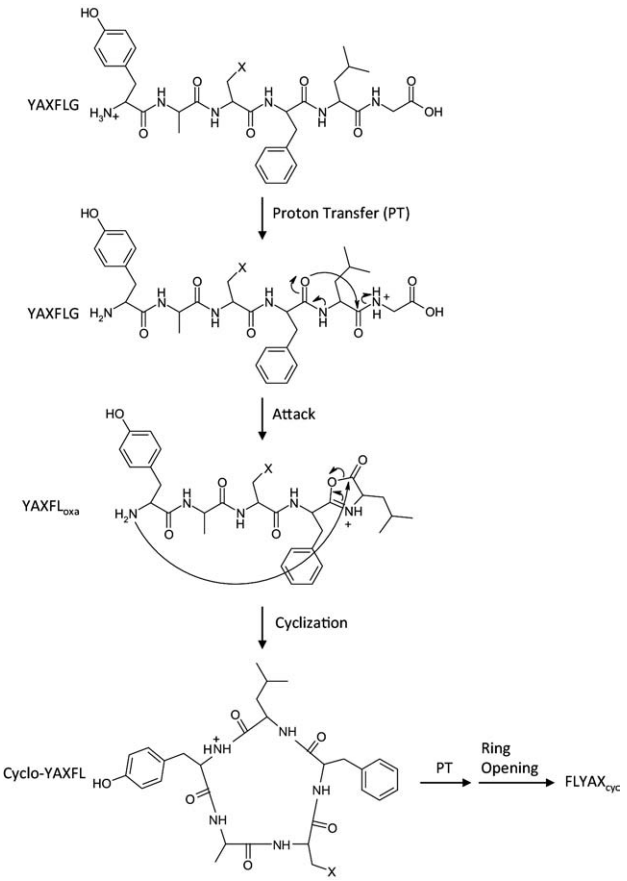
1044-0305/10/$32.00
doi:10.1016/j.jasms.2010.02.011

Published online February 10, 2010
Received November 29, 2009
Revised February 2, 2010
Accepted February 3, 2010

carbon center of the amide bond to form a cyclic isoimide, which is a nonclassic *b* ion [20, 25–27]. Alternatively, another nonclassic *b* ion can arise via attack of the side-chain amide nitrogen on the carbon center of the amide bond leading to a six-membered ring structure (glutarimide) such as described by Jonsson et al. [25] and Farrugia and coworkers [20]. It has also been shown that more complicated cyclic structures can form, such as a five-membered pyroglutamate ring when the Q residue is located on the N-terminus [27, 28]. Lastly, for peptides containing acidic amino acid residues such as Asp (D) and Glu (E), charge-remote peptide fragmentation pathways have been invoked to explain selective cleavages, primarily breaking the amide bond to the C-terminal side of the residues of interest [24, 29–37] through the formation of a cyclic anhydride.

In the present study, we explored the propensity for selective opening of a putative macrocyclic *b* ion or *b* ion intermediate and resulting of sequence scrambling due to possible influence/mediation by side-chain nucleophiles. Our focus here was on CID of b_5 ions derived from model peptides with sequence YAXFLG. The amino acid labeled X, contained either an acidic, basic, or amide side chain. Throughout our experiments, two major observations/criteria were utilized in the identification of cases where scrambling of sequence (via formation of macro-cycle) and selective opening is evident: (1) elimination of internal residues from b_5^+, and (2) high tendency for neutral losses of the X residue with or without additional residues. Scheme 1 illustrates the pathway from the linear peptide AFLP through the formation of the oxazolone containing b_5^+ ion as it undergoes further cyclization and scrambling, ultimately opening selectively because of the amino acid at position X. One or multiple cyclic structures are generated as a result of the selective opening, often times depending on the side chain involved in the pathway.



Scheme 1. General pathway showing the selective opening of the YAXFLG peptide, illustrating formation of the peptide macrocycle and selective opening to the X residue on the C-terminus. The subscript "cyc" indicates that one or multiple cyclic structures are likely present, such as a more conventional linear form and/or other possible unknown macrocyclic structures.

Experimental

Peptide Synthesis and Preparation

All model peptides were prepared using conventional solid-phase synthesis techniques [38] employing 9-fluorenylmethoxycarbonyl (Fmoc) amino acid loaded Wang resin, Fmoc-protected amino acids, and a custom-built, multiple reaction vessel peptide synthesis apparatus. 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, St. Louis, MO, USA) and deionized H_2O to produce final concentrations of 10^{-5}–10^{-4} M.

