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A comparison of negative and positive ion time-of-flight post-source decay mass spectrometry for peptides containing basic residues

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Dedicated to professor J.L. Beauchamp on the occasion of his 60th birthday for his many seminal contributions to gas-phase ion chemistry and mass spectrometry.

Abstract

Nine peptides containing highly basic residues were studied by post-source decay (PSD) in a reflectron time-of-flight (TOF) mass spectrometer. Although these compounds produced more abundant yields of protonated ions, $[M + H]^+$, by matrix-assisted laser desorption ionization (MALDI), deprotonated ions, $[M–H]^-$, were formed in sufficient intensities for study by tandem mass spectrometry (MS/MS). PSD was conducted in both the positive and negative ion modes. Peptide backbone cleavage involving the y-ion series is generally seen in both modes and the two Dalton difference in mass between $y''_n{}^+$ and $y_n{}^-$ can be useful in identifying these ions. For negative ions, PSD also generated $c_n{}^-$ and (to a lesser extent) $a_n{}^-$, while $b_n{}^+$ are produced from positive ions. When a peptide contains a mixture of acidic and basic residues, the negative PSD spectra are more complex and the locations of acidic residues dictate some fragmentations. The most extensive and abundant production of $c_n{}^-$ occurs in peptides with no acidic residues. This suggests that the mechanism for c-ion formation does not involve a deprotonated side chain, but may invoke a mobile deprotonation site along the peptide amide backbone or may possibly involve a charge remote cleavage. Negative ion PSD of basic peptides yields structurally informative spectra that complement the positive data. Even highly basic peptides, such as ACTH (11–24), can be studied in the negative ion mode. (Int J Mass Spectrom 222 (2003) 363–381)

Keywords: MALDI; Post-source decay; Basic peptides; Negative ions

1. Introduction

Peptides and proteins containing basic amino acid residues (i.e., arginine, lysine, histidine) are known to play important roles in enzyme catalysis mechanisms [1,2]. They are major sources of protons for proton transfer reactions such as the bovine pancreatic RNAse hydrolysis of RNA, which involves a histidine residue [3,4]. Furthermore, in the case of tryptic digests cleavage of the peptide chain occurs at basic residues [5].

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Historically, mass spectrometric studies of peptides have involved positive ions [6]; these reports dwarf the relatively few negative studies. However, it has recently been shown that the analysis of phosphopeptides can benefit from examination in both positive and negative ion modes [7,8]. In addition, Harrison [9] has used electrospray ionization (ESI) to produce deprotonated peptide ions, $[M–H]^-$, while Beauchamp [10], Bowie [11–14] and their co-workers have produced peptide $[M–H]^-$ by fast atom bombardment (FAB). In these studies, collision-induced dissociation (CID) was used to fragment $[M–H]^-$ and the dissociation mechanisms were explored. Negative ion CID spectra were generally found to be as informative as the positive ion spectra [12]. In addition, our group has shown that negative mode post-source decay (PSD) time-of-flight (TOF) analysis of peptides can provide complementary structural information to positive ion studies [15,16]. Negative mode PSD has been effective on peptides with neutral amino acid chains, as well as peptides containing acidic residues [16].

The protonated and deprotonated ions from a peptide differ by both polarity and charge location. Charge location is especially important because it can dramatically impact the structural information that is obtained by tandem mass spectrometry (MS/MS). Charge-directed fragmentation is a major mode of peptide dissociation. For example, Harrison and Yalcin [17] and Wysocki and co-workers [18,19] reported that protonation of an amido nitrogen along the peptide backbone causes the (O=C)–NHR bond to weaken, leading to cleavage at the stressed site. In the positive ion mode, sequence-determining fragments are believed to result predominantly when the peptide backbone is protonated. The mobile proton model suggests that initial ionization may occur on the side chain of basic residues [20] and that the proton may migrate to other sites upon ion activation [17]; the location of these specific sites drives the fragmentation patterns. The probability of a proton being attracted to a basic residue is high, thus causing greater fragmentation adjacent to basic residues [21–23]. The locations of basic sites can complicate peptide dissociation; for example, if a basic residue is at or near the C-terminal, then more C-terminal ions may be observed than N-terminal ions and vice versa [23]. Arginine residues are particularly problematic because their highly basic side chains can sequester the proton and limit its mobility across the peptide backbone. Consequently, in positive mode analysis, the presence of arginine in a peptide may lead to spectra dominated by arginine-containing fragments and to a significant reduction in the amount of sequencing information obtained [23–27].

Basic residues have other implications upon positive ion mass spectrometry. The gas-phase basicities of the major basic residues decrease in the order of arginine (Arg, R) $>$ lysine (Lys, K) $\approx$ histidine (His, H) [28–31]. In a study of synthetic tryptic peptides by positive mode matrix-assisted laser desorption ionization (MALDI), this basicity ordering explains the preferred protonation and enhanced detection of peptides containing a C-terminal arginine residue relative to peptides with a lysine residue [24]. In addition, arginine-containing peptides are known to undergo preferential cleavage at the C-terminal side of aspartic acid residues [32–34], while peptides containing C-terminal lysine and at least one arginine yield a rearrangement that results in the loss of the terminal lysine [35]. Also, arginine residues may participate in gas-phase zwitterion formation, which can complicate dissociation patterns. It is believed that bradykinin ions (which have N- and C-terminal arginine residues) can exist as stable zwitterions in the gas phase [36–39], as can arginine itself [40].

As the studies discussed before indicate, basic residues play a major role in the dissociations of protonated peptide ions and their effects can at times be detrimental to obtaining sequence information. In contrast, for the negative ion mode, the side chains of basic residues should not be charge sites. Thus, major differences in fragmentation may be observed in comparing the two modes. The present study will explore this using PSD of $[M + H]^+$ and $[M–H]^-$ produced by MALDI. All three common basic amino acids are represented in the peptides examined:

ACTH (11–24)	**K**PVG**KKRR**PV**K**VYP
$(KG_2)_4$	**K**GG**K**GG**K**GG**K**GG
$(K_2G_4)_2$	**KK**GGGG**KK**GGGG
K_4G_8	**KKKK**GGGGGGGG
Bradykinin	**R**PPGFSPF**R**
des-Arg^1-bradykinin	PPGFSPF**R**
des-Arg^9-bradykinin	**R**PPGFSPF
Tyrosine protein kinase	**RR**LIEDNEYTA**R**G
Histidine-containing renin inhibitor	**H**PF**H**LLVY

2. Experimental

All experiments were performed on a Bruker Daltonics (Billerica, MA) Reflex III TOF mass spectrometer, which is equipped with a two-stage reflectron. The instrument has an effective flight path of 2.9 m and sample ionization by MALDI involves a Laser Science (Franklin, MA) model VSL-337ND-S nitrogen laser emitting at 337 nm.

