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Conformations of Protonated Gas-phase Bradykinin Ions: Evidence for Intramolecular Hydrogen Bonding

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The post-source decay of bradykinin, Lys[1]-bradykinin, des-Arg[1]-bradykinin, des-Arg[9]-bradykinin and [D-Phe[7]]-bradykinin $[M + H]^+$ ions was examined in order to assertain the influence of secondary structure on peptide ion dissociation. Fragment ions corresponding to the elimination of H_2O and HN=C=NH are observed in the product ion mass spectra of Lys[1]-bradykinin and des-Arg[1]-bradykinin but not in the spectra of bradykinin or des-Arg[9]-bradykinin. Cleavage reactions at the Phe–Ser and/or Ser–Pro bonds are observed for all peptide $[M + H]^+$ ions with the exception of des-Arg[9]-bradykinin. The product ions arising from the processes described above are rationalized in terms of the intramolecular solvation of the protonated guanidino groups of the arginines. The strongest intramolecular interaction appears to be a proton bridge between the guanidino groups of the *N*- and *C*-terminal arginines in bradykinin. In addition, increased abundances of fragment ions in the vicinity of Ser–Pro may be attributed to intramolecular solvation of the protonated *C*-terminal guanidino group by the Ser-Pro portion of the molecule. This self-solvation of the ionizing proton leads to a gas-phase peptide conformation that is supported by solution-phase NMR studies at elevated temperatures and in non-polar solvents but which is different from the conformation in polar solvents. Copyright © 1999 John Wiley & Sons, Ltd.

KEYWORDS: matrix-assisted laser desorption/ionization; post-source decay; hydrogen bonding; bradykinin; secondary structure

INTRODUCTION

Tandem mass spectrometry is a powerful technique for the structural characterization of peptides owing to the sensitivity of the method (generally not more than femtomoles of sample are required) and the wealth of information that is obtained.[1] The instrumentation cost required for tandem mass spectrometry has been reduced considerably by the introduction of reflection time-of-flight (R-TOF) methods of separating and analyzing post-source decay (PSD) product ions of the intact peptide, typically the protonated peptide $[M + H]^+$ formed by matrix-assisted laser desorption/ionization (MALDI).[2] The combination of MALDI and R-TOF permits structural characterization using high-sensitivity and relatively simple, low-cost instrumentation.

Peptide sequencing using tandem mass spectrometry is accomplished by forming $[M + H]^+$ ions with a distribution of internal energies such that some fraction of the ions dissociate. Alternatively, stable $[M + H]^+$ ions can be activated to energies above the dissociation threshold by collisions with background gases or by the absorption of photons. In general, peptide $[M + H]^+$ ions fragment randomly along the peptide backbone resulting in a series of fragment ions separated by masses corresponding to the amino acid sequence. Questions concerning the influence of amino acid composition, location of the charge site(s) and interactions between charge sites on ion dissociation have motivated considerable research.[3–8] For example, gas-phase dissociation reactions of organo-alkali ions $[M + X]^+$ (M = small peptides and X = Na^+, K^+, Rb^+ or Cs^+) have been studied extensively and arguments made as to the site of cation binding and whether the binding sites mimics that of the solution phase ionic species.[4–6] Of equal interest to the analysis of larger peptides is the

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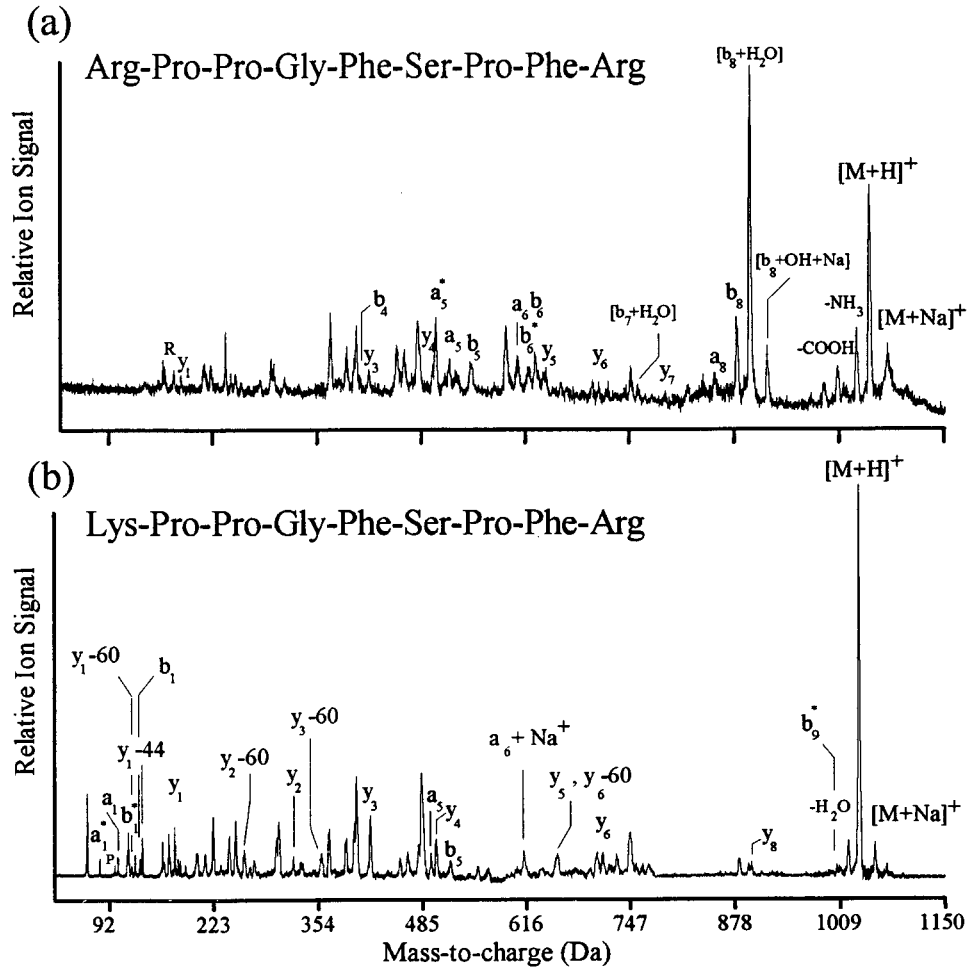


Figure 1. PSD mass spectra obtained for (a) bradykinin (M_r 1059.6 Da) $[M + H]^+$ ions and (b) Lys^1-bradykinin (M_r 1031.6 Da) $[M + H]^+$ ions generated using the 337 nm MALDI matrix α-cyano-4-hydroxycinnamic acid.