Mass Spectrometry

Collection of all ESI mass spectra was performed utilizing a Finnigan LCQ-Deca ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA). Each of the peptide solutions was infused into the ESI-MS instrument using the incorporated syringe pump at a flow rate of 5 μL/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole, and octapole 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. Helium was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The $(M + H)^+$ ions were isolated for the initial CID stage (MS/MS) using an isolation width of 1.2 to 1.8 mass to charge (*m/z*) units. Product ions selected for subsequent CID (MS^n experiments) were isolated using widths of 1.2–1.5 *m/z* units. For each stage, the width was chosen empirically to produce the best compromise between high precursor ion intensity and ability to isolate a single isotopic peak. The (mass) normalized collision energy (as defined by Thermo) was set between 2% and 25%, which corresponds roughly to 0.55–0.68 V tickle voltage applied to the end cap electrodes with the current instrument calibration. The

activation Q, which defines the frequency of the applied rf potential, was set at 0.30. In all cases, the activation time employed was 30 ms. Spectra displayed represent the accumulation and averaging of at least 30 isolation, dissociation, and ejection/detection steps.

Results and Discussion

In earlier studies, *b*-type scrambling was evident by not only the elimination of internal amino acid residues of a set of peptides to generate smaller *b*- and *a*-type ions, but also in the similarity of product ion distributions for *b* ions derived from precursor peptides and their permuted isomers [18, 39]. A similar approach was taken in the present study, with the main focus being whether or not *b*-type scrambling occurs with peptides containing amino acids with various nucleophilic side chains. CID (MS/MS) was used to generate b_5^+ from the protonated, model hexapeptides, using the sequence YAXFLG where X = K, E, D, N, Q, and G (as a control). The b_5 product ion was then subjected to a subsequent CID (MS^3 stage) step to probe for generation of cyclic isomers and sequence scrambling (Figure 1).

CID of the b_5 ion from the control peptide, YAGFLG, showed no preference for the loss of the internal G residue (−57 u), as illustrated by Figure 1a. In fact, a product ion corresponding to elimination of G was not observed. Fragmentation of b_5^+ for the control peptide yielded a dominant a_5 ion (*m/z* 524) and product ions at *m/z* 534, 507, 481, and 389. The *m/z* 507 corresponds to the a_5-17 or a_5^* ion (−45 u). The other product ions at *m/z* 534, 481, and 389 are a result of losses of water (−18 *m/z*), A (−71 *m/z*), and Y (−163 *m/z*), respectively. The product ion spectrum generated from b_5^+ of YAGFLG is very similar to those produced by CID of b_5^+ derived from permuted sequence isomers of YAGFL-NH_2 in our previous study [18]. There, the similarity of product distributions, despite the different sequences of the precursor peptides, and the loss of internal residues, was used as evidence for generation of the cyclic intermediate and resulting sequence scrambling. Because no preferential elimination of G was observed from b_5^+ derived from protonated YAGFLG, this sequence position was used for substitution by the amino acid residues of interest in this study.

Analysis of YAKFLG peptide, where the third amino acid residue is lysine, was first used to probe the effect(s) of incorporation of an amino acid with a basic, nucleophilic side group on potential ring-opening and generation of non-direct sequence ions. The CID spectrum of b_5^+ from YAKFLG peptide is shown in Figure 1b. The dominant fragment ion observed is at *m/z* 605 (water loss, −18 u). Other fragment ions include *m/z* 595 (a_5^+), 580, 495, 488, 424, 389, and 345 corresponding to elimination of CO (−28 *m/z*), CH_3CHNH (−43 *m/z*), K (−128 *m/z*), Y imine (−135 *m/z*), KA (−199 *m/z*), YA (−234 *m/z*), and FL + H_2O (−278 *m/z*), respectively. The second most dominant fragment ion observed (at about 40% relative intensity) is an ion that involves loss of the internal lysine residue (*m/z* 495). The K containing peptide macro-cycle ring opening could be explained by the attack of the side chain and formation of a caprolactam ring such as described by Yalcin and Harrison [21], or even by oxazolone ring formation facilitated by the Lys side chain (through proton transfer): both reactions would place the K residue on the C-terminus of the resulting product ion. Loss of (internal) K, alone and with A, strongly suggests that *b*-type scrambling and ring opening is directly influenced by the presence of the basic residue.

