A Study of b_1+H_2O and b_1-Ions in the Product Ion Spectra of Dipeptides Containing N-Terminal Basic Amino Acid Residues

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The product ion spectra of approximately 200 dipeptides were acquired under low-energy conditions using a triple quadrupole mass spectrometer. The spectra of dipeptides containing an N-terminal arginine (R), histidine (H), or lysine (K) were observed to yield a $b_1 + H_2O$ ion corresponding to the protonated basic amino acid. This was equivalent to the y_1-ion in the corresponding C-terminal isomer. The formation of a $b_1 + H_2O$ ion was not a significant fragmentation channel in any dipeptides analyzed including those containing a C-terminal basic amino acid unless they also contained an N-terminal basic amino acid. Occurring simultaneously and under equal energy conditions an apparent b_1-ion was formed, which has its corresponding C-terminal equivalent in the y_1-H_2O ion. Energy resolved mass spectrometry (ERMS), deuterium labeling, and accurate mass experiments as well as data reported were used to show the relationships between the b_1+H_2O and b_1-ions in the dipeptides containing an N-terminal basic amino acid and the y_1 and y_1-H_2O ions in the corresponding C-terminal isomers. (J Am Soc Mass Spectrom 2007, 18, 1414–1422) © 2007 American Society for Mass Spectrometry

Collision induced dissociation (CID) in a triple quadrupole mass spectrometer utilizing electrospray ionization (ESI) is a widely used technique for obtaining product ion spectra for the characterization of peptides. The spectrometric information in the product ion spectrum acquired at a single collision energy is limited and can be significantly enhanced utilizing energy resolved mass spectrometry (ERMS), where product ion spectra are acquired at increasingly greater collision cell energies. This results in an increase in the internal energy of the precursor ion, which leads to further dissociation. A plot of relative intensity versus collision energy of selected fragment ions leads to the generation of breakdown curves [1, 2]. Evaluation of these breakdown curves can provide information on fragmentation mechanisms such as distinguishing between competitive and consecutive fragmentation pathways, the stability of product ions, and identification of first and subsequent generation product ions [3–8]; identification of isomers and tautomers [9–11]; and development of library searchable product ion spectra [12, 13]. Since the internal energy of the precursor ion is not rigorously known, breakdown curves provide a qualitative picture of a fragmentation mechanism.

Using these analytical tools, our laboratory analyzed ~200 dipeptides and have made some noteworthy observations in the product ion spectra of dipeptides containing N-terminal arginine (R), histidine (H), and lysine (K) and their C-terminal isomers. We used the single letter abbreviations for amino acids in dipeptides throughout this paper.

The first was the observation of a fragment ion that corresponded to the mass of the protonated molecule $(M + H)^+$ of the basic amino acid. This was observed at m/z = 175 for arginine, m/z = 156 for histidine, and m/z = 147 for lysine. This was true for A_1A_2 isomers RD, RF, HD, HA, HK, HE, HP, HF, KV, KW, KI, KL, KS, KE, KM, KH, KT, KD, KA, and KF. This could only happen by transfer of the C-terminal hydroxyl group to the amide bond of the dipeptide most likely involving a cyclic intermediate leading to a b_1+H_2O ion. This was not observed in the other dipeptides we analyzed or was a minor fragmentation channel.

The second was what appeared to be a loss of H_2O from the above protonated basic amino acids which would lead to an unusually stable b_1-acylium ion. This was observed at m/z = 157 for dipeptides with an arginine residue, m/z = 138 for dipeptides with a histidine residue, and m/z = 129 for dipeptides with a lysine residue. This applied to both the C-terminal and N-terminal isomers. In the case of the C-terminal isomers, loss of H_2O appeared to be from a y_1-ion. However, there were numerous reports in the literature that demonstrated that amino acid acylium ions lose CO exothermically to form the corresponding a_1-immonium ion (H_2N^+=RH) and are not observed [14–18].

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We will present ERMS, accurate mass, deuterium labeling studies, and results from previous studies reported to demonstrate that dipeptides containing an N-terminal basic amino acid undergo a rearrangement involving a cyclic intermediate to form the corresponding protonated molecule $(M+H)^+$ of the respective basic amino acid, and that the b_1+H_2O ion from A_1A_2 dipeptides and the y_1-ion from A_2A_1 dipeptides, which are equivalent, are formed by different mechanisms. We will also show, using ERMS data, that the fragment ions at m/z = 157, m/z = 138, and m/z = 129 originated from the corresponding dipeptide and were not the result of a second generation fragmentation which would be indicative of loss of H_2O from a b_1+H_2O or y_1-ion.

Experimental

Synthesis

The dipeptides were synthesized using a Symphony Multiple Synthesizer (Protein Technologies, Inc., Tucson, AZ) and their identity confirmed by NMR as well as mass spectrometry.