influence of higher order structure on the ionization of the compound and on the dissociation chemistry. Clearly, the solvent plays a very important role in determining the higher order structure of large peptides in solution but gas-phase ions lack the stabilizing influence of solvent. Hence the important intramolecular interactions of gas-phase ions are (1) intramolecular hydrogen bonding between amino acid residue side-chains and (2) cation-induced conformational changes. For example, Hunt *et al.*[9] suggested that the ionizing proton is internally solvated by intramolecular hydrogen bonds involving amide linkages; hence the location of the charge site can directly influence the structure of the gas-phase ion. Intramolecular solvation of the proton lowers the total energy of the ionized peptide system by $\sim$ 7–8 kcal mol^{-1} per hydrogen bond (1 kcal = 4.184 kJ) More recent studies by the groups of Williams, Lebrilla and Fenselau underscore this point.[10] Any similarities between gas-phase and solution-phase conformations may reflect the intrinsic stability conferred to the peptide by non-covalent interactions, whereas differences may arise if solvent molecules play an important role in the determining secondary or higher order structure and chemical reactivity.[11]

The results of several studies have been interpreted as evidence that the gas-phase conformation of a peptide ion affects the nature of dissociation product ions observed in tandem mass spectrometric experiments.[12,13] For example, Thorne *et al.*[12] showed that changing Arg^1 to Lys^1 in the peptide bradykinin $[M + H]^+$ ion (H_2N–Arg^1–Pro^2–Pro^3–Gly^4–Phe^5–Ser^6–Pro^7–Phe^8–Arg^9–OH) markedly decreased the *C*-terminal rearrangement product $[b_8 + H_2O]^+$ ion. The changes observed in the abundance of fragment ions may have arisen from changes in the site of protonation (e.g. Lys^1-bradykinin is preferentially protonated at the *C*-terminal arginine owing to the lower basicity of lysine) or differences in the *N*-terminal conformation of the two peptide ions. A recent study by Glish and co-workers[14] attributed the differences in dissociation reactions of peptide ions containing arginine (e.g. bradykinin) to charge-remote fragmentation reactions. They also suggested that the fragmentation reactions of the arginine-containing peptides may be due to some higher order structure of the gas-phase ions. Glish and co-workers suggested that the greater amount of energy required to dissociate arginine-containing peptides is due to interactions between the arginine side-chain and adjacent amino acid residues. Probably the most compelling evidence of the influence of higher order structure has been the recent work of the Williams and co-workers[10a] in which they attributed conformational

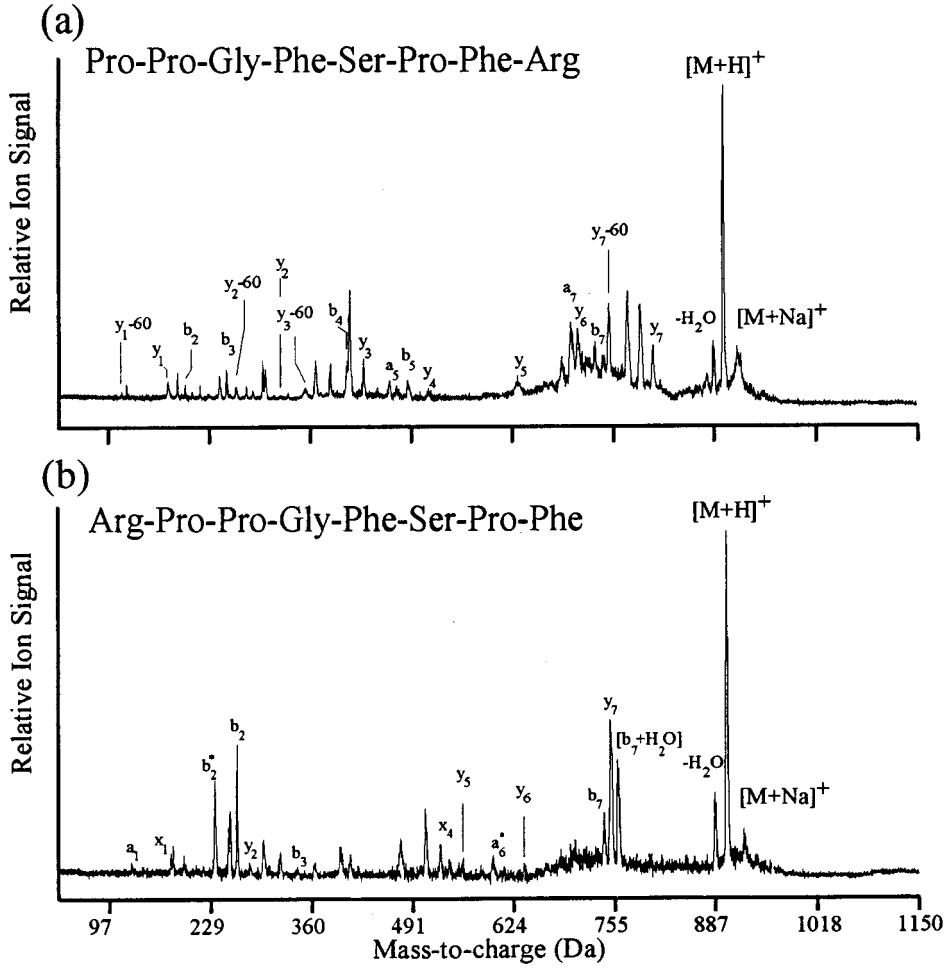


Figure 2. PSD mass spectra obtained for (a) des-Arg^1-bradykinin (M_r 903.4 Da) $[M + H]^+$ ions and (b) des-Arg^9-bradykinin (M_r 903.4 Da) $[M + H]^+$ ions generated using the 337 nm MALDI matrix α-cyano-4-hydroxycinnamic acid.

changes to the formation of salt bridges. Furthermore, Williams and co-workers suggested that the structure of bradykinin $[M + H]^+$ ions is best represented as a protonated guanidino group of Arg^1 and a zwitterionic Arg.[9] The stabilization of the charged species is accounted for by the formation of salt bridges. Bowers and co-workers[15] used ion mobility measurements and molecular mechanics to characterize bradykinin ion in terms of geometric size. Their results suggest that both guanidino groups of the arginines are strongly involved in the charge-carrying capacity as are four of the carbonyl oxygens.