The CID spectra for b_5^+ derived from YADFLG and YAEFLG, peptides with amino acids in position X that feature acidic side chains, are shown in Figure 1c and d, respectively. These peptides demonstrate apparent selective ring opening, although to a lesser extent than did those with the basic residue containing YAKFLG. For example, the fragment ion resulting from the loss of the internal D residue in YADFLG was present at only about 20% relative intensity, and at 10% for the fragment ion resulting from the loss of E for YAEFLG. Both neutral losses (D and E) are significantly less prominent than were the analogous elimination of K from b_5^+ derived from YAKFLG (40%). For YAEFLG, the dominant fragment ion observed is *m/z* 606, correlating to a water loss of −18 u. Additional fragment ions include *m/z* 596 (a_5^+), 579 (a_5-17 or a_5^{*+}), 535, 495, 459, 424, 364, and 348, corresponding to elimination of CO (−28 *m/z*), CO + NH_3 (−45 *m/z*), A + H_2O (−89 *m/z*), E (−129 *m/z*), F + H_2O (−165 *m/z*), EA (−200 *m/z*), FL (−260 *m/z*), and LY (−276 *m/z*). respectively. The a_5-17 (or a_5^*) ion appears as the second most dominant fragment ion observed (at about 60% relative intensity). The next major fragment ion is the loss of F + H_2O (*m/z* 459) at about 30% relative intensity. As the phenylalanine residue is not only internal to the peptide but also proximal to the glutamic acid residue, this could indicate that the fragmentation pathway leading to the formation of this ion involves macro-cycle formation and selective opening at the F residue, however, with some influence by the side chain of E, as indicated by the loss of water in addition to F. This structure would potentially have a cyclic structure on the N-terminus of the ion as a result of side-chain attack and ring opening to the F residue. In the CID spectrum of b_5^+ derived from YADFLG (Figure 1d) the dominant fragment ion is a_5^+ (*m/z* 582). This is a notable difference from the glutamic acid containing peptide where the loss of water was the dominant fragment ion. However, the loss of water (*m/z* 592) is the second most abundant fragment ion at about 70% relative intensity.

For b_5^+ from YADLFG, other fragment ions include *m/z* 565 (a_5-17 or a_5^{*+}), 564, 539, 497, 495, 447, 445, 429, 424, 417, and 350 corresponding to elimination of CO + NH_3 (−45 *m/z*), CO + H_2O (−46 *m/z*), A (−71 *m/z*), L (−113 *m/z*), D (−115 *m/z*), Y (−163 *m/z*), F + H_2O (−165 *m/z*), Y + H_2O (−181 *m/z*), DA (−186 *m/z*), F + CO + H_2O (−193 *m/z*), and FL (−260 *m/z*), respectively. The major fragment ion resulting in loss of residue masses is