Mass Spectrometry

A TSQ 7000 triple quadrupole mass spectrometer, with an API2 source, was interfaced to a SpectraSystem P4000 gradient pump (Thermo Fisher Scientific, San Jose, CA) for the acquisition of product ion spectra and ERMS data. Samples were diluted in H_2O/acetonitrile (50:50) with 0.1% formic acid (HCOOH) and infused into the mobile phase stream, at 10 μL/min, using a Harvard Model 11 syringe pump (Harvard Apparatus, Inc., Holliston, MA). The mobile phase consisted of 63% acetonitrile (0.1% HCOOH) and 37% H_2O (0.1% HCOOH) and was pumped at a flow rate of 0.2 mL/min. The mass spectrometer was operated in the positive ion mode using electrospray ionization (ESI). The spray voltage was 4.5 kV and the capillary temperature was 250 °C. The sheath gas was set to 50 psi and the auxiliary gas to 0 units. The electron multiplier was optimized for a gain of 300,000 (1330 V).

Product ion spectra were obtained by using Q1 to focus selected precursor ions $(M+H)^+$ into the collision cell (Q2) which was maintained at 2 mTorr with argon (Ar). The offset voltage for Q2 was −22 V, and Q3 was scanned from 25 to 400 u in 1 s. Resolution for Q1 and Q3, which were tuned and calibrated with MRFA/apomyoglobin, was maintained at 0.7 FWHM.

Energy-resolved mass spectrometric (ERMS) data were acquired while infusing solutions of the dipeptides using the conditions described above for acquiring single energy product ion spectra. An 18-segment scan sequence was set up in which the collision cell offset voltage was incremented from 7 to 40 V in 3 V steps and then 40 to 70 V in 5 V steps. Each segment was scanned from 25 to 400 u in 1 s for a 30 s duration. Spectra were averaged over each segment and used to generate breakdown curves for selected ions that were plotted versus the most intense ion in each segment.

Accurate mass measurements for RF and KV were made using a TSQ Quantum Ultra AM (Thermo Fisher Scientific, San Jose, CA) in the internal lock mass mode. Auto tune, calibration, and high-resolution tuning were accomplished using polytyrosine. Ammoniated polyethylene glycol homologues (PEG) were used for accurate mass calibration. Product ions of PEG homologue $[HO(CH_2CH_2O)_4NH_5]^+$ at m/z = 195.1227 $(C_8H_{19}O_5)^+$, and m/z = 133.0859 $(C_6H_{13}O_3)^+$ were used as internal lock masses. Additional instrument conditions: scan width = 0.8 m/z, scan rate = 100 u/s, collision energy = 16 V (0.5 mTorr argon), vaporizer temperature = 300 °C, capillary temperature = 270 °C, spray voltage = 3000 V, sheath gas = 50, and auxiliary gas = 10, Q1 resolution = 0.7 FWHM, Q3 resolution = 0.1 FWHM, global mass tolerance = 0.1 u, and chromatography filter = 5 s. Loop injections were made into a mobile phase stream consisting of 63% acetonitrile (0.1% HCOOH) + 37% H_2O (0.1% HCOOH).

Accurate mass measurements for HF were made using the above instrument conditions except tyrosine internal lock masses at m/z = 165.0546 $(C_9H_9O_3)^+$ and 136.0757 $(C_8H_{10}N_1O_1)^+$ were used.

Results and Discussion

Rearrangement of Dipeptides Containing N-Terminal Basic Amino Acids

The product ion spectra of dipeptides containing a basic (R, H, or K) N-terminal amino acid contain fragment ions corresponding to the protonated basic amino acids. This is illustrated in the product ion spectra for dipeptides RF, HF, and KV (Figure 1). The fragment ions corresponding to the protonated molecules were observed at m/z = 175, m/z = 156, and m/z = 147, respectively, for arginine, histidine, and lysine. The product ion spectra for the deuterated analogues of RF, HF, and KV showed deuterated fragment ions at m/z = 183, m/z = 161, and m/z = 153, corresponding to the deuterated analogues of the respective basic amino acids. This necessarily involved the transfer of the C-terminal hydroxyl group to the amide bond of the dipeptide. Rearrangement to an oxazolidin-5-1 intermediate was proposed by Thorne et al. [19] for bradykinin, angiotensins, and related analogues. They utilized ^{18}O-labeling experiments to show that the B'_{n-1}+OH fragment ion in the spectra of the peptides they studied incorporated a C-terminal oxygen forming essentially a truncated peptide minus the C-terminal amino acid. In Thorne's examples, the greatest tendency to form a B'_{n-1}+OH ion was when the peptide contained a C-terminal arginine (peptides containing other basic amino acid residues were not investigated). For the dipeptides we studied, a C-terminal basic amino acid suppressed rearrangement to formation of the N-termi-