In this work, we explored the PSD of bradykinin $[M + H]^+$ ions formed by MALDI in detail. Using the aforementioned studies as a starting point, the influence of the *N*- and *C*-terminal arginine residues on the observed ionic products was examined by comparing the PSD product ion mass spectra of bradykinin with those of three structural analogues: Lys^1-bradykinin (M_r 1032.6 Da), des-Arg^1-bradykinin (M_r 903.4 Da) and des-Arg^9-bradykinin (M_r 903.4 Da). In addition, we report the PSD of [D-Phe^7]-bradykinin (M_r 1110.7 Da). All sequence-specific and internal fragment ions are reported. We assume that peptide $[M + H]^+$ ion metastable decomposition occurs via the generally accepted mechanisms.[9,16–19] The ionic products are rationalized in terms of gas-phase interactions involving the *N*- and *C*-terminal amino acid residues. Finally, the known solution phase conformation and conformations suggested on the basis of ion mobility measurements are compared with our proposed gas-phase ion conformation(s).

EXPERIMENTAL

The two-stage R-TOF mass spectrometer has been described in detail elsewhere (M. E. Gimon-Kinsel, G. R. Kinsel, D. C. Barbacci and D. H. Russell, *J. Am. Soc. Mass Spectrom.* submitted for publication). Briefly, the peptide $[M + H]^+$ ions were generated using the standard 337 nm MALDI matrix α-cyano-4-hydroxycinnamic acid. The positive ions were extracted from the ion source by applying a large positive voltage to the repeller plate. A fraction of the peptide $[M + H]^+$ ions undergo PSD in the field-free flight tube prior to entering the two-stage reflectron. The lower molecular mass PSD product ions are separated in time and space from the precursor peptide $[M + H]^+$ ion by systematically reducing the voltage applied to the two-stage reflectron. The resultant product ions scans were stitched together to form the complete metastable ion

Table 1. Post-source decay of $[M + H]^+$ ions to yield b-type and y-type sequence ions

Peptide	X–X[a]	b_m ion	(%)[b]	y_{n-m} ion	(%)[b]	$(b_m + y_{n-m})/\sum(b_m + y_{n-m})$ (%)
Bradykinin						
(M_r 1059.6 Da)	Phe^8–Arg^9	b_8	6	y_1	0.4	43
	Pro^7–Phe^8	b_7	—	y_2	—	0
	Ser^6–Pro^7	b_6	2	y_3	1	20
	Phe^6–Ser^6	b_5	1	y_4	1	14
	Gly^4–Phe^5	b_4	1	y_5	1	14
	Pro^3–Gly^4	b_3	—	y_6	0.5	3
	Pro^2–Pro^3	b_2	—	y_7	0.4	3
	Arg^1–Pro^2	b_1(R)	0.4	y_8	—	3
Lys^1-bradykinin						
(M_r 1032.6 Da)	Phe^8–Arg^9	b_8	—	y_1	2	15
	Pro^7–Phe^8	b_7	—	y_2	1	8
	Ser^6–Pro^7	b_6	—	y_3	3	23
	Phe^5–Ser^6	b_5	1	y_4	2	23
	Gly^4–Phe^5	b_4	—	y_5	1	8
	Pro^3–Gly^4	b_3	—	y_6	1	8
	Pro^2–Pro^3	b_2	—	y_7	—	0
	Lys^1–Pro^2	b_1	1	y_8	1	15
Des-Arg^1-bradykinin						
(M_r 903.4 Da)	Phe^7–Arg^8	b_7	2	y_1	1	12
	Pro^6–Phe^7	b_6	—	y_2	0.4	2
	Ser^5–Pro^6	b_5	2	y_3	3	19
	Phe^4–Ser^5	b_4	2	y_4	0.5	10
	Gly^3–Phe^4	b_3	1	y_5	3	15
	Pro^2–Gly^3	b_2	1	y_6	6	27
	Pro^1–Pro^2	b_1	—	y_7	4	15
Des-Arg^9-bradykinin						
(M_r 903.4 Da)	Pro^7–Phe^8	b_7	5	y_1	—	14
	Ser^6–Pro^7	b_6	0.4	y_2	1	4
	Phe^5–Ser^6	b_5	—	y_3	—	0
	Gly^4–Phe^5	b_4	1	y_4	—	3
	Pro^3–Gly^4	b_3	1	y_5	2	8
	Pro^2–Pro^3	b_2	9	y_6	0.4	26
	Arg^1–Pro^2	b_1	—	y_7	17	46
[D-Phe^7]-bradykinin						
(M_r 1110.7 Da)	Phe^8–Arg^9	b_8	10.5	y_1	0.1	60
	Phe^7–Phe^8	b_7	1.2	y_2	0.2	8
	Ser^6–Phe^7	b_6	0.1	y_3	0.2	2
	Phe^5–Ser^6	b_5	0.1	y_4	0.5	3
	Gly^4–Phe^5	b_4	0.2	y_5	0.3	3
	Pro^3–Gly^4	b_3	—	y_6	1.8	10
	Pro^2–Pro^3	b_2	0.2	y_7	2.1	13
	Arg^1–Pro^2	b_1	0.1	y_8	—	1

[a] Amide bond broken.
[b] Per cent PSD product ion current.

spectrum. Note that no precursor ion selection was implemented in these experiments. Hence peptide $[M + H]^+$, $[M + Na]^+$ and prompt fragment ions generated in the source could enter the reflectron and act as precursor ion to the reflected product ions observed as the voltages are decreased. In the linear mode, no prompt fragment ions were observed, eliminating the need to consider such ions as potential precursors. In this work the post-source decay (PSD) product ions produced by each peptide were collected under nearly identical experimental conditions (e.g. 15.0 kV repeller place voltage, 10^{-8} Torr vacuum pressure (1 Torr = 133.3 Pa) and 8–12 mJ cm^{-2} desorbing laser irradiance).

RESULTS

Figure 1 shows the PSD product ion mass spectra of (a) bradykinin and (b) Lys^1-bradykinin $[M + H]^+$ ions obtained using the experimental method described above. Sequence ions are denoted using the nomenclature proposed by Roepstorff and Fohlmann[20] and later modified by Biemann.[21] The major fragment ion (m/z 904.3) in the PSD product ion mass spectrum of bradykinin $[M + H]^+$ is assigned to sequence ions y_8 and or $[b_8 + H_2O]^+$, which have identical masses. On the basis of isotopic labeling data obtained for bradykinin by Gaskell and co-workers,[7] we assigned the m/z 904

Table 2. Post-source decay of bradykinin $[M + H]^+$ ions to yield internal fragment ions