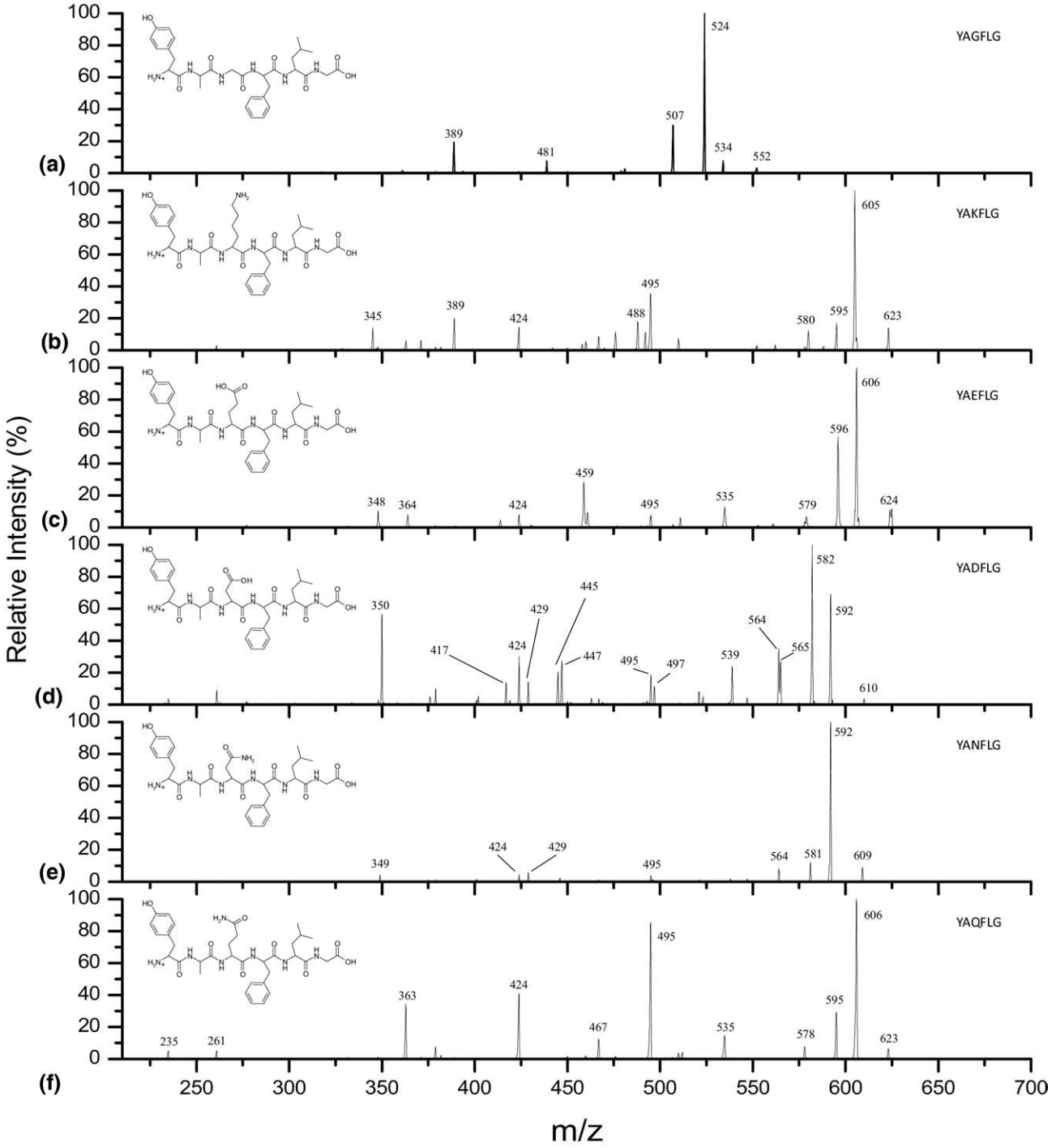


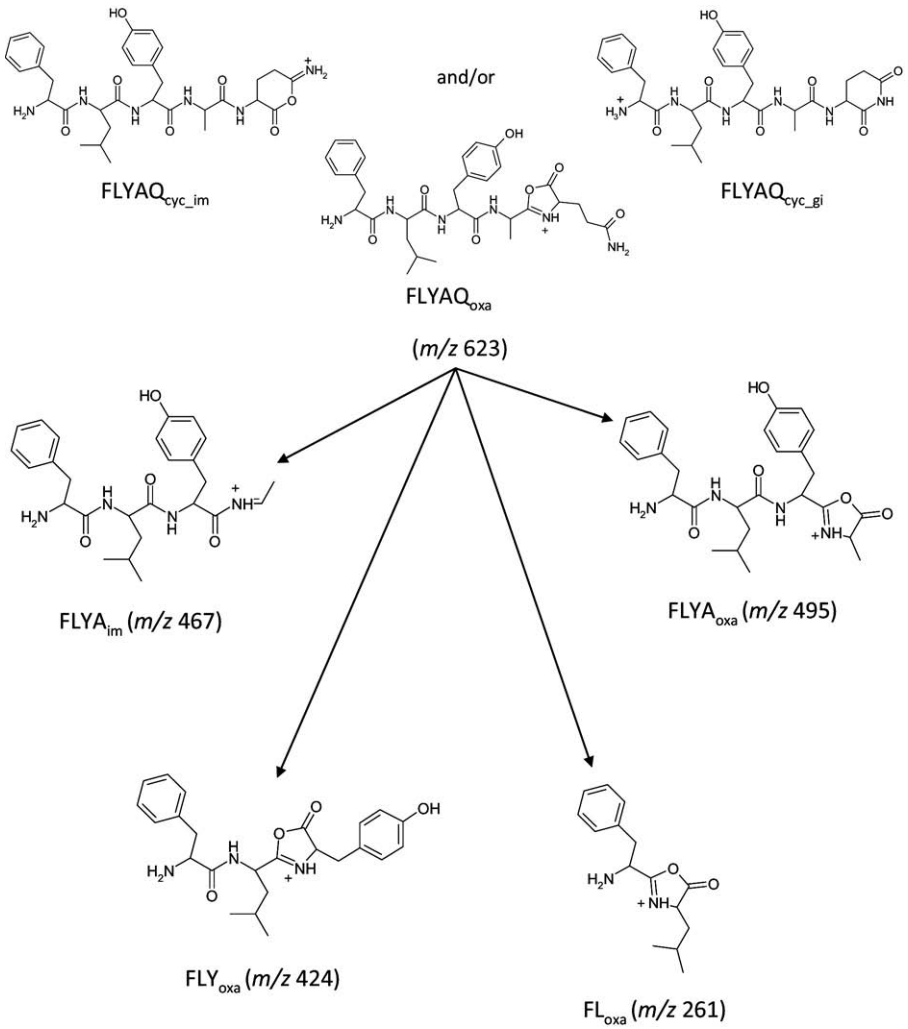
Figure 1. CID of b_5^+ (MS^3 of the model peptide sequence YAXFLG, where X is G (**a**), K (**b**), E (**c**), D (**d**), N (**e**), or Q (**f**). Sequences and structures of each precursor peptide are shown with their respective spectra.

the ion at *m/z* 350, which corresponds to the loss of both phenylalanine and leucine, and appears at about 60% relative intensity. This fragment ion could be explained as a direct sequence fragment (b_3^+), and can be attributed to the "aspartic acid effect" invoked in other, earlier studies [29, 30, 35, 36]. For peptides with acidic residues, attack by the side chain is proposed to result in formation of a cyclic anhydride at the C-terminal end of a product ion, and, in the case of the opening of the putative macro-cycle, this would more than likely be the case.