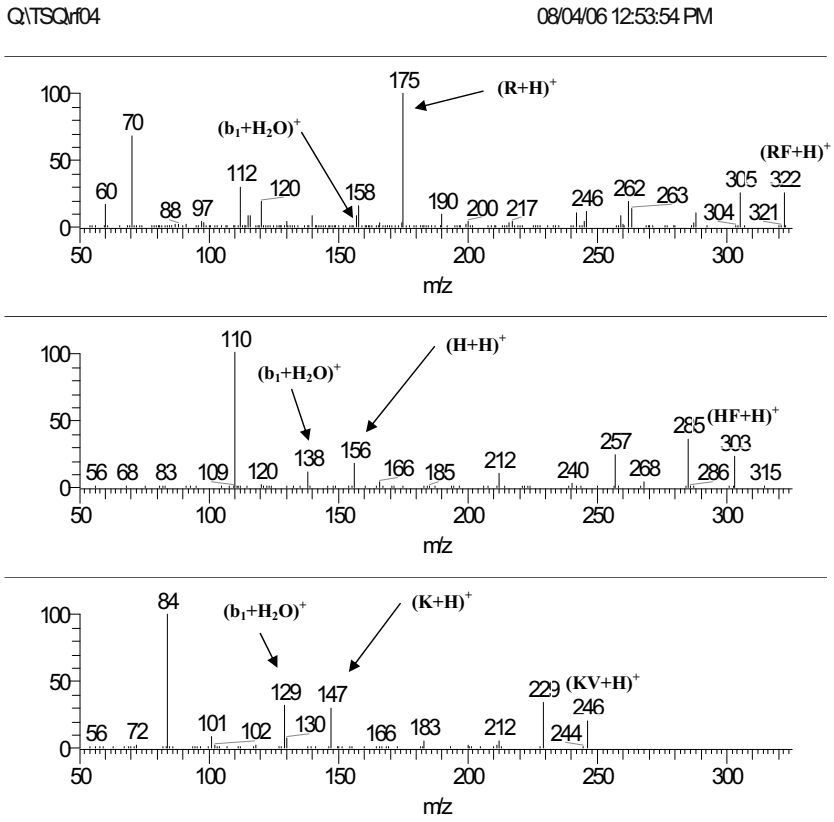


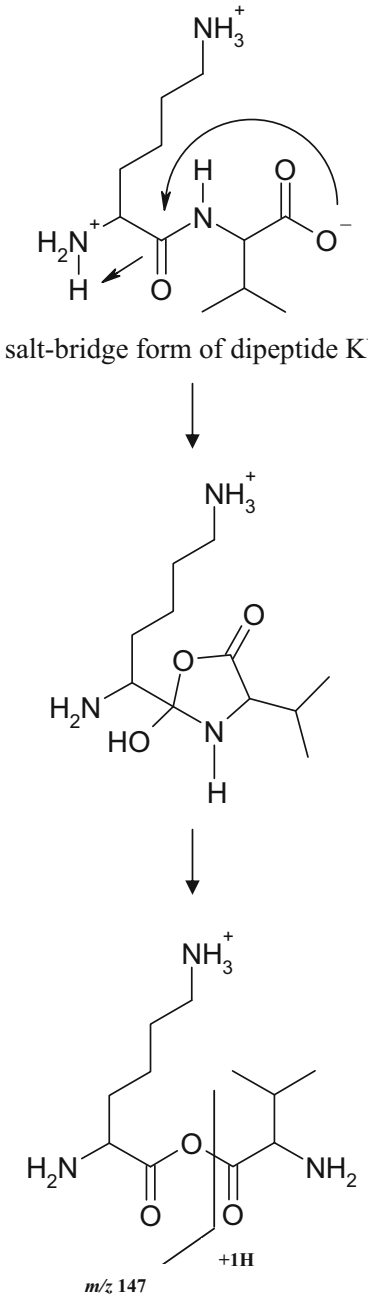
Figure 1. Product ion spectra of RF (top), HF (middle), and KV (bottom).

nal amino acid unless, of course, the N-terminal amino acid was also basic as in the case of HK and KH.

Gonzalez et al. [20] studied the $(b_{n-1} + H_2O)/b_{n-1}$ ratio for a series of arginine containing petapeptides with arginine at positions $n = 1$ through $n = 5$. This ratio increased correspondingly from 6 to 9 and then collapsed to zero for the pentapeptide containing arginine at the C-terminal. Salt bridge formation between the protonated guanidinyl side-chain of arginine and the peptide backbone with subsequent rearrangement to form the $b_{n-1} + H_2O$ ion was used to explain these observations in product ion spectra resulting from metastable decompositions and CID. The ratio was less if lysine was substituted for arginine at $n = 4$ of the pentapeptides because it has a lower gas-phase basicity.

Although Thorne and Gonzalez proposed similar mechanisms, neither introduced the idea of the mixed anhydride intermediate proposed concurrently by Feng and Farrugia. Feng et al. [21] concluded that the cyclic oxazolidin-5-one intermediate proposed by Thorne had an activation barrier that was too high to achieve under low-energy CID conditions. They studied the fragmentation patterns for a series of lithiated dipeptide pairs (A_1A_2 and A_2A_1) containing hydrophobic and uncharged hydrophilic amino acids under low-energy conditions using an ion trap mass spectrometer and found that the intensity of the $b_1 + OH + Li$ ion in A_1A_2 dipeptides was equal to the intensity of the $y_1 + H + Li$ ion in A_2A_1 dipeptides even though the spectra were not always identical. This led them to propose a mechanism for the fragmentation of metal cationized dipeptides that was initiated by formation of a salt bridge leading to a mixed anhydride intermediate.