Ions were moved from the source to the flight tube via delayed extraction [41] with an extraction delay of 250 ns. For PSD experiments, a gated pulse was used to selectively allow the peptide's quasi-molecular ions to enter the flight tube, while deflecting matrix ions and other contaminants. The mass window for this parent ions selection was ca. ±15 Da. For both polarities the initial magnitude of the accelerating voltage was 20 kV. Switching between positive and negative ion modes was simply a matter of switching the polarities of the voltages; however, in order to obtain the maximum ion signal, the negative mode generally required a laser power about 10% higher than the positive mode. For negative ion PSD, the reflectron voltage was stepped from −21 to −2.8 kV in nine stages, with fibrinopeptide B used for PSD calibration [42]. For positive ion PSD, the initial reflectron voltage was +23 kV, which was stepped down to +0.7 kV in 14 stages and calibrated with ACTH (18–39) [43]. The mass spectrum at each PSD voltage step was the sum of 100–200 laser shots. Bruker XMASS software combined the spectra from the individual voltage steps to form a complete PSD mass spectrum. Uncertainties in mass measurements for ions in the final calibrated PSD mass spectra were on the order of ±1 Da.

All compounds were commercially available and purchased from Sigma (St. Louis, MO) with the exception of the lysine- and glycine-containing dodecapeptides. These were custom synthesized by Quality Controlled Biochemicals (Hopkington, MA) and prepared as a 200 μM solution in a 70/30 mixture of acetone/water with 0.1% trifluoroacetic acid. All compounds were used as received without further purification. For commercially available compounds, preparation consisted of dissolving the peptide in water to a concentration of 1 mg/mL and mixing 2 μL aliquots with 5 μL of α-cyanohydroxycinnamic acid or 2,5-dihydroxybenzoic acid matrices dissolved in methanol. A 1 μL aliquot of each final mixture was deposited on the sample plate and allow to air dry prior to insertion of the plate into the ion source of the mass spectrometer.

3. Results

3.1. Ion formation by MALDI

For the basic peptides under study, MALDI yields weaker $[M–H]^-$ signals compared to $[M+H]^+$. However, the $[M–H]^-$ intensities from all peptides were still sufficient for study, which is undoubtedly due to the carboxylic acid group of the C-terminus offering a ready site for deprotonation. To illustrate this, Fig. 1 shows the MALDI/TOF mass spectra of ACTH (11–24) in both the positive and negative ion modes. This is the most basic peptide studied here, with 6 of its 13 residues being highly basic arginine or lysine, yet $[M–H]^-$ readily forms without chemical modification to the peptide. Plus, an advantage of the negative mode is its lack of alkali metal adducts. Even without employing sample cleanup procedures, $[M–H]^-$ is essentially the only negative quasi-molecular ion to form by MALDI. In contrast, $[M + Na]^+$ and $[M + K]^+$ sometimes compete with $[M + H]^+$ for positive ion yield.

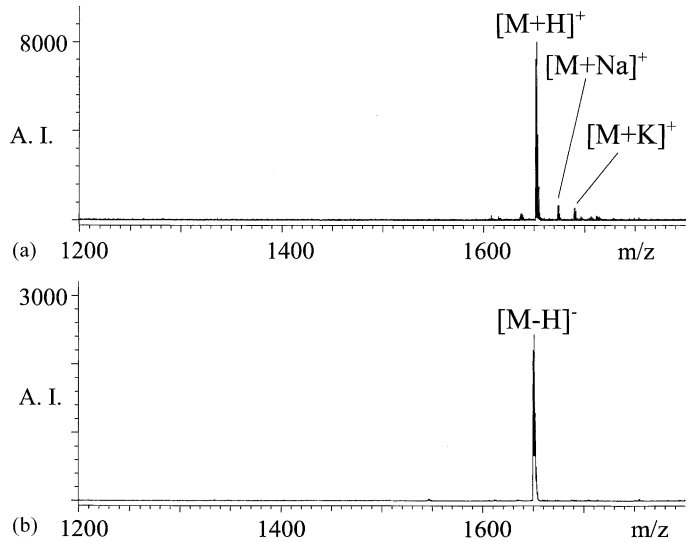


Fig. 1. MALDI/TOF mass spectrum of ACTH (11–24) in the (a) positive mode and (b) negative mode.

3.2. Peptide cleavage nomenclature

The nomenclature employed in this work for contrasting negative and positive ions was recently used by Harrison [9]. The standard symbols a, b, c, x, y, and z denote cleavages of the peptide backbone. Primes refer to lost or added hydrogens from the ionic fragment; before the cleavage symbol denotes lost of hydrogens and after the symbol indicates added hydrogens. For example, the positive mode's $y''_n{}^+$ and $c''_n{}^+$ contain two hydrogens more than the negative mode's $y_n{}^-$ and $c_n{}^-$. For the a-, b-, and x-ion series, we observed the same masses in both the positive and negative modes. The masses of b-ions are a topic in Section 4.

3.3. Adrenocorticotropic hormone fragment (11–24)

The 13 residue peptides ACTH (11–24) is strongly basic with four lysine residues and two arginine residues. These basic residues are distributed throughout the peptide, but a basic array of KKRR is also present in the middle of the sequence. Fig. 2 shows the negative and positive MALDI PSD spectra of this peptide, while Table 1 lists all fragment ion assignments in both modes.

Negative PSD (Fig. 2a) reveals primarily N-terminal fragments, displaying an almost complete series of $c_n{}^-$, $n = 3$–12, with additional and generally weaker yields of $a_n{}^-$, $n = 9$–14, and $b_n{}^-$, $n = 10$, 11, 13, and 14. Although cleavage between Lys^6 and Arg^7 is intense, overall there is no obvious preference for cleavage at basic residues. This is as expected because basic residues are not charge sites in the negative mode.

Positive PSD produces a more intense signal (due to the more abundant formation of $[M + H]^+$ by MALDI) and a more complex spectrum. C- and N-terminal fragments form, including $b_n{}^+$, $n = 1$–8 and 10–14, and $y''_m{}^+$, $m = 2$–5 and 7–13; in both cases, the absent fragmentation involves cleavage on the C-terminal side of Pro^9. As Fig. 2b illustrates, there are also many unassigned peaks, especially below m/z 700, which can hinder interpretation of the spectrum.