The CID spectra of b_5^+ derived from YANFLG and YAQFLG, which feature amide (polar) side groups, are shown in Figure 1e and f. These peptides demonstrate

significantly different fragmentation behavior when compared to one another, with the glutamine containing peptide yielding a very dominant loss of the internal Q residue (about 85% relative intensity) and the asparagine containing peptide yielding a very small loss of the internal N residue (5% relative intensity). For YANFLG, the dominant fragment ion observed is *m/z* 592, which is attributed to loss of NH_3 (17 u) and likely corresponds to elimination from the amide side group. Other fragment ions include *m/z* 581 (a_5^+), 564 (a_5-17 or a_5^{*+}), 495, 429, 424, and 349 corresponding to elimination of CO (−28 *m/z*), CO + NH_3 (−45 *m/z*), N (−114 *m/z*), Y + NH_3 (−180 *m/z*), NA (−185 *m/z*), and FL (−260 *m/z*), respectively. The a_5^+ ion appears as the second most dominant fragment ion observed (at about 15% relative intensity). All other fragment ions appear at less than 15% relative intensity, including ions involving losses of internal N and NA: product ions generated by these neutral losses both appear at about 5% relative intensity.

In the CID spectrum of b_5^+ derived from YAQFLG (Figure 1f) the dominant fragment ion is a result of the loss of NH_3 (*m/z* 606). This is similar to the asparagine containing peptide (YANFLG). However, the second most abundant fragment ion produced from b_5^+ derived generated by CID of YAQFLG appears at *m/z* 592; this product is the result of loss of the internal Q residue (at about 85% relative intensity). Other fragment ions include *m/z* 595 (a_5^+), 578 (a_5-17 or a_5^*), 535, 495, 467, 424, 363, 261, and 235 corresponding to elimination of CO (−28 *m/z*), CO + NH_3 (−45 *m/z*), A + NH_3 (−88 *m/z*), Q (−128 *m/z*), Q + CO (−156 *m/z*), AQ (−199 *m/z*), FL (−260*m/z*), YAQ (−362 *m/z*), and QFL (−388 *m/z*), respectively. The fragment ions that appear to be a result of apparent selective ring opening based on the preference for the internal residue losses include *m/z* 535, 495, 467, 424, and 261. Possible structures of the ions at *m/z* 495, 467, 424, and 261 and the potential selectively opened b_5 ions from which they may originate are shown in Scheme **2**.



Scheme 2. Diagram illustrating the four major non-direct sequence ions resulting from either the cyclic isoimide terminated FLYAQ or the oxazolone terminated FLYAQ.

The fragment ions attributed to the losses of FL, YAQ, and QFL could potentially be direct sequence ions; however, upon further evaluation of permuted isomers of YAQFLG where Q is placed in every amino acid position of the b_5 ion, the preference to form these ions is still present (as discussed below). The fragmentation behavior exhibited by both peptides with amide (polar) side group suggests, through the observed losses of the internal residues (N and Q), that there is a significant influence by side chains on opening of the putative cyclic *b* ion or *b* ion intermediate.

As is apparent in the spectra shown in Figure 1, the selective opening and resulting fragmentation pathways of the peptide macro-cycle are dependent on the side-chain group of the amino acid at position X. This is most evident in the preferential, if not dominant, loss of the internal residues placed in position X. Because Q appeared to have the greatest impact on selective ring opening (Figure 1f), this residue was placed within the permuted isomers in each of the five possible sequence positions of the b_5 ion (total of five peptides). The resulting CID spectra from b_5^+ derived from permuted sequence isomers are shown in Figure 2. The CID spectra generated from b_5^+ from each of the permuted sequence isomers contain the same fragment ions with roughly the same relative intensity. Neutral losses, excluding ones necessary to form *a*-type ions, observed in the CID spectra of the isomers include: 17 u (NH_3), 128 u (Q), 260 u (FL), 88 u (A + NH_3), 199 u (QA), 156 u (Q + CO), 362 u (YAQ), and 388 u (QFL). As in our previous study, the similarity of the product ion spectra in Figure 2 provides strong evidence for generation of a macrocyclic b_5 ion. In this case, however, the macrocycle appears to be opened selectively due to an attack by the side-chain group of the amino acid X.