Farrugia et al. [22] reported that their product ion spectra for protonated GR and RG, acquired using a quadrupole ion trap mass spectrometer, were identical. Relating gas-phase pathways to condensed phase pathways led them to also propose a modification of Thorne's mechanism involving the concept of a salt bridge. This resulted in the formation of a mixed anhydride intermediate that was common to both GR and RG, which then fragmented to yield identical spectra. Our product ion spectra for KV and VK showed the same fragment ions but the spectra were easily distinguishable because they appeared at distinctly different intensities. This was always the case for the C-terminal isomers (A_2A_1) of the dipeptides containing N-terminal basic amino acids (A_1A_2) that we studied, most of which were available to us. This is in agreement with recently published work by Wee et al. [23] who also used an ion trap mass spectrometer. They argued that variation in intensity in the spectra was due to incomplete isomerization to Farrugia's mixed anhydride. Some of these differences in the product ion spectra may be attributed to the longer time scale for rearrangements to occur and lower energy of the ion trap mass spectrometer compared with beam type instruments like the triple quadrupole mass spectrom-



Scheme 1

eter we used. It is also possible that there were two different mechanisms being observed—one for the formation of protonated lysine from KV and another for the formation of protonated lysine from VK.

Scheme **1** depicts the fragmentation of KV to form protonated lysine based on the mechanism proposed by Farrugia. The clean shifts from the fragment ion at *m/z* = 147, for protonated lysine in KV, to *m/z* = 153, for deuterated lysine in deuterated KV, indicated that there was complete H/D exchange as predicted by the mobile proton theory [24–26]. Dipeptide KV is shown in a salt-bridge form in Scheme **1** with the side-chain protonated. Csonka et al. [24] have demonstrated that the favored sites for protonation of lysine containing dipeptides are at the α-amino or ε-amino groups. They have also demonstrated that under low-energy conditions and within the time frame of a typical mass spectrometer the proton can reside on the carbonyl oxygen or nitrogen of the amide bond with protonation at the oxygen thermodynamically favored. This makes the carbonyl carbon electrophilic and subject to nucleophilic attack by the carboxyl OH. Rearrangement to the mixed anhydride follows from which both $b_1 + H_2O$ and y_1-ions can be formed in equal or varying amounts from a single mechanism.

The ERMS data for dipeptides KV and VK showed that as $(M + H)^+$ decreased in intensity the fragment ion at *m/z* = 147, which represents protonated lysine, increased, and was a first generation product ion for both dipeptides (Figure 2). However, the appearance potential of *m/z* = 147 was lower in VK- a y_1-ion (12 V) than it was in KV- a $b_1 + H_2O$ ion (15 V). This observation suggested the possibility of two mechanisms for the formation of protonated lysine from VK and KV.

VK did not contain a fragment ion at *m/z* = 118 corresponding to protonated valine under low-energy CID conditions. The reason for this can be rationalized by analyzing the ERMS data for VK (Figure 3). Loss of H_2O from $(M + H)^+$, at *m/z* = 228, of the dipeptide is a low-energy process and typically does not involve backbone cleavages [27]. The onset for loss of H_2O in HF and FH occurred at about 10V for both dipeptides (Figure 4). Sequential losses of ammonia dominated the high mass region of arginine containing dipeptides. It was also interesting to note the loss of NH_3 was primarily a second generation product in VK (246 > 147 > 130 in Figure 3) and a first generation product in the product ion spectra of KV (246 > 229 in Figure 5). These data also suggested the possibility of more than one competing mechanism as a possible alternative to fragmentation from a common intermediate.

The a_1-y_1 path proposed by Paizs et al. [28, 29] is one such mechanism. This mechanism involves cleavage of the dipeptide backbone and is charge directed, as are most backbone cleavages [18, 25, 30–32]. The fragmentation of VK to form protonated lysine by this mechanism is depicted in Scheme **2**. Here protonation of the amide nitrogen is necessary to yield the hydrogen bonded imine-lysine complex with a departing molecule of CO. Although thermodynamically the least favored site of protonation, it is necessary for backbone cleavages. Hydrogen bonding to the α-amino nitrogen and the carboxyl group will stabilize the charge on the amide nitrogen while weakening this bond. Imine basicity parallels amino acid basicity and the departing lysine, in this case, protonates to produce a y_1-ion. If the imine has a higher proton affinity than the departing acid, the

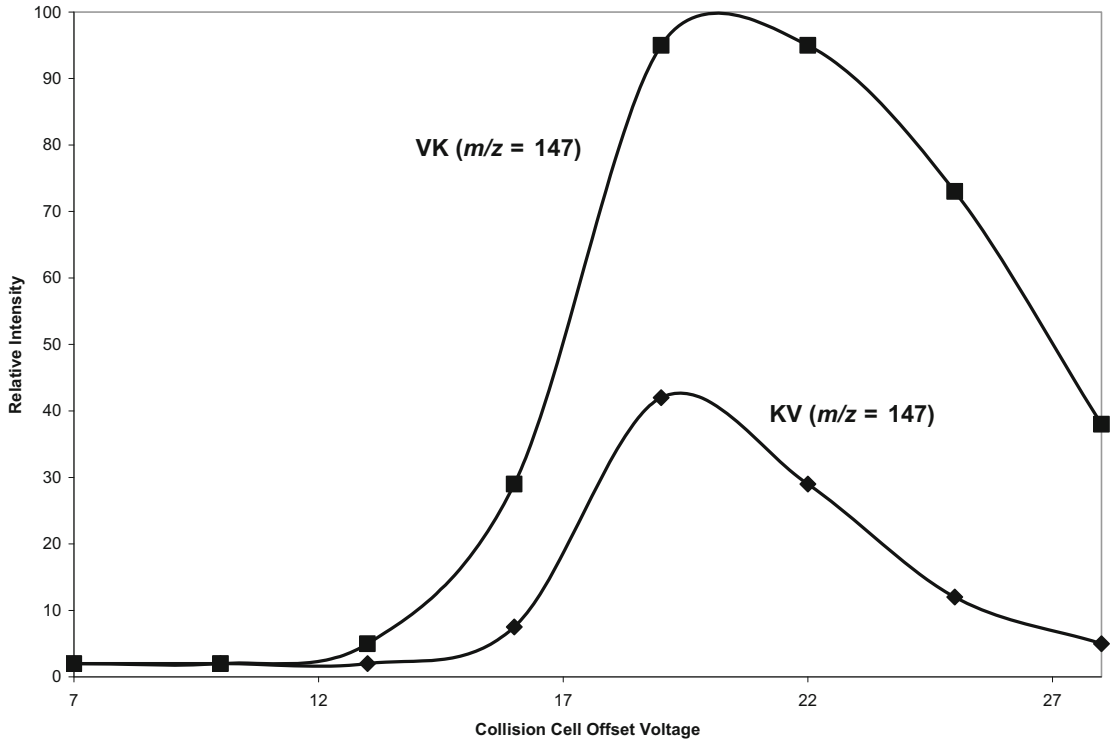


Figure 2. Breakdown curves for 246 > 147 transition in KV and VK.

corresponding immonium ion is formed instead of or in addition to the y_1 ion. The ERMS data for VK and KV (Figure 2) showed that the a_1-y_1 pathway is lower energy than the mixed anhydride pathway under our experimental conditions. This distinction was also true for HF and FH but was less obvious for RD and DR. The appearance potential for protonated arginine in RD and DR were both about the same as proton-

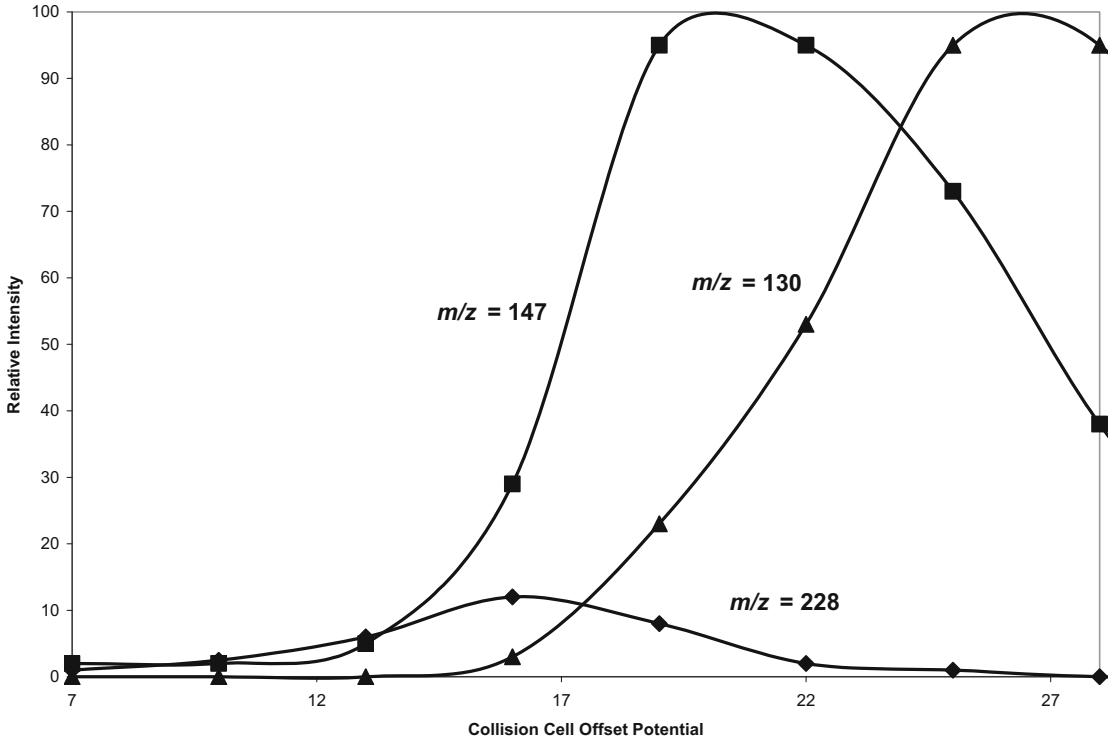


Figure 3. Breakdown curves for VK showing loss of NH_3 (m/z = 130) from $(K+H)^+$ at m/z = 147 and loss of H_2O (m/z = 228) from protonated KV.

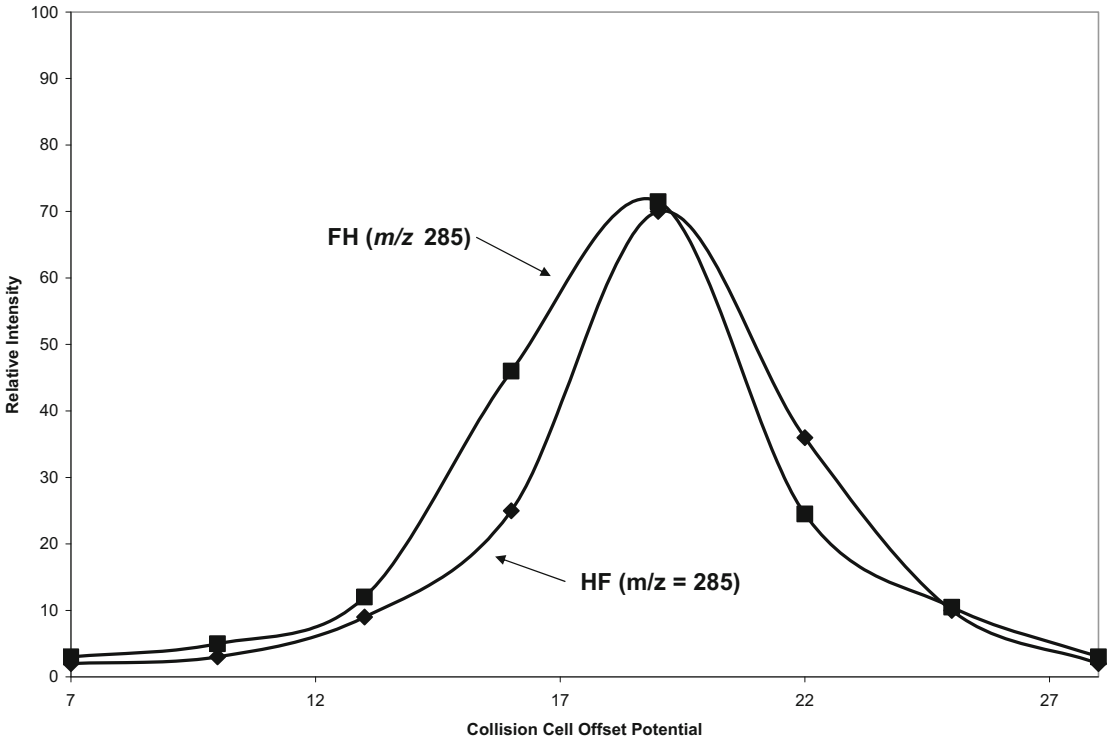


Figure 4. Breakdown curves for 303 > 285 transitions (loss of H_2O) in HF and FH.

ated lysine in KV. This was unexpected since the highly resonance-stabilized protonated guanidinyl group, with additional stabilization by hydrogen bonds to the α-amino and carbonyl groups that effectively sequesters the proton, should fragment at higher°energy°[33].

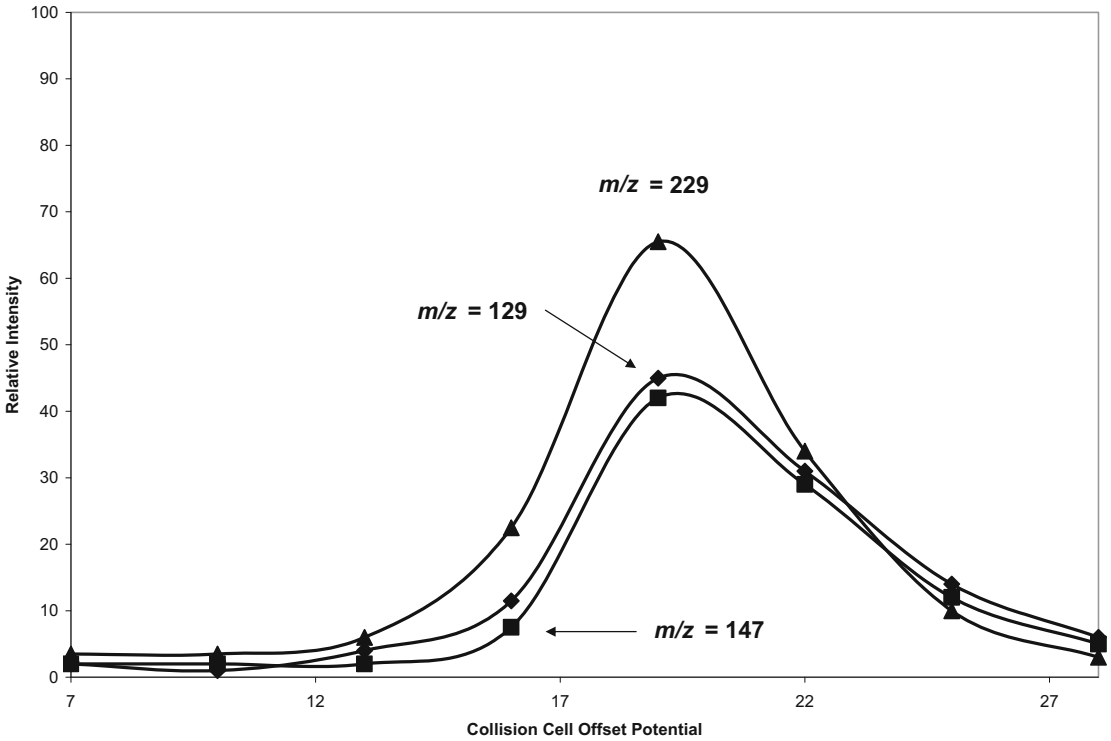
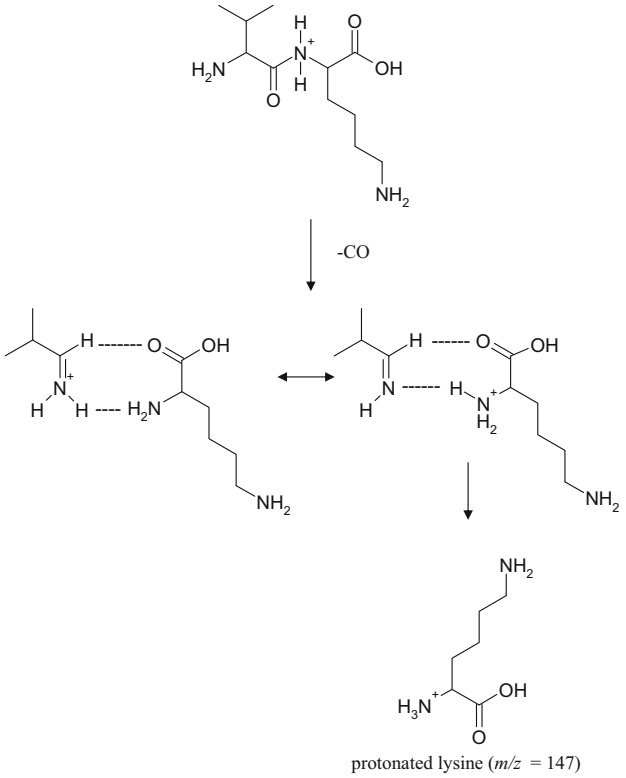


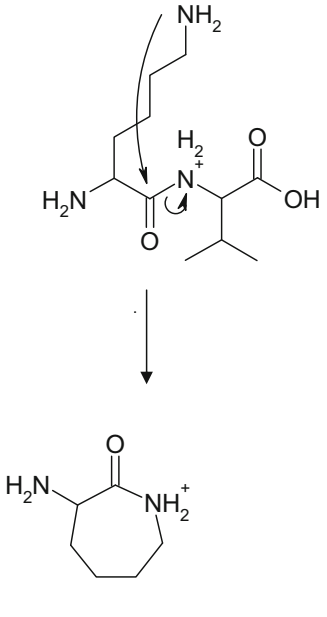
Figure 5. Breakdown curves for KV showing concurrent loss of NH_3 (m/z = 229), formation of $(K + H)^+$ at m/z 147, and the b_1-ion at m/z 129.



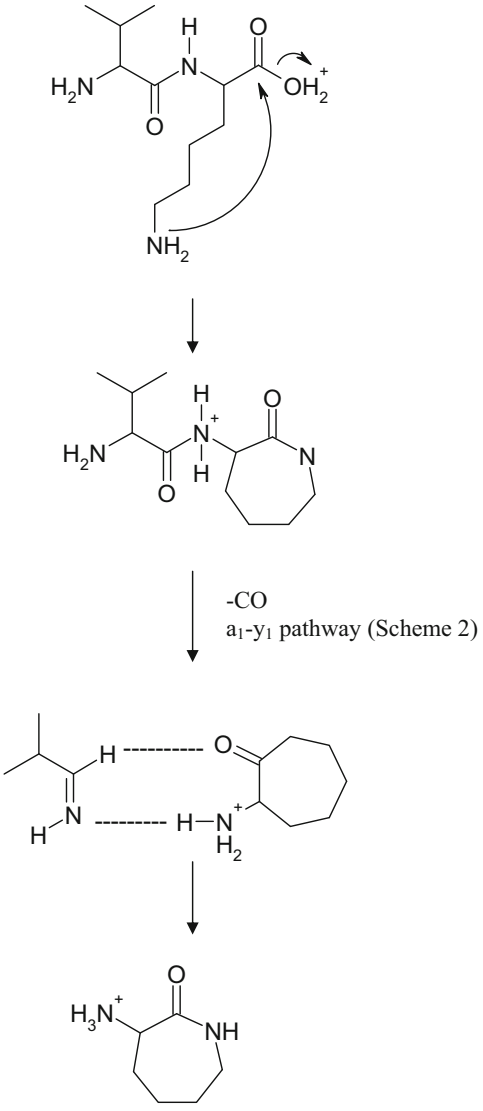
Scheme 2

Formation of b_1-Ions That Are Not Acylium Ions

The ERMS data for KV (Figure 5) also showed that, in addition to the ion at m/z = 147 representing protonated lysine, the fragment ion at m/z = 129 was also a first generation product ion of the dipeptide rather than loss of water from protonated lysine $(K + H)^+$, which would lead to an unstable acylium ion. Additionally, the product ion spectrum of lysine did not show a significant loss of H_2O. Yalcin and Harrison [16] presented data to support the formation of α-amino-ε-caprolactam to explain the formation of the fragment ion at m/z = 129 in the tripeptide GKG. Under CID conditions, the proton on the ε-amino side-chain of lysine side-chain amino group can migrate to the amide bond oxygen (more basic) or nitrogen. Harrison proposed proton migration to the amide nitrogen, which would weaken the amide bond forming a hydrogen bound complex between valine and α-amino-ε-caprolactam with the proton going to the species with the highest gas-phase basicity α-amino-ε-caprolactam in this case. This is depicted in Scheme **3** for KV.



Scheme 3

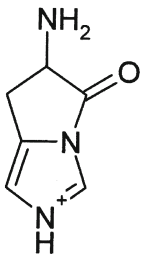


Scheme 4

Table 1. Accurate mass data for arginine in RF, histidine in HF, and lysine in KV

	Basic amino acid				
Dipeptide	Formula	$Mass_{calc.}$	$Mass_{exp.}$	Δu	ppm
RF	$C_6H_{15}N_4O_2^+$	175.1190	175.1196	+0.0006	3
HF	$C_6H_{10}N_3O_2^+$	156.0768	156.0767	–0.0001	1
KV	$C_6H_{15}O_2N_2^+$	147.1128	147.1138	+0.0010	7

Similar mechanisms were reported by Wysocki et al. [24] and Farrugia et al. [18] for apparent b_1-ions of histidine. In Wysocki's mechanism, intramolecular solvation of an imidazole hydrogen by the oxygen in the amide bond led to enhanced cleavage in peptides containing an H–P bond such as angiotensin. For a dipeptide containing an N-terminal histidine, this facilitates transfer of the imidazole hydrogen to the amide bond with subsequent cleavage forming the fragment ion at m/z = 138 (**1**).



1
$m/z = 138$

Farrugia et al. discussed formation of b_n-ions in di- and tripeptides containing histidine and glycine residues and their methyl esters and emphasize the prevalence of b_1-ions when histidine is at the N-terminal. This observation was made for all of the dipeptides containing an N-terminal basic amino acid studied here. They point out that proton transfer by the initially protonated histidine side chain can occur at either hetero-atom of the amide bond leading to (**1**). Histidine and lysine b_1-ions underwent loss of CO to form the corresponding immonium ion.

It should be noted here that a corresponding y_1-ion was formed in the product ion spectrum of VK. ERMS data for KV and VK showed that the formation of m/z 147 and 129 are concurrent events in both dipeptides but occurred at a slightly lower energy in VK, supporting the possibility of different mechanisms. Loss of water from VK (Figure 3) was observed as a low-energy process that was not observed in KV. One possible mechanism for the formation of m/z 129 from VK is presented in Scheme **4**. Nucleophilic attack by the ε-amino group on the carbonyl carbon of the carboxyl results in the loss of water followed by fragmentation at the amide bond by way of the a_1-y_1 pathway. The α-amino-ε-caprolactam protonates, leaving the imine and CO as neutral byproducts.

A weak apparent b_1-ion was also observed for arginine in the spectrum of RF at m/z = 157. Studies of the gas-phase basicity of amino acids [30, 34] have shown arginine to be the most basic amino acid, almost as basic as guanidine. Fragmentation of arginine and arginine containing dipeptides is directed by protonation of the highly resonance-stabilized guanidinyl side chain. Dookeran et al. [15] showed the major fragmentation channel of arginine to be formation of the fragment ion at m/z = 70 resulting from decarboxylation of protonated proline, formed by loss of neutral guanidine, from protonated arginine. Csonka et al. [33] have presented theoretical work to support Dookeran's experimental results. Our ERMS data for RF supported the fragmentation sequence $290 > 175(R + H)^+ > 116(P + H)^+ > 70$ with the ion at m/z = 70 the dominant fragment ion at higher energies.

Determination of Exact Mass

Accurate mass data for the fragment ions at m/z = 175, m/z = 156, and m/z = 147 were consistent with the calculated exact mass for the basic amino acids in RF, HF, and KV (Table 1) [35–37].

Conclusions

The formation of $b_1 + H_2O$ and b_1-ions discussed in this paper applied to all of the dipeptides analyzed with N-terminal basic amino acids and was not generally observed in the C-terminal isomers DR, DH, AH, EH, VK, WK, IK, LK, SK, EK, TK, DK, AK, and FK except in the instances where both amino acids were basic (HK and KH). There was one possible exception with the observation of a fragment ion at m/z = 166 (7% relative intensity at −22 V) in FH, which may correspond to protonated phenylalanine, but this was the only instance. These fragmentation channels were not observed or were minor for the other peptides studied that did not contain a basic amino acid residue. Unfortunately, being able to identify a b_1+H_2O ion in a dipeptide containing an N-terminal basic amino acid will not enable it to be distinguished from its C-terminal isomer because this isomer will readily yield a y_1-ion which, of course, is equivalent to a b_1+H_2O ion. Additionally, y_1-H_2O ions corresponding to b_1-ions were also detected in the product ion spectra of the C-terminal

isomers. These dipeptides can easily be distinguished by synthesis and recording the spectra in a searchable library. However, low-energy mechanistic studies will not readily enable a priori differentiation.

Acknowledgments

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