For ACTH (11–24), complete sequence data involving cleavage at every residue is not obtained in either mode. However, total sequence information can be obtained by considering spectra from both the negative and the positive ion modes.

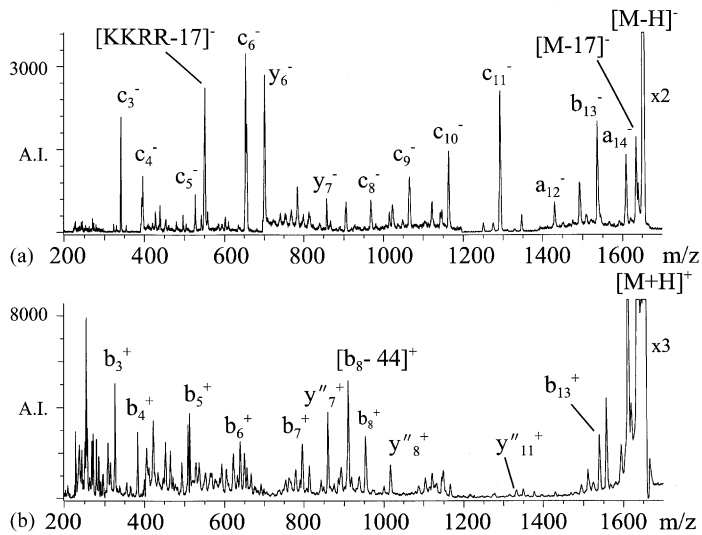


Fig. 2. PSD spectra of ACTH (11–24) in the (a) negative mode and (b) positive mode.

3.4. Bradykinin and its des-arginine analogues

Bradykinin, a hormone-like peptide, possesses two basic arginine residues, one at each terminus. The fragmentation behavior in the negative mode is summarized in Table 2. Bradykinin displays a dominant loss of 30 Da from $[M–H]^-$ peak, which is believed to involve elimination of CH_2O from the serine side chain [13,44,45]. This elimination is observed in both the PSD spectrum and in MALDI spectrum without PSD analysis. In addition, serine-containing PSD products display loss of CH_2O from the serine side chain. Also prominent in the negative ion spectrum is the loss of the C-terminal arginine residue. Members of the a-, b-, c-, and y-series are produced but no complete backbone ion series forms.

Fragmentation of bradykinin ions in the positive mode yields some a-, b- and y″-ions, as well as loss of 17 Da from these ion series. Elimination of 17 Da is probably loss of NH_3 from the arginine side chains at Arg^1 and Arg^9 [15,43,46]. There is also abundant formation of $[a_1-17]^+$ and y''^+_8, which involve cleavages adjacent to the basic Arg^1 residue. (It is also possible that the peak assigned as y''^+_8 may be $[b_8+18]^+$, which is the assignment used in a study by Gimon-Kinsel et al. [47].) In addition, internal ions are observed from cleavages at serine and proline residues, such as $[PGFS]^+$, $[PGFS-18]^+$ and $[PPGFS]^+$.

The removal of the N-terminal arginine to yield des-Arg^1-bradykinin allows examination of the role that this residue plays in the PSD behavior of bradykinin (see Table 3). In the negative mode a much simpler spectrum is produced (relative to that of bradykinin), where the largest peak derives from the loss of 30 Da from the molecular ion. Other serine-containing fragments in the PSD spectrum also exhibit CH_2O elimination. While fragmentation from bradykinin occurs throughout the peptide backbone, des-Arg^1-bradykinin has a greater tendency to fragment at residues on the C-terminal end of the peptide. However, there is a prominent peak that corresponds to x_6^-; this is unusual because x-ions are rarely observed in our negative ion spectra.

Our positive ion PSD spectrum of des-Arg^1-bradykinin is in good agreement with previous work of Rouse et al. [43]. A complete series of a-, b- and y-ions are detected, along with a complete series involving 17 Da losses from the y-ions.

The removal of bradykinin's C-terminal arginine forms des-Arg^9-bradykinin. The PSD data is

Table 1
Fragmentation of ACTH (11–24)[a]

	ACTH (11–24)													
	K, $n = 1$	**P**, $n = 2$	**V**, $n = 3$	**G**, $n = 4$	**K**, $n = 5$	**K**, $n = 6$	**R**, $n = 7$	**R**, $n = 8$	**P**, $n = 9$	**V**, $n = 10$	**K**, $n = 11$	**V**, $n = 12$	**Y**, $n = 13$	**P**, $n = 14$
Negative ion														
a_n^-									w	w	w	w	w	m
b_n^-										w	w		m	w
c_n^-			s	w	w	s	w	w	w	m	s	w		—[b]
$y_{(15-n)}^-$	—[b]		w	w	w	s	w	w	m	w	w		w	
Internal ion[c]					s, KKRR-17									
Positive ion														
b_n^+	s	w	m	w	w	w	w	w		w	w	w	m	s
$y''^+_{(15-n)}$	—[b]	w	w	w	w	m	w	m		w	w	w	w	

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of the parent ion.
[c] The internal ion is composed of the underlined residues.

Table 2
Fragmentation of bradykinin[a]

	Bradykinin								
	R, $n = 1$	**P**, $n = 2$	**P**, $n = 3$	**G**, $n = 4$	**F**, $n = 5$	**S**, $n = 6$	**P**, $n = 7$	**F**, $n = 8$	**R**, $n = 9$
Negative ion									
a_n^-	s	m	w		w				
b_n^-				w	w	w		m	
c_n^-			w	m	w				–[b]
$[c_n - 30]^-$						w	w	w	s
$y_{(10-n)}^-$	–[b]		w	w		w	s	w	w
$[y_{(10-n)} - 30]^-$	s	w	m	w	m	s			
Positive ion									
a_n^+	w				w	w		w	w
$[a_n - 17]^+$	s	m	w		w				
b_n^+	w	w		w	w	w			
$[b_n - 17]^+$	w	w	w	w	w	w			
$y''^+_{(10-n)}$	–[b]	s	w	w	w	w	w		w
$[y''_{(10-n)} - 17]^+$	s	w	w				w	w	
Internal ions[c]			w, PGFS						
			w, PGFS-18						
		w, PPGFS							

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of the parent ion.
[c] The internal ions are composed of the underlined residues.

summarized in Table 4. The negative PSD spectrum of this peptide is again dominated by a loss of 30 Da (CH_2O elimination). Also observed is an intense c_4^- from the cleavage between Gly^4 and Phe^5. An intense y_3^- peak is seen, corresponding to a cleavage between Ser^6 and Pro^7, along with a complete series of y-ions. All y-ions that contain Ser^6 also show loss CH_2O. While one low intensity internal ion is present,

Table 3
Fragmentation of des-Arg^1-bradykinin[a]

	des-Arg^1-bradykinin							
	P, $n = 1$	**P**, $n = 2$	**G**, $n = 3$	**F**, $n = 4$	**S**, $n = 5$	**P**, $n = 6$	**F**, $n = 7$	**R**, $n = 8$
Negative ion								
b_n^-					w		w	s
c_n^-			m					–[b]
$[c_n - 30]^-$					w	w	w	s
$y_{(9-n)}^-$	–[b]			w	w	w	w	m
$[y_{(9-n)} - 30]^-$	s		w	m	m			
$x_{(9-n)}^-$			s					
Positive ion								
a_n^+	s	w	w	s	m	w	w	w
b_n^+	w	w	m	m	s	w	w	m
$y''^+_{(9-n)}$	–[b]	m	w	w	m	m	w	m
$[y''_{(9-n)} - 17]^+$	m	m	w	w	m	s	m	w

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of the parent ion.