Scheme 3 shows the proposed competing pathways leading to either the conventional or selective ring opening of the YAQFLG peptide. Ring opening to several possible cyclic structures, including several isomeric six membered rings and the oxazolone species, is likely to occur, which is consistent with studies probing the structure of b_2 ions performed with *N*-acyl amino

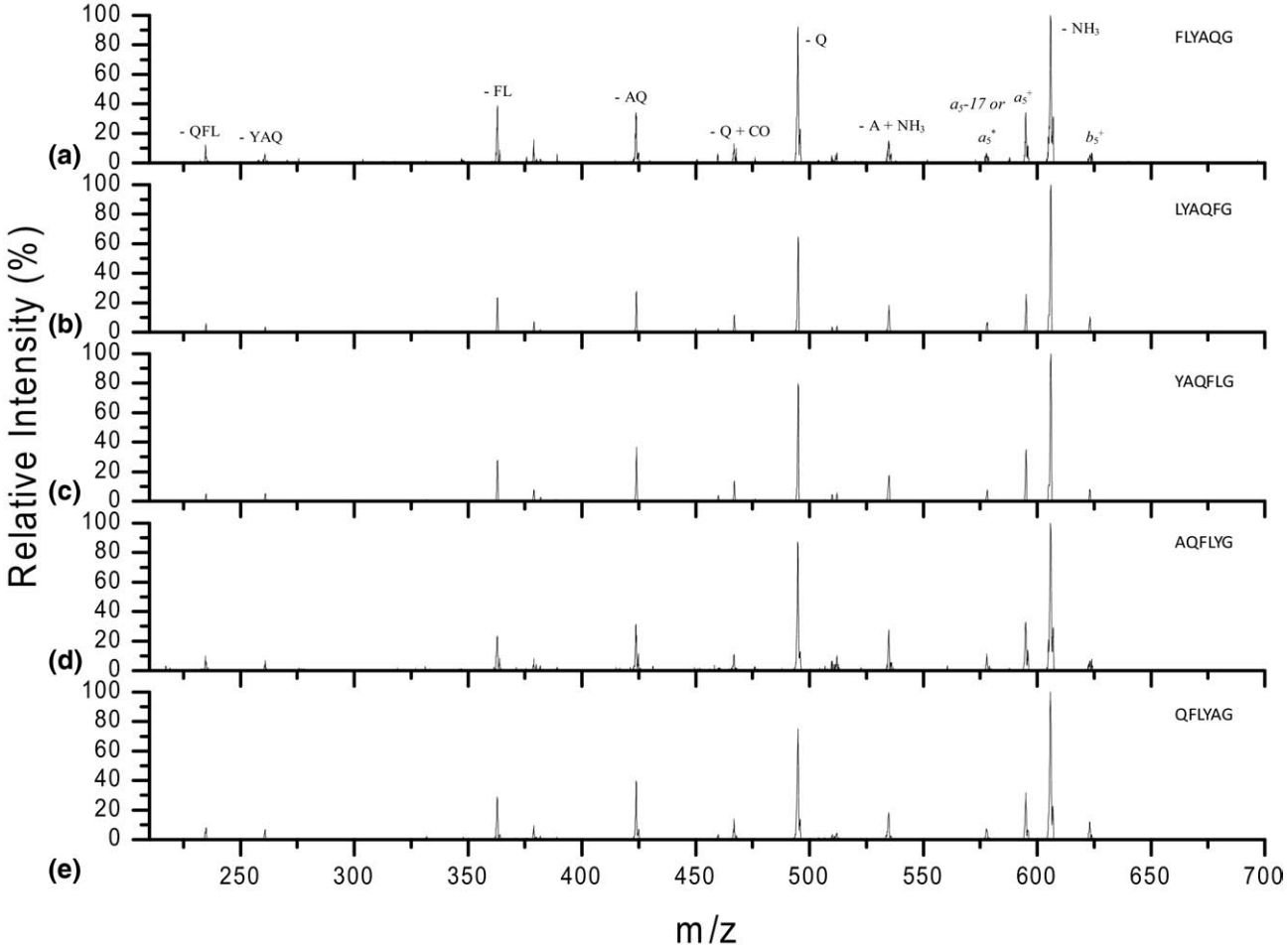
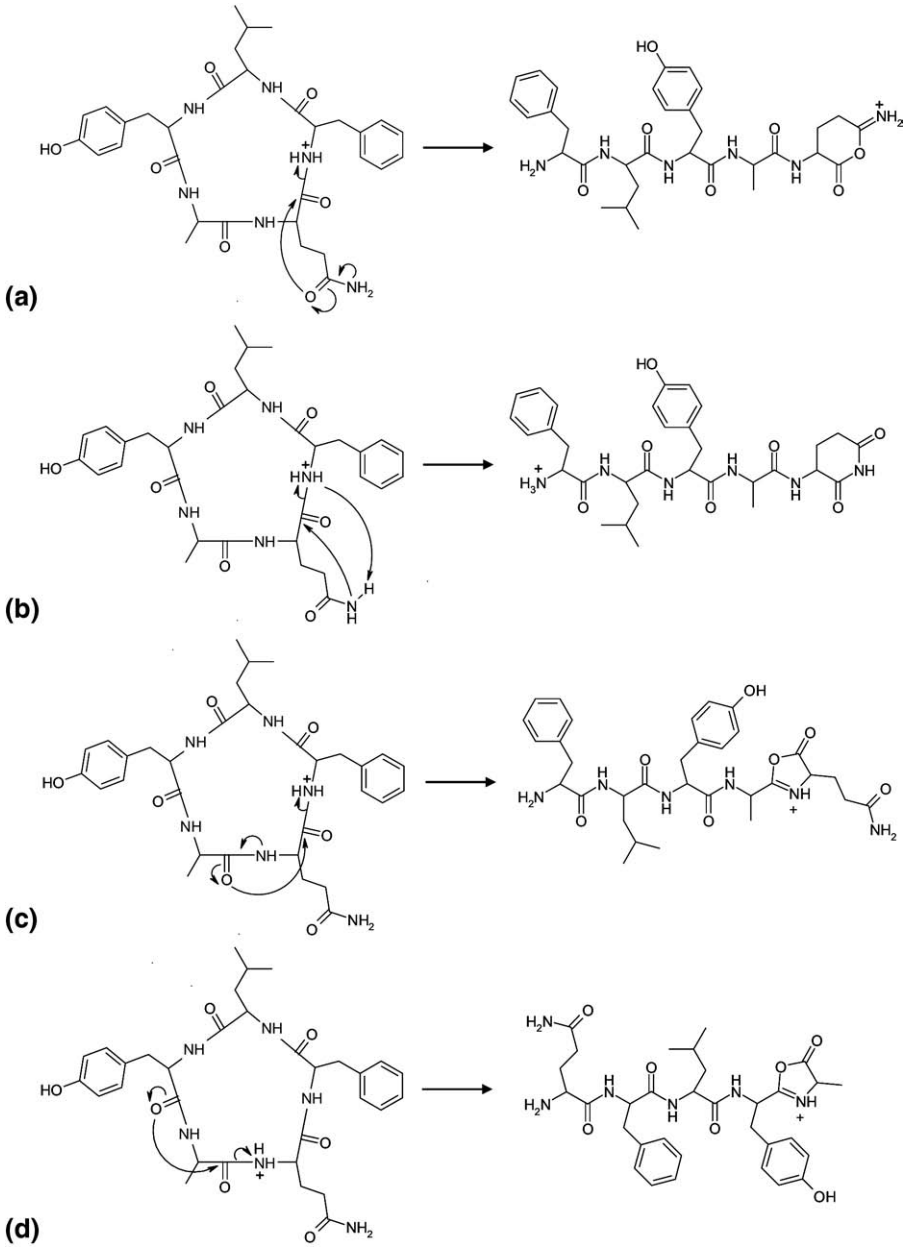