Peptide	Product ion	Sequence[a]	*m/z*	(%)[b]
RPPGFSPFR + H_2O	$(b_8y_8)_7 + H_2O$	PPGFSPF + H_2O	747.6	2
	$(b_8y_7)_6$	PGFSPF	632.6	2
	$(b_7y_8)_6$	PPGFSP	583.0	1
	$(b_6y_8)_5$, $(b_7y_7)_5$	PPGFS, PGFSP	485.6	3
	$(b_6y_8)_5$, $(b_7y_7)_5 - 18$	(PPGFS, PGFSP) − 18	467.4	1
	$(b_9y_3)_3$	PFR	400.9	2
	$(b_7y_6)_4$, $(b_6y_7)_4$	GFSP, PGFS	388.6	1
	$[(b_7y_6)_4, (b_6y_7)_4] - 18$	(GFSP, PGFS) − 18	370.8	2
	$(b_7y_5)_3$, $(b_8y_4)_3$	FSP, SPF	332.6	0.3
	$[(b_7y_5)_3, (b_8y_4)_3] - 18$	(FSP, SPF) − 18	315.0	0.5
	$(b_9y_2)_2$	FR	303.8	1
	$(b_5y_7)_3$	PGF	301.8	1
	$(b_4y_8)_3$	PPG	251.6	0.5
	$(b_8y_3)_2$	PF	244.6	0.4
	$(b_6y_5)_2$	FS	235.2	1
	$(b_5y_6)_2$	GF	204.5	1
	$(b_7y_4)_2 - 18$	SP − 18	167.6	0.4
	$(b_9y_1)_1$	R	156.6	0.4
	$(b_4y_7)_2$	PG	155.2	0.3
	$[(b_3y_7)_1, (b_2y_8)_1, (b_7y_3)_1] - 28$	P − 28	69.9	0.1

[a] Using single letter amino acid residue nomenclature.
[b] Per cent PSD product ion current.

ion signal to the $[b_8 + H_2O]^+$ ion. An ion signal corresponding to this type of rearrangement product (*m/z* 876) is noticeably absent from the PSD product ion mass spectrum of Lys[1]-bradykinin $[M + H]^+$ ions. In addition to sequence ions, a large number of 'internal' fragment ions are observed (i.e fragments that contain neither the original *N*- nor *C*-terminal amino acid residues). The internal fragment ions can be represented by a two-letter code as $(b_l y_m)_n$ where the subscripts *l* and *m* denote the amide bonds cleaved to form the *C*- and

Table 3. Post-source decay of Lys[1]-bradykinin $[M + H]^+$ ion to yield internal fragment ions

Peptide	Product ion	Sequence[a]	*m/z*	(%)[b]
PPGFSPFR + H_2O	$(b_9y_8)_8$	PPGFSPFR	886.6	1
	$(b_8y_8)_7 + H_2O$	PPGFSPF + H_2O	748.0	3
	$(b_8y_8)_7$	PPGFSPF	730.0	2
	$(b_8y_7)_6$	PGFSPF	633.4	1
	$(b_7y_8)_6$	PPGFSP	583.2	1
	$(b_7y_8)_6 - 18$	PPGFSP − 18	564.2	1
	$(b_6y_8)_5$, $(b_7y_7)_5$	PPGSF, PGFSP	485.7	8
	$(b_8y_5)_4$	FSPF	478.8	1
	$[(b_6y_8)_5, (b_7y_7)_5] - 18$	(PPGFS, PGFSP) − 18	485.7	1
	$(b_9y_3)_3$	PFR	401.2	5
	$(b_5y_8)_4$	PPGF	397.8	1
	$(b_6y_7)_4$, $(b_7y_6)_4$	PGFS, GFSP	389.0	1
	$[(b_6y_7)_4, (b_7y_6)_4] - 18$	(PGFS, GFSP) − 18	371.0	3
	$(b_7y_5)_3$, $(b_8y_4)_3$	FSP, SPF	332.1	2
	$(b_9y_2)_2$	FR	303.6	4
	$(b_5y_7)_3$	PGF	301.5	3
	$(b_4y_8)_3$	PPG	252.9	3
	$(b_8y_2)_2$	PF	245.2	2
	$(b_5y_6)_2$	GF	205.8	2
	$(b_3y_8)_2$	PP	196.3	0.5
	$(b_7y_4)_2$	SP	185.2	1
	$(b_7y_4)_2 - 18$	SP − 18	167.8	1
	$(b_9y_1)_1$	R	156.5	0.3
	$(b_4y_7)_2$	PG	155.1	2
	$[(b_5y_5), (b_8y_2)_1] - 28$	F − 28	120.4	1
	$(b_2y_8)_1$, $(b_3y_7)_1$, $(b_7y_3)_1$	P	97.8	0.3
	$[(b_2y_8)_1, (b_3y_7)_1, (b_7y_3)_1] - 28$	P − 28	69.8	1

[a] Using single letter amino acid residue nomenclature.
[b] Per cent PSD product ion current.

Table 4. Post-source decay of des-Arg^1-bradykinin $[M + H]^+$ ion to yield internal fragment ions

Peptide	Product ion	Sequence[a]	*m/z*	(%)[b]
PPGFSPFR + H_2O	$(b_8y_7)_7$	PGFSPFR	789.0	6
	$(b_8y_7)_7 - 18$	(PGFSPFR) − 18	771.0	7
	$(b_8y_6)_6$	GFSPFR	692.7	4
	$(b_6y_7)_5 - 18$	PPGFS − 18	467.8	1
	$(b_8y_3)_3$	PFR	400.8	7
	$(b_5y_7)_4$, $(b_6y_6)_4$	PGFS, GFSP	388.6	1
	$[(b_5y_7)_4, (b_6y_6)_4] - 18$	(PGFS, GFSP) − 18	371.4	1
	$(b_6y_5)_3$, $(b_7y_4)_3$	FSP, SPF	332.2	0.4
	$(b_8y_2)_2$	FR	303.5	2
	$(b_4y_7)_3$	PGF	301.9	2
	$(b_7y_3)_2$	PF	244.5	2
	$(b_5y_5)_2$	FS	235.1	1
	$(b_4y_6)_2$	GF	205.0	0.3
	$(b_6y_4)_2$	SP	184.6	1
	$[(b_4y_5)_1, (b_7y_2)_1] - 28$	F − 28	120.3	0.4

[a] Using single letter amino acid residue nomenclature.
[b] Per cent PSD product ion current.

N-terminus, respectively, of the fragment ion and *n* denotes the number of amino acid residues contained in the fragment ion.[20] Finally, note the overall increase in number and abundance of fragment ions observed in the Lys^1-bradykinin PSD product ion mass spectrum as compared with bradykinin.

Figure 2 shows the PSD product ion mass spectra of (a) des-Arg^1-bradykinin and (b) des-Arg^9-bradykinin $[M + H]^+$ ions. The arginine residue is located at the *C*-terminus in des-Arg^1-bradykinin and at the *N*-terminus in des-Arg^9-bradykinin. The PSD product ion mass spectrum of des-Arg^1-bradykinin $[M + H]^+$ ions is dominated by a series of fragment ions between *m/z* 700 and 800 and a fragment ion at *m/z* 401. Several low-abundance, low-mass fragment ions are also observed. A much cleaner PSD product ion mass spectrum is produced by des-Arg^9-bradykinin $[M + H]^+$ ions. The dominant ions are at *m/z* 757 ($[b_7 + H_2O]^+$), 748 (y_7), 739 (b_7), 505, 254 (b_2), 250, and 237 ($b_2 - 17$).