Table 4
Fragmentation of the des-Arg^9-bradykinin[a]

	des-Arg^9-bradykinin							
	R, $n = 1$	**P**, $n = 2$	**P**, $n = 3$	**G**, $n = 4$	**F**, $n = 5$	**S**, $n = 6$	**P**, $n = 7$	**F**, $n = 8$
Negative ion								
b_n^-						w		s
$[b_n - 30]^-$							m	m
c_n^-	w		w	s	w	w		–[b]
$[c_n - 30]^-$						w	w	s
$y_{(9-n)}^-$	–[b]	w	w	w	w	w	s	w
$[y_{(9-n)} - 30]^-$	s	w	m	w	m	w		
Internal ion[b]						w, FSP		
Positive ion								
a_n^+				w	w	m		w
$[a_n - 17]^+$	s	s		w	m	s		w
b_n^+	m	s	w	w	w	m	w	s
$[b_n - 17]^+$	m	s	w	m	w	w		
$y''_{(9-n)}{}^+$	–[b]	m	m	w		m	s	w
Internal ion[c]		m, PP						
				w, PGF				
				s, PPGFS				

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of the parent ion.
[c] The internal ion is composed of the underlined residues.

$[FSP]^-$, the simplicity of the spectrum matches that of des-Arg^1-bradykinin.

Positive ion fragmentation for des-Arg^9-bradykinin yields a complete series of N-terminal b-ions, along with several a-ions. Both series show further elimination of NH_3 (17 Da). Cleavages around the highly basic Arg^1 residue are particularly prominent. For C-terminal ions, the complete series of $[y_m - 17]^-$ forms; in the lower mass end of the spectrum, they are more intense than the y_m^- series. The most prominent cleavage is between Ser^6 and Pro^7 to produce $[a_6 - 17]^-$ and $y''_2{}^+$. Our positive ion PSD spectrum of des-Arg^9-bradykinin is also in good agreement with previous work of Rouse et al. [43].

3.5. Glycine and lysine dodecapeptides

The PSD spectra of three dodecapeptides containing only lysine and glycine were obtained in the negative mode and compared to our previous studies in the positive mode [21]. For $(KG_2)_4$, basic lysine residues are separated by two glycine residues; $(K_2G_4)_2$ has two adjacent lysines in a different repeat sequence; and in K_4G_8, the lysine residues are concentrated at the N-terminus. Fig. 3 gives the negative ion PSD mass spectra of these peptides and Table 5 summarizes the observed fragmentations.

The negative ion PSD spectrum of $(KG_2)_4$ (Fig. 3a) reveals complete sequence information in the form of a series of c_n^-, $n = 1$–11. Abundant C-terminal fragmentation also occurs as is evidenced by an almost complete series of y_m^-, $m = 3$–11. The intense peaks at y_5^-, y_6^-, y_8^-, and y_9^- correspond to cleavages adjacent to the basic residues; however, abundant Gly–Gly cleavages are also seen.

As Fig. 3b illustrates, complete sequence information for $(K_2G_4)_2$ is also attainable in the negative mode through N-terminal c_n^-, $n = 1$–11, and C-terminal y_m^-, $m = 3$–11. While the most intense fragment peak is c_9^-, involving cleavage adjacent to a basic lysine residue, intense signals are again found from Gly–Gly cleavages.

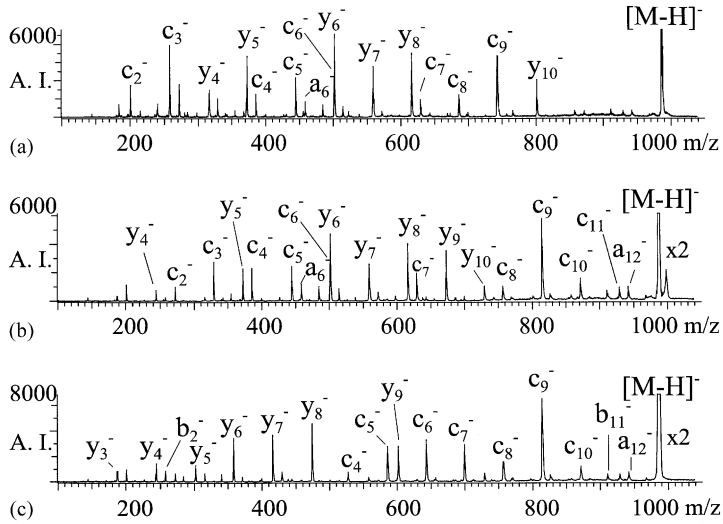


Fig. 3. Negative mode PSD spectra of $[M-H]^-$ from (a) $(KG_2)_4$, (b) $(K_2G_4)_2$ and (c) K_4G_8.

The trend continues for K_4G_8 in Fig. 3c as complete sequencing is attainable through the c_n^-, n = 1–11, and y_m^-, m = 3–10. Many low intensity b_n^- also form. The dominant peak in this spectrum is again c_9^-, although here it involves cleavage between two glycine residues.

In the positive mode, all three dodecapeptide ions generate a complete series of b_n^+, n = 1–12. PSD of $[M+H]^+$ for $(KG_2)_4$ also reveals an almost complete series of y''^+_m, m = 3–11. Positive ions from $(K_2G_4)_2$ and K_4G_8 exhibit y''^+_m, m = 5–11 [21]. The presence of such high intensity y''^+_m, m = 9–11, indicates a favorable fragmentation pathway involving the glycine component of the peptide, while the abundant early b-series suggest labile fragmentation occurring adjacent to the more basic lysines. Enhanced cleavage adjacent to lysine residues is particularly noteworthy for $[K_4G_8+H]^+$; in contrast, no enhanced cleavage at lysine is found for the corresponding deprotonated ion, $[K_4G_8–H]^-$.