Figure 2. CID spectra of b_5^+ (MS^3 of the permuted isomers of peptide sequence YAQFLG. Sequences were strategically altered to illustrate scrambling and apparent selective ring opening. Each precursor peptide sequence is shown with the respective spectra. The *m/z* values for the relative losses can be seen in Figure 1 for the YAQFLG peptide.



Scheme 3. Possible pathways demonstrating the opening of the YAQFLG peptide macro-cycle. Pathways (**a**) and (**b**) show the selective opening and formation of the cyclic isoimide and glutarimide to the C-terminus to yield the non-classical b_5 ions. Pathway (**c**) illustrates the opening via the conventional oxazolone pathway. Pathway (**d**) shows another classical b_5 pathway, but with the Q residue opening to the N-terminus.

acid esters [20], and are illustrated in Scheme 3**a**, **b**, **c**. Scheme 3**d** shows the conventional opening to the Gln residue on the N-terminus, which is a likely precursor to describe the loss of NH_3 in which formation of pyroglutamine (or another cyclic structure formed via side-chain attack) may occur during the CID of the b_5^+. This suggestion is supported by late stage CID spectra (MS^4 and MS^5). Figure S-1 in the supplemental data, which can be found in the electronic version of this article, shows the MS^4, MS^5, and MS^6 data generated from the b_5 ion. CID of b_5^+–NH_3 (MS^4 yields a dominant fragment ion in which the loss of Ala is evident (*m/z* 535) which would be the C-terminal residue (as illustrated in the proposed structure in Figure S-1), along with several other sequence ions consistent with a N-terminal containing cyclic structure involving Gln. The CID of the *m/z* 535 ion (MS^5 spectrum) demonstrates a major loss of CO (*m/z* 507), but the second most dominant ion involves the loss of Tyr (*m/z* 372), which would be the next amino acid on the C-terminus. It is believed that the pyroglutamine or other cyclic structure on the N-terminus would inhibit further formation