Table 1 summarizes the relative abundance of the b-type and y-type sequence ions observed for bradykinin and related analogues (including [D-Phe^7]-bradykinin). Tables 2–5 contain the internal fragment ions identified in the PSD product ion mass spectra of bradykinin Lys^1-bradykinin, des-Arg^1-bradykinin and des-Arg^9-bradykinin, respectively.

DISCUSSION

N-Terminal *vs. C*-terminal fragmentation

CID fragmentation products of bradykinin, Lys^1-bradykinin, des-Arg^1-bradykinin and des-Arg^9-bradykinin $[M + H]^+$ ions formed by fast atom bombardment ionization have been reported previously.[7] The ions examined in this study are formed by the fragmentation reactions of MALDI-generated $[M + H]^+$ ions and are assumed to be the result of unimolecular processes. In addition, our work was focused on the structure or conformation of gas-phase bradykinin $[M + H]^+$ ions that have relatively small

Table 5. Post-source decay of des-Arg^9-bradykinin $[M + H]^+$ ions to yield internal fragment ions

Peptide	Product ion	Sequence[a]	*m/z*	(%)[b]
RPPGFSPF + H_2O	$[(b_6y_7)_5, (b_7y_6)_5] + H_2O$	(PPGFS, PGFSP) + H_2O	505.7	2
	$(b_8y_4)_4$	FSPF	478.8	4
	$(b_5y_7)_4$	PPGF	399.4	1
	$(b_6y_6)_4$, $(b_7y_5)_4$	PGFS, GFSP	389.3	2
	$[(b_6y_6)_4, (b_7y_5)_4] - 18$	(PGFS, GFSP) − 18	371.2	1
	$[(b_7y_4)_3, (b_6y_5)_3] - 18$	(FSP, SPF) − 18	314.3	1
	$(b_5y_6)_3$	PGF	301.8	2
	$(b_4y_7)_3$	PPG	251.6	4
	$(b_3y_7)_2$	PP	194.6	1

[a] Using single letter amino acid residue nomenclature.
[b] Per cent PSD product ion current.

but sufficient amounts of internal energy to undergo unimolecular dissociation on the microsecond timescale. We will present arguments based upon the premise that the conformation of gas-phase bradykinin $[M + H]^+$ ions is determined by intramolecular interactions, e.g. hydrogen bonding between the charge-carrying amino acid residues and basic sites of the peptide. Changes in the conformation of the gas-phase ion can be accomplished by altering the amino acid composition of the peptide. We probe the conformational changes that occur in bradykinin analogue that lack an *N*-terminal or *C*-terminal arginine (e.g. Lys^1-, des-Arg^1- and des-Arg^9-bradykinin) and by altering the amino acid residues in the Ser–Pro portion of the peptide.

In general, the presence of an amino acid residue with a highly basic side-chain such as arginine has a strong influence on the dissociation reactions of the peptide $[M + H]^+$ ions, that is, the positive fragment ions chiefly observed are those which contain the basic residue.[22] Assuming that the *N*-terminal and *C*-terminal arginine residues have similar basicities, we expect the *N*- and *C*-terminal sequence ions to appear in roughly equal abundances. The bradykinin $[M + H]^+$ MI mass spectrum contains the b-type sequence ion series including the b_8, b_6, b_5, b_4 and b_1 ions, but the b_7, b_3, and b_2 ions are absent, presumably owing to the presence of Pro^7, Pro^3 and Pro^2. Protonation at proline residues typically result in the formation of y-type fragment ions.[9] The y-type ion series includes y_1, y_3, y_4, y_5, y_6 and y_7 but y_2, formed by cleaving Pro^2–Pro^3, is missing. The b-type sequence ions are slightly more abundant than the y-type fragment ions. This observation reflects the facile loss of the *C*-terminal arginine residue as a neutral to form the b_8 sequence ions ($\sim 6\%$ of the PSD product ions). It is evident from these data that arginine plays a significant role in the fragmentation of bradykinin; however, the absence of b_7, b_3 and b_2 and also y_2 fragment ions illustrates that proline residues also have an important role in the fragmentation process.

The influence of the *N*-terminal side-chain basicity on the PSD of the bradykinin $[M + H]^+$ ions is illustrated by the PSD product ion mass spectrum generated from Lys^1-bradykinin $[M + H]^+$ ions. The lysine side-chain has a lower gas-phase basicity than the arginine guanidino group (228.7–230.3 kcal mol^{-1}),[23] as opposed to 245.2 ± 0.5 kcal mol^{-1},[24] respectively). The b_1 and b_5 sequence ions are the only ions observed that contain the *N*-terminal amino acid residue. An almost complete series of y-type sequence ions (y_7 is missing) is consistent with the ionizing proton being anchored to the *C*-terminus of the peptide (i.e. Arg^9).

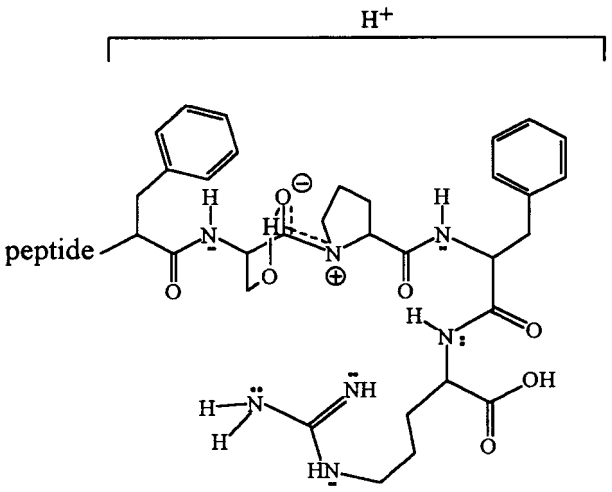
PSD of des-Arg^1-bradykinin $[M + H]^+$ ions leads primarily to the production of y-type sequence ions, indicating that the ionizing proton is preferentially located on the *C*-terminal fragment (again probably on the Arg^9 side chain). Conversely, the influence of the *C*-terminal arginine amino acid residue can be investigated by examining the PSD of des-Arg^9-bradykinin $[M + H]^+$ ions. The observed abundances for b- and y-type sequence ions are roughly equal. This observation appears to be counter intuitive; one would expect the b-type ion to outnumber the y-type fragment ions owing to the strongly basic *N*-terminal arginine. The loss of the *N*-terminal arginine to yield y_7 ions (PPGFSPF) now appears to be highly favored.

Observation of $[y - 60]^+$ fragment ions

A series of moderately abundant ion signals corresponding to a loss of *m/z* 60 from several y-type sequence ions (e.g. $y_2 - 60$, $y_3 - 60$ and $y_6 - 60$) is observed in the Lys^1-bradykinin $[M + H]^+$ ion PSD spectrum. Moreover, we observe $y_1 - 60$, $y_2 - 60$, $y_3 - 60$ and $y_7 - 60$ in the des-Arg^1-bradykinin $[M + H]^+$ PSD mass spectrum. Loss of *m/z* 60 from cationized peptides $[M + X]^+$, where X=Na^+ and Li^+, has been reported by Tang *et al.*[25] for peptides that contain a *C*-terminal arginine residue. The authors' proposed mechanism involved cyclization of a portion of the *C*-terminal arginine side-chain accompanying the loss of H_2O and HN=C=NH. The same reaction was examined by Jennings and co-workers[26] and they also suggested that the neutral loss corresponds to H_2O plus NH=C=NH. The complete absence of this decomposition pathway in the bradykinin $[M + H]^+$ PSD product ion mass spectrum suggests that the *N*-terminal arginine (or at least structural features remote to the reaction site) must be inhibiting reaction.

Fragmentation in the vicinity of the serine residue

The presence of Ser–Pro residues can alter the solution-phase structures of peptides and proteins owing to specific hydrogen bonds and/or electrostatic interactions with other amino acid residue side-chains. The most common structural feature associated with the presence of the Ser–Pro residues is the increased preference for type I β-turns.[27] In terms of gas-phase ion structure, the presence of Ser–Pro residues could increase the ionic character of the imide bond, effectively increasing the basicity of Pro (**I**). We performed *ab initio* calculations (using a progression of basis sets STO-3G → 3–



I

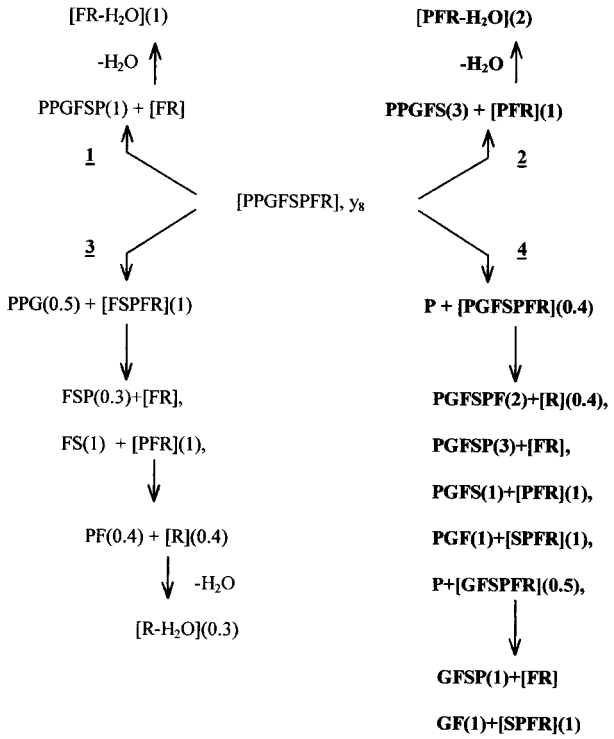
21G → 6–31G*) on the hydrogen bonding between the Ser hydroxyl group and the keto oxygen of Pro using model compounds and found stabilization energies of 6–7 kcal mol^{-1}.[28] It is interesting to consider the effect of changing the *N*-terminal and *C*-terminal amino acid residues on fragmentation reactions occurring in the vicinity of serine. Approximately 34% (see Table 1) of all b- and y-type sequence ions, e.g. $(b_m + y_{n-m})/\sum(b_m + y_{n-m})$, observed in the PSD mass spectrum of bradykinin $[M + H]^+$ ions are formed by breaking the Ser^6–Pro^7 and/or Phe^5–Ser^6 amide linkages. Changing the *N*-terminal arginine residue to a lysine residue results in a slight increase (from 34 to 46%) in sequence ions arising from this source. Removing the *N*-terminal arginine of bradykinin does not change significantly the relative abundance of fragmentation occurring in the vicinity of the serine residue. The fragmentation reactions occurring at Phe^4–Ser^5 and Ser^5–Pro^6 account for ~29% of the dissociating des-Arg^1-bradykinin $[M + H]^+$ ions. The results discussed above might lead to the supposition that serine residues are intrinsically reactive; however, the dramatic change that occurs when the *C*-terminal arginine residue is removed argues against this explanation. Only 4% of the b- and y-type sequence ions in the des-Arg^9-bradykinin $[M + H]^+$ PSD mass spectrum involve cleavage of either the Ser^6–Pro^7 or Phe^5–Ser^6 bonds. This observation is interesting in the light of the fact that substitution of lysine for the *N*-terminal arginine in Lys^1-bradykinin or the complete absence of the *N*-terminal arginine in des-Arg^1-bradykinin appears to have little effect on reactions occurring in the vicinity of serine. The important difference between des-Arg^9-bradykinin and the other bradykinin analogues is the absence of a *C*-terminal arginine residue. Clearly, then, the reactions occurring in the vicinity of the serine residue cannot be explained simply as an 'intrinsically' reactive site. It would appear that *C*-terminal arginine is influential in determining the relative amount of fragmentation at the Ser–Pro and/or Phe–Ser linkages. Changing the proline to D-phenylalanine in [D-Phe^7]-bradykinin yields a similar significant decrease in fragmentation reactions in the vicinity of serine as removal of the *C*-terminal arginine. Only 5% of the b- and y-type sequence ions result from breaking the Phe^5–Ser^6 and/or Ser^6–Phe^7 bonds.

Reactions leading to formation of internal fragment ions, $[b_l y_m]^+$

In addition to the b- and y-type sequence ions, a large number of internal fragment ions are observed in the bradykinin $[M + H]^+$ ion PSD product ion mass spectrum (see Table 2). It appears that a major portion of the internal fragment ions are formed by a sequence of reactions that possibly involve the initial formation of an excited (high internal energy) y_8 ion (*m/z* 904.3) followed by intramolecular transfer of the ionization proton to reactive sites where charge-site induced cleavage reactions occur creating $[b_l y_m]_n$ fragment ions or other y-type sequence ions (Scheme 1). If the *N*-terminal fragment carries the charge, internal fragment ions are generated (species to the left in each product pair). If the *C*-terminal fragment carries the charge, smaller y-type sequence ions are generated (species to the right in each product pair). The internal fragment ions can decompose by loss of CO to form $(a_l y_m)_n$ ions or loss of H_2O if the fragment contains the serine residue. The y-type sequence ions can fragment further to produce smaller internal fragment or y-type sequence ions or lose H_2O. Note that three internal fragment ions have the same mass and contain the same amino acid residues but differ in residue sequence (e.g. PPGFS or PGFSP, PGFS or GFSP and FSP or SPF). For any given pair of isomass internal fragment ions, we are not able to distinguish whether either one or both of these internal fragment ions are present. Regardless of these isomass ions identities, it appears that cleavage of the Ser^6–Pro^7 imide linkage forming the $(b_6 y_8)_5$ ion (PPGFS, 3%) and the y_3 ion (PFR, 1%) and/or the Pro^2–Pro^3 imide linkage forming the proline-immonium ion (P) and y_7 (PGFSPFR, 0.4%) are favored reaction pathways (Scheme 1, pathways 2 and 4, respectively). In addition, the ionizing proton appears to be mobile on the y_7 ion (an unusual observation because the y_7 ion contains a *C*-terminal arginine residue). Fragment ions are observed corresponding to cleavages at all amide linkages with the exception of Gly^4–Phe^5 and the internal fragment ions are slightly more abundant than the corresponding y-type sequence ions. The proline immonium ion (P) is not observed in the PSD product ion mass spectrum; however, a signal corresponding to loss of CO, P-28, is present at *m/z* 70 (0.1%). The remaining internal fragment ion $[(b_8 y_8)_7 + H_2O]^+$ must arise from a different source. This is nominally a y-type ion and can be described as $[b_8 + H_2O - Arg^1]^+$, which can be formed by loss of the *N*-terminal arginine residue from the $[b_8 + H_2O]^+$ ion.

Several internal fragment ions are also observed in the Lys^1-bradykinin PSD product ion mass spectrum (Table 3). A reaction flow chart can also be used to account for the observed internal fragment ions (Scheme 2). As is the case for bradykinin, the formation of a high internal energy y_8 ion (*m/z* 904.3) appears to be required to initiate the series of reactions. The internal fragment ion(s) PPGFS and/or PGFSP (*m/z* 486) are the most abundant internal fragment ion(s) (8%). The presence of one (or both) of these fragments suggests that cleavage *N*-terminal to Pro^7 and/or Pro^3 is a favored process. The y_7 ions formed by cleavage of the Pro^2–Pro^3 bond appear to be very reactive (note that no y_7 ion signal is observed in the Lys^1-bradykinin PSD product ion mass spectrum) and internal fragments arise from dissociation at every amide linkage (Scheme 2, reaction pathway 5). The relative abundance of internal fragment ions arising from Lys^1-bradykinin y_7 ion decomposition is higher relative to those attributed to decomposition of the bradykinin y_7 ions. This observation may reflect that the y_7 ions derived from the two sources have different internal energies.

The internal fragment ions observed when the *N*-terminal arginine residue is removed are shown in Table 4. The des-Arg^1-bradykinin $[M + H]^+$ ion is nominally the same as the putative y_8 sequence ion produced by fragmentation of either bradykinin or Lys^1-bradykinin $[M + H]^+$ ions. The PSD product ion mass spectrum of des-Arg^1-bradykinin is dominated by internal frag-



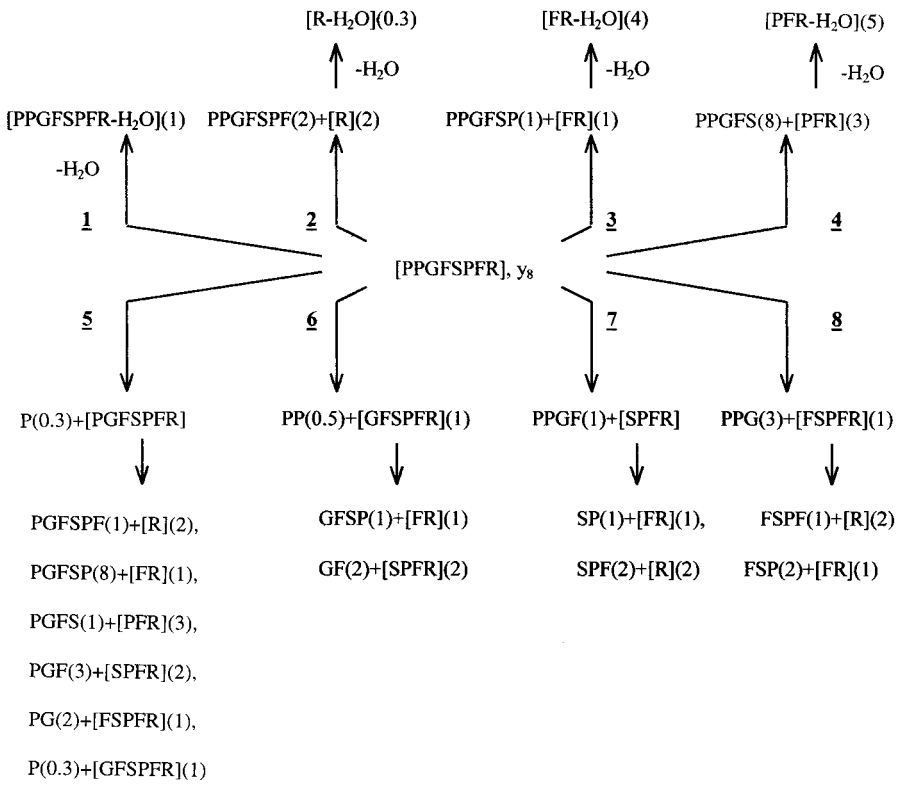
Scheme 1. Reactions yielding internal fragment ions observed in the PSD product ion spectrum of bradykinin $[M + H]^+$ ions. Relative abundances observed appear in parentheses and dominant reaction pathways in bold.

ment ions that can be rationalize in terms of fragmentation of a single ionic species, a high internal energy y_7 ion [PGFSPER] (see Scheme 3). The y_7 ion is one of the ionic species from which we suggest the majority of the internal fragment ions are derived in the metastable decay of bradykinin (Scheme 1, reaction pathway 4) and Lys[1]-bradykinin $[M + H]^+$ (Scheme 2, reaction pathway 5). Only one very low-intensity internal fragment ion signal in the PSD product ion mass spectrum of des-Arg[1]-bradykinin can be attributed to decomposition by another mechanism. This ion has m/z 468 and is assigned to $(b_6V_7)_5 - 18$ (i.e. PPGFS $- H_2$)O.

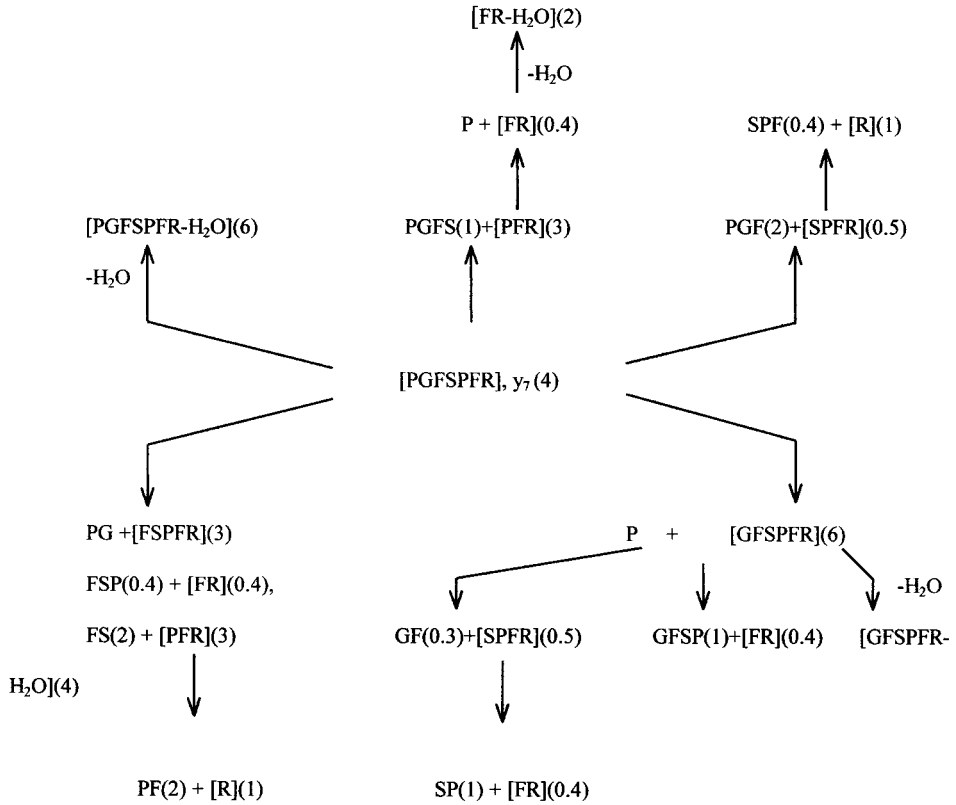
When the *C*-terminal arginine residue is removed, few internal fragment ions are observed in the PSD product ion mass spectrum (see Scheme 4). This observation suggests that the des-Arg[9]-bradykinin $[M + MH]^+$ ions from the MALDI are relatively stable (low internal energy) ionic species. Arguments can be made in support of this assertion based on the observed stability of the $[b_8 + H_2O]^+$ rearrangement product ion in the bradykinin $[M + H]^+$ PSD product ion mass spectrum. The PSD of the des-Arg[9]-bradykinin $[M + H]^+$ ion that is observed may be attributed to differences in internal energies between the $[M + H]^+$ ion generated directly during MALDI and the $[b_8 + H_2O]^+$ rearrangement product ion of bradykinin $[M + H]^+$ ions.

Solution-phase conformation *vs.* gas-phase conformation

The solution-phase secondary structure and biological activity of bradykinin have been thoroughly investigated.[29] The three proline residues Pro[2], Pro[3] and Pro[7] are responsible for introducing some conformational rigidity into the solution-phase peptide. In an acidic

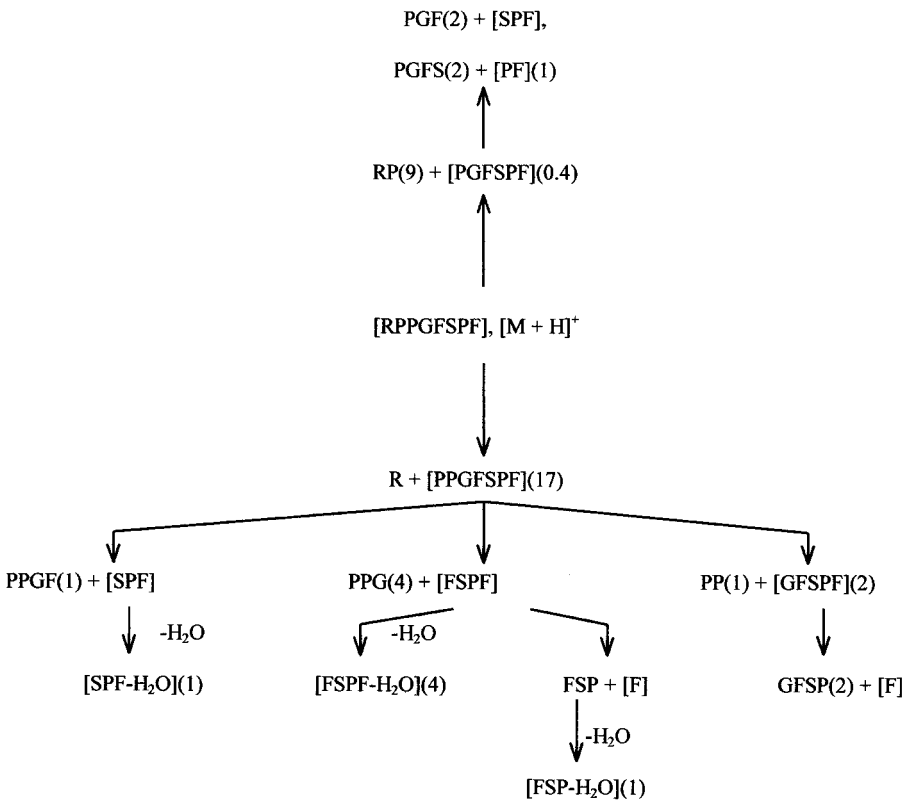


Scheme 2. Reactions yielding internal fragment ions observed in the PSD product ion spectrum of Lys[1]-bradykinin $[M + H]^+$ ions. Relative abundances observed appear in parentheses and dominant reaction pathways in bold.



Scheme 3. Reactions yielding internal fragment ions observed in the PSD product ion spectrum of des-Arg^1-bradykinin $[M + H]^+$ ions. Relative abundances observed appear in parentheses and dominant reaction pathways in bold.

solution, peptides exist as cations with the positive charge located either at the amino terminus or on the side-chains of basic amino acid residues such as arginine or lysine. Solvent molecules organize to delocalize and stabilize the charge on the peptide. The circular dichroism (CD) studies reported by Cann *et al.*[29] suggest that in