3.6. Tyrosine protein kinase substrate 13 (TPK-13)

The synthetic tridecapeptide TPK-13 contains three basic arginine residues (Arg^1, Arg^2, and Arg^{12}) and three acidic residues (Glu^5, Asp^6 and Glu^8). Table 6 summarizes the data obtained. In the negative mode, ions from the b-, c-, and y-series are seen, along with $[b_n-17]^-$ and $[y_m-17]^-$. The PSD products are of low intensity but the most abundant ions generally involve cleavage adjacent to an acidic residue (e.g., c_5^- and y_7^-). As can be seen from Fig. 4a, the spectrum is unusually sparse between *m*/*z* 900 and 1500 and there are numerous peaks below *m*/*z* 900 that do not correspond to any commonly identifiable cleavage.

Fragmentation of $[M+H]^+$ reveals a complete series of b-ions, although many are of low intensity. C-terminal ions y''^+_m, m = 3–12, also form. The positive ion data is more structurally informative than the negative PSD spectrum. However, the positive ion spectrum is still difficult to interpret because, as seen from Fig. 4b, there are many unassigned peaks that do not correspond to typical backbone cleavages and are not obviously related to common neutral losses. Again, there is a sparse region in the spectrum but for positive ions it lies between *m*/*z* 1000 and 1400. In addition, the molecular ion region is cluttered with peaks from small neutral eliminations, such as the loss of 17 Da (NH_3) and 42 Da.

Table 5
Fragmentation of glycine–lysine dodecapeptides[a]

	$(KGG)_4$											
	K, $n=1$	**G**, $n=2$	**G**, $n=3$	**K**, $n=4$	**G**, $n=5$	**G**, $n=6$	**K**, $n=7$	**G**, $n=8$	**G**, $n=9$	**K**, $n=10$	**G**, $n=11$	**G**, $n=12$
Negative ion												
a_n^-			w		w	w		w	w			w
b_n^-						w		w	w			w
c_n^-	w	m	s	w	m	m	w	w	s	w	w	–[b]
$y_{(13-n)}^-$	–[b]	w	m	s	s	m	s	s	m	w		
Positive ion												
a_n^+		w	w		w	w		w	w		w	
b_n^+	s	m	m	s	m	s	m	w	w	s	w	m
$y''_{(13-n)}^+$	–[b]	s	m	w	m	w	s	w	w	w		
	$(K_2G_4)_2$											
	K	**K**	**G**	**G**	**G**	**G**	**K**	**K**	**G**	**G**	**G**	**G**
Negative ion												
a_n^-				w	w	w			w	w		w
c_n^-	w	w	m	m	m	m	m	w	s	w	w	–[b]
$y_{(13-n)}^-$	–[b]	w	w	s	s	m	s	m	w	w		
Positive ion												
a_n^+								w	w	w	w	w
b_n^+	s	w	w	w	w	m	m	m	w	w	w	w
$y''_{(13-n)}^+$	–[b]	m	m	w	w	w	w	m				
	K_4G_8											
	K	**K**	**K**	**K**	**G**	**G**	**G**	**G**	**G**	**G**	**G**	**G**
Negative ion												
a_n^-							w	w	w	w		w
b_n^-		w	w	w	w	w	w	w	w		w	w
c_n^-	w	w	w	w	m	m	m	w	s	w	w	–[b]
$y_{(13-n)}^-$	–[b]		w	m	m	m	m	w	w	w		
Positive ion												
a_n^+	w	w	w	w	w		w	w	w	w	w	
b_n^+	s	s	m	m	w	w	w	w	w	w	w	m
$y''_{(13-n)}^+$	–[b]	m	s	s	w	w	w	w		w		

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of $[M–H]^-$.

The profusion of peaks below *m/z* 700 in the spectra of both polarities is unusual and is not observed for other peptides involved in this study. These are actual fragment ions present in the PSD spectrum, not electronic or chemical noise. The presence of multiple acidic and basic residues in this peptide may be leading to numerous complex rearrangements and cleavages for these parent ions.

3.7. Histidine-containing renin inhibitor peptide

This renin inhibitor has two histidine residues, one at the N-terminus, and one mid-sequence. As is seen in Fig. 5 and Table 7, the negative PSD spectra contains complete series of both c- and y-ions. Thus, this peptide can be readily sequenced using the negative ion data. Dominating the spectrum are c_5^-, from

Table 6
Fragmentation of TPK-13[a]

	TPK-13 (13)												
	R, $n = 1$	**R**, $n = 2$	**L**, $n = 3$	**I**, $n = 4$	**E**, $n = 5$	**D**, $n = 6$	**N**, $n = 7$	**E**, $n = 8$	**Y**, $n = 9$	**T**, $n = 10$	**A**, $n = 11$	**R**, $n = 12$	**G**, $n = 13$
Negative ion													
b_n^-	s	m	w	w	w	m	w	w	w		w	s	s
$[b_n - 17]^-$		s	w	m	w	m		w			w		s
c_n^-	w	w			s	w			w	w	w	w	–[b]
$y_{(14-n)}^-$	–[b]	m	w	w	w	m	s	w	w		w		
$[y_{(14-n)} - 17]^-$	s	w				m	m	w					
Internal ions[c]						m, DNEY							
					w, EDNEY-18								
Positive ion													
b_n^+	w	w	w	w	m	m	w	w	w	w	w	w	s
$y''_{(14-n)}{}^+$	–[b]	w	w	w	w	m	s	w	w	w	w		

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of the parent ion.
[c] The internal ions are composed of the underlined residues.

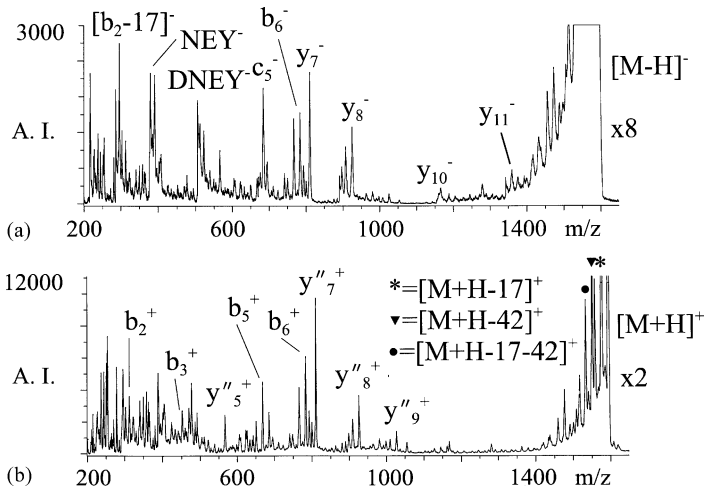


Fig. 4. PSD spectra of TPK-13 in the (a) negative mode and (b) positive mode.

cleavage at Leu[5]–Leu[6], and y_5^-, which cleaves between His[4]–Leu[5]. Another prominent ion is y_4^-, which involves C-terminal cleavage at histidine.

The positive PSD spectrum also contains complete sequence information through the b- and y-series, as well as an almost complete (but low intensity) series of a-ions. The a_1^+ and b_2^+ ions, which involve cleavage near His[1], are intense. For both the negative and positive modes, high quality spectra can be readily obtained and allow complete sequencing of the peptide.

4. Discussion

4.1. Effects of charge location on fragmentation

The dominant backbone fragmentations in the positive PSD spectra are b- and y″-ions. In contrast, the negative PSD spectra showed primarily c- and y-ions, although some a- and b-ions also form. These fragmentations generally occur throughout the peptide backbone. This is consistent with previous studies

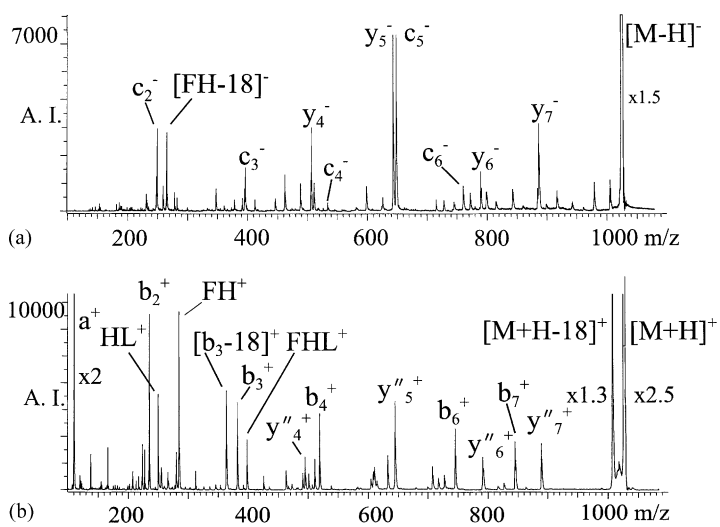


Fig. 5. PSD spectra of the histidine-containing renin inhibitor peptide in the (a) negative mode and (b) positive mode.

Table 7
Fragmentation of the histidine-containing renin inhibitor peptide[a]

	Histidine- containing peptide							
	H, $n = 1$	**P**, $n = 2$	**F**, $n = 3$	**H**, $n = 4$	**L**, $n = 5$	**L**, $n = 6$	**V**, $n = 7$	**Y**, $n = 8$
Negative ion								
c_n^-	w	m	w	w	s	w	w	–[b]
$y_{(9-n)}^-$	–[b]	m	w	s	m	w	w	w
Internal ions[c]			m, FH-18					
Positive ion								
a_n^+	s	w	w	w	w	w	w	
b_n^+	w	s	m	m	m	m	w	s
$y''^+_{(9-n)}$	–[b]	w	w	m	w	w	w	w
			s, FH					
			m, HL					
			w, FH					

[a] Intensities where "w" is weak (<30% of the base peak), "m" is medium (30–65%), and "s" is strong (>65%).
[b] Corresponds to the mass of the parent ion.
[c] The internal ions are composed of the underlined residues.

[21,43], which have found that for singly protonated peptides, PSD yields fragments at almost every residue. In comparison to PSD, both high [43] and low [21] energy CID generally have more fragmentation that is dictated by the location of the charge sites.

Charge site location differs between $[M + H]^+$ and $[M–H]^-$. Intrinsic basicity considerations indicate that the side chains of arginine, lysine, and histidine and the N-terminal amino group will be sites of protonation in the positive mode [31,48,49]. In the negative mode, higher acidity groups such as aspartic acid, glutamic acid and the C-terminal carboxylic acid group are sites of deprotonation; although less acidic, alternative deprotonation sites are OH-containing residues such as serine, threonine and tyrosine [50]. (However, for both modes, it should be noted that the charge site may become mobile upon ion activation and shift to other locations on the peptide chain.) Cleavages at potentially charged residues do not generally dominate the PSD spectra, although they occasionally occur. For example, bradykinin and its analogues exhibit enhanced cleavage adjacent to serine residues in the negative mode and arginine in the positive mode. As another example, K_4G_8 shows enhanced fragmentation adjacent to the potentially protonated lysine residues in the positive mode; in the negative mode, where the lysine residues are unlikely to be charged, cleavages adjacent to lysine are present but of the same general abundances as cleavages elsewhere on the peptide backbone.

Elimination of neutrals from amino acid side chains also reflect differing charge locations. In the negative mode, bradykinin and its analogues gave abundant loss of 30 Da from serine-containing fragments and from the deprotonated molecular ions. This elimination of CH_2O from serine-side chains has been found in other negative ion studies [13,44,45] and the mechanism is believed to involve deprotonation of the $–CH_2OH$ group to produce $–CH_2O^-$. In the positive mode, deprotonation of the serine side chain is highly improbable and no CH_2O elimination is observed.

As another example of charge site effect on side chain cleavages, in the positive PSD spectra, ACTH (11–24) and bradykinin and its analogues yield pronounced fragments having a loss of 17 Da. This corresponds to elimination of NH_3 from a protonated arginine side chain [15,43,46]. For TPK-13, the other peptide under study that contained arginine,

elimination of 17 Da is seen in the molecular ion region but not in the backbone fragmentation; however, the positive PSD spectrum for this peptide is markedly cluttered and difficult to interpret. In the negative PSD spectra, the arginine side chain is no longer protonated and the only ions incorporating loss of 17 Da are from backbone cleavage. However, TPK-13 may be unique because it has three arginine residues; its most significant backbone cleavage ions that include 17 Da loss also incorporate at least two arginine residues. In addition, TPK-13 has three acidic residues and 17 Da loss may, in some cases, correspond to elimination of OH from the carboxylic acid side chains (rather than loss of NH_3 from an arginine side chain).

4.2. *b- and y-ions*

The b- and y-series of ions are formed from cleavage of amide (O=C)–NH linkages along the peptide backbone. The mechanisms for b- and y-formation have been extensively investigated in both the positive and the negative modes. For the peptides under study here, in positive mode PSD, both b- and y-ions formation are often equally dominant, while the negative mode generally shows more y-ions than b-ions.

In the positive mode, "mobile" protons may migrate to various amido N atoms (and possibly O atoms) along the peptide backbone; the presence of this proton weakens the amide bond [19,20,51]. For the positive y-series, which are really $y''_n{}^+$, the ionizing proton remains on the C-terminal side of the amide bond and a proton migrates from the N-terminal section as the amide bond cleaves. In the positive b-series, attack by nearby nucleophilic sites on the weakened amide bond can result in a cyclic oxazolone $b_n{}^+$ structure that incorporates the N-terminal portion of the peptide [52–54].

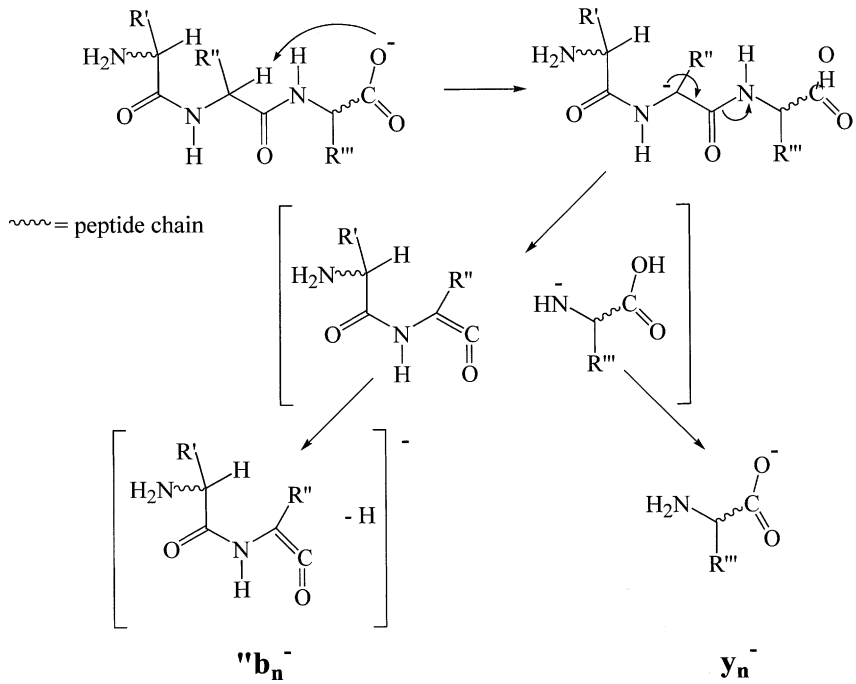
Notable in our data is the presence of $b_1{}^+$ that is a low intensity product for all the peptides under study. For peptides with a lysine residue at the N-terminus, Yalcin and Harrison [55] have shown that $b_1{}^+$ is a protonated α-amino-ε-caprolactam rather than a linear acylium ion; that is, a seven-membered ring cyclic structure is formed by attack of the amino group from lysine on the C-terminal carbonyl carbon. Ab initio calculations on $b_2{}^+$ containing arginine, lysine, and histidine residues suggest that the ions have cyclic structures that involve the basic residue's side chain [56]. Thus, it is possible that the $b_1{}^+$ observed in the present study for peptides with basic N-terminal residues may also have a cyclic structures that incorporate the basic side chain.

In the negative mode, fragmentation involving the amide bond has been studied by Bowie and co-workers [11–14] and Harrison [9]. Cleavage of the amide bond to form $y_n{}^-$ and $''b_n{}^-$ is a competitive mechanism and is illustrated in Scheme 1, which was adapted from the work of Bowie and co-workers [11–14]. (However, that this group refers to $y_n{}^-$ as α-ions and $''b_n{}^-$ as β-ions.)

The mechanism of Scheme 1 results in a negative b-ion that contains two fewer hydrogens than the corresponding positive b-ion. However, in the present study, b-ions are found to have the same masses in both the positive and negative modes. This has also been observed in earlier PSD studies in our laboratory [15,16] and in various MS/MS studies by Bradley et al. [57,58] and Yang and Wilkins [59]. In contrast, several other studies [9,60–63], including other work in our laboratory [64], have found $''b_n{}^-$ to have two hydrogens less than the $b_n{}^+$ series. In addition, a recent study of multiply charged negative ions in our laboratory [65] suggests that two series of b-ions may exist in the negative mode, $''b_n{}^-$ and $b_n{}^-$.

The types of residues present in the peptide may play a role in determining which b-series occurs in the greatest abundance. The current work study suggests that the presence of basic residues on the N-terminal side of the peptide may lead to the more highly hydrogenated (i.e., more saturated) form of the ion, $b_n{}^-$. The possibility exists that the nature of the basic residues present in these peptides allows the formation of zwitterionic intermediates that facilitate fragmentation without hydrogen transfer. Further investigations into b-ion formation by negative mode MS/MS are currently underway in our laboratory.

Extensive and abundant series of y-ions can be found in both the positive and the negative PSD



Scheme 1.

spectra. However, the positive ions, y''^{+}_n, are two Daltons more massive than the corresponding negative ions, y_n^-. This mass differentiation is useful in interpreting the spectra because it allows more facile identification of y-ions.

4.3. c-Ions

A noteworthy feature of the negative ion PSD spectra is the c_n^--series, which involves cleavage of N–C^{α} linkages along the peptide backbone. These ions are two Daltons lower in mass than the c''^{+}_n that are formed in positive mode MS/MS spectra of peptides. Also, in positive mode PSD and CID experiments, c-ions are rarely found. Instead, they are most often produced by methods that have been described as "energy-sudden desorption" [66], including FAB [67,68] and in-source decay (ISD) MALDI/TOF [66,69–73]. Ions from the c-series are also prevalent in the electron capture dissociation (ECD) spectra of peptides [74–76]. A common feature of FAB, ISD, and ECD is that fragmentation occurs rapidly, on the ionization timescale and prior to complete energy randomization [66]. For both ISD [66] and ECD [75], the proposed mechanism for c-ion formation involves cleavage alpha to a transient hypervalent site of the type HO–$C^{\bullet}$, which is formed by hydrogen transfer to a carbonyl oxygen on the peptide backbone.

The peptides under study here do not form c-ions by positive mode PSD. However, all peptides showed c_n^- formation by negative PSD. Complete or near complete c_n^- series were found for ACTH (11–24), the glycine–lysine dodecapeptides, and the histidine-containing renin inhibitor peptide; a common feature of these peptides is their lack of residues containing side chains with carboxylic acid or alcohol functional groups that might be deprotonated. In contrast, the peptides with acidic side chains (i.e., bradykinin and its analogues and TPK-13) showed less intense and less complete series of c_n^- but more complex fragmentation related to the acidic site. In addition, we reported very little c-ion formation in

previous studies of highly acidic peptides that were investigated by PSD on $[M–H]^-$ [16] and low energy CID on $[M - nH]^{n-}$ [65]. Also, c-ions have not been found in the numerous high energy CID studies of peptide negative ions by Bowie and co-workers [11–14].

The present study is not the only report of negative c-ion formation, however. Harrison [9] observed c_n^- in the low energy CID of $[M–H]^-$ from di- to penta-peptides containing only residues with hydrogen or alkyl side chains (i.e., glycine, alanine, and leucine). Also, although the peaks in the published spectra are not labeled as such, c_n^- may be present in two low energy CID studies of moderate size peptides in a QTOF mass spectrometer [62,77]. It appears that c_n^- are found in the dissociation of $[M–H]^-$ from basic and neutral peptides, but are not commonly produced from acidic peptides. Our previous studies of acidic peptide negative ion dissociation [16,65] revealed that fragmentation is dominated by b- and y-type cleavages adjacent to the acidic residues; thus, charge-directed fragmentation is occurring adjacent to deprotonated side chains. Consequently, a driving force in c_n^- formation for basic and neutral peptides may be the *absence* of acidic side chains (i.e., deprotonated sites).

The mechanism for negative c-ion formation has not yet been established and it is unclear how closely it relates to the positive mode mechanism. We are currently in the process of studying simpler peptide systems in order to gain a better understanding of this mechanism. However, several pieces of information are known. First, the near absence of c_n^- in the MS/MS spectra of acidic peptides indicates that a deprotonated carboxylic acid group is not a major mechanistic participant. This also suggests a lack of involvement (aside from charge migration) from the deprotonated C-terminal carboxylic acid group. Second, side chain functional groups are not involved in the mechanism because c-ions are produced in the vicinity of glycine residues, where the side chain is only a hydrogen atom. Third, observation of the smallest member of the series, c_1^-, means that a c-ion can incorporate a single residue. Fourth, observation of the largest possible c-ion for several peptides means that the corresponding neutral loss can be as small as CHR(C=O)OH. Thus, based upon these third and fourth points, there is no expectation of a cyclic structure incorporating portions of two or more residues for either the c_n^- or its corresponding neutral elimination. Fifth, based upon the work of Harrison [9] there is reason to believe that a deprotonated amide nitrogen may be involved in the mechanism. Harrison studied the dissociation of $[AAG–H]^-$, which had deuterium labeling at the labile hydrogen sites (i.e., hydrogens attached to oxygen and nitrogen). The N-terminal portion of the peptide that involves c_1^- would have three of these initial labels. The CID spectra revealed that, while a considerable population of c_1^- retained three deuteriums, a significant amount of c_1^- had only two deuteriums. This indicates that some hydrogen scrambling has occurred between a site on a portion of the peptide that became the N-terminal ion and a site on the C-terminal neutral portion. Sixth, Harrison [9] found that c-ion production is a higher energy process than is generation of a-, b-, or y-ions. This is consistent with our results showing c-ions to be the major N-terminal fragments in PSD, which is a relatively high energy technique [21,78]. The fact that c_n^- formation requires higher energies is also consistent with a mechanism utilizing a deprotonated amide nitrogen. The proposed mechanism for b- and y-ions formation (Scheme 1) involves a deprotonated methylene carbon along the peptide backbone. However, only slightly more energy is required to abstract a proton from the amide nitrogen. (For a CH group adjacent to a carbonyl function and an NH group adjacent to a carbonyl, the difference in $\Delta G^\circ_{\text{acid}}$ is only on the order of 5 kcal/mol [79].) Therefore, c-ions may be preferred products when backbone amide hydrogens are abstracted, while b- and y-ions may be favored when backbone methylene hydrogens are abstracted.

Another possibility is that c_n^- formation is independent of the charge site; that is, a charge-remote fragmentation [80]. Charge-remote fragmentation is known to occur during PSD [78,81]. In support of a charge-remote process, for the ISD of several peptides, Brown et al. [70] found that the positive and negative mode dissociation were very similar with c-ions

occurring at both polarities. Takayama and Tsugita [72] studied the positive and negative ISD of porcine pancreastatin (33–49) and found that cleavage of the N–C^{α} bond dominated both spectra, although in the positive mode this yielded z-ions while the negative mode gave exclusively c-ions. These reports suggest that in ISD, where ion formation by MALDI and dissociation occur virtually simultaneously, N–C^{α} bond cleavage may be independent of the charge state and charge site of the molecular ion. However, it should be noted that an argument against a charge-remote mechanism is the fact that c_n^- were also observed in Harrison's low energy CID studies of deprotonated peptides [9]. Although charge-remote fragmentation can occur during low energy CID, it is relatively rare in low energy processes [80].

The possibility of $M^{\bullet -}$ providing a slight contribution to the c_n^- formation should also be considered. In general, we found that MALDI produced ions one Dalton in higher in mass than $[M–H]^-$ at intensities that were ca. 10–20% elevated over the intensities expected from the native ^{13}C and ^{2}D isotopic contributions. (The exception to this was the glycine–lysine dodecapeptides where, within experimental error, the experimental data matched the theoretical intensities for the isotopic peak cluster.) The presence of an $M^{\bullet -}$ radical species might lead to the formation of the c-series by a transient hypervalent radical mechanism similar to that proposed for ISD [66] and ECD [75].

5. Conclusions

Basic peptides readily form negative ions, $[M–H]^-$, by MALDI. Although not as abundant as the corresponding positive ions, $[M+H]^+$, these negative ions are of sufficient intensity for PSD studies. The y-ion series is generally seen in both positive and negative modes and the two Dalton difference in mass between $y_n''^+$ and y_n^- can be useful in identifying these ions. Production of c_n^- and, to a lesser extent, a_n^- are also notable in negative mode PSD, while b_n^+ are produced from positive ions. When a peptide contains a mixture of acidic and basic residues, the negative PSD spectra are more complex and the locations of acidic residues dictate some fragmentations. The most extensive and abundant production of c_n^- occurs in peptides with no acidic residues. Thus, the mechanism for c-ion formation does not involve a deprotonated side chain, but may invoke a mobile deprotonation site at amide sites of the peptide backbone or may possibly involve a charge remote cleavage. In summary, negative ion PSD of basic peptides yields structurally informative spectra that complement the positive data. Even highly basic peptides, such as ACTH (11–24), can be studied in the negative ion mode.

Acknowledgements

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