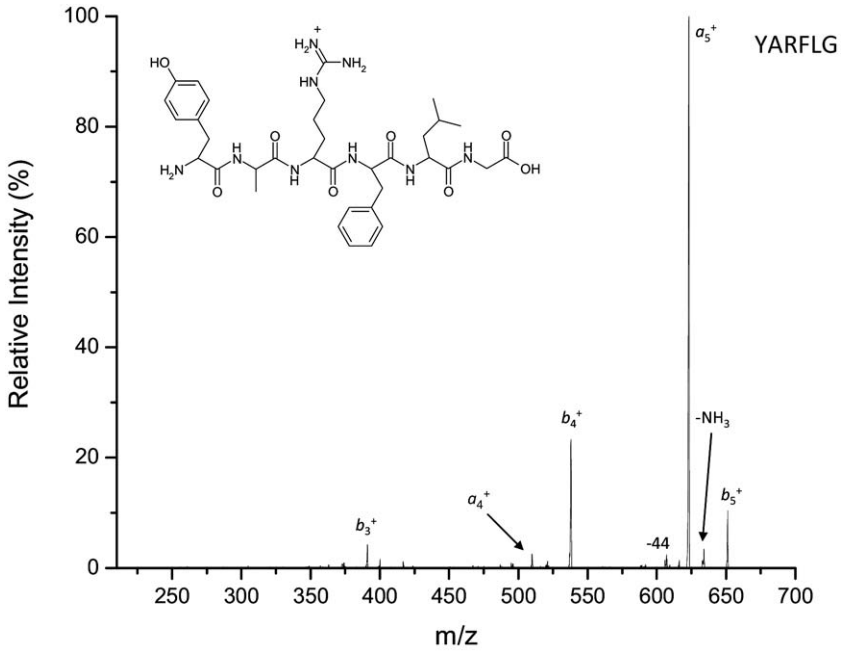


Figure 3. CID spectra of b_5^+ (MS^3 of the peptide sequence YARFLG. The Arg containing peptide was the only peptide of the series that did not demonstrate scrambling and selective ring opening.

of the peptide macrocycle, which could potentially be observed with the b_4 ion. This inhibition, along with the sequential losses of C-terminal residues, support the structure proposed with the MS^4 spectrum.

One additional amino acid was evaluated in the YAXFLG peptide sequence, Arg (R). The CID of the b_5^+ for the peptide YARFLG demonstrated inhibition of sequence scrambling, eliminating any tendency for selective ring opening, and is shown in Figure 3. The dominant fragment ion is the a_5^+, but also observed are direct sequence ions of b_4^+, a_4^+, and b_3^+. In addition, the typical NH_3 loss is observed as well as the loss of 44, which is due to the loss of part of the side chain. It is hypothesized that the basic nature of the R side chain leads to sequestering of the proton that would be needed for a charge directed fragmentation, and instead amide proton mobilization would be required for formation of the fragment ions. Amide proton mobilization has previously been demonstrated through the use of a probe for proton sequestering (pyridine on the N-terminus) for a glycyl-glycine methyl ester [40]. The general influence of R on apparent inhibition of cyclization and sequence scrambling is being investigated further and will be reported at a later date.

Conclusions

To summarize, the objective of this study was to determine whether selective opening of a putative macrocyclic *b* ion or *b* ion intermediate occurs during CID of b_5^+ for peptides containing amino acid residues with basic, acidic, or amide (polar) side chains. Many notable observations were made during the study of the CID of the b_5 ion of the YAXFLG peptides. One observation was the peptides containing the polar (amide) side chains (Q, N) consistently demonstrated dominant losses of ammonia followed by either the residue of interest for the Q containing peptide or formation of the a_5^+ for the N containing peptide. While CID of the b_5 ion of the asparagine (N) containing peptides also produces a_5 and a_5-17 (or a_5^*) ions, these species were less abundant for glutamine containing peptides. The CID of the acid side chain containing peptides (E, D) both led to dominant losses of water, although the aspartic acid containing peptide showed a larger tendency to form the a_5^+ than the b_5–H_2O. Peptides with acid side chains both show losses of the residue of interest, although to a lesser extent than the peptides with amide side chains, particularly the glutamine containing peptide. The lysine (K) containing peptide CID spectrum shows a dominant loss of water, followed by loss of the internal K residue. Overall, the trend for apparent preferential opening of the cyclic b_5 as determined by the overall propensity to lose the internal amino acid residue of interest (X) is Q > K > D > N ~ E and the trend for formation of the a_5 ion is D > E > Q > N ~ K. In sharp contrast to the other nucleophilic side chain containing amino acids, presence of an internal arginine residue appears to completely any peptide macrocycle formation and resulting sequence scrambling. Due to the basicity of the Arg (R) side chain, proton sequestration becomes a likely possibility and reason for the sequence scrambling inhibition.

Acknowledgments

The authors acknowledge support for this work by a grant to M.V.S. from the National Science Foundation (CAREER-0239800).

Funds to purchase the LCQDeca mass spectrometer were provided by Wichita State University and the State of Kansas NSF EPSCoR program. S.O. thanks Wichita State University for support of her undergraduate research. S.M. acknowledges Hospira, Inc. for financial support of his graduate research. The authors also extend sincere thanks to Bela Paizs, Alex Harrison, and Ben Bythell for stimulating and helpful discussions.

Appendix A
Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2010.02.011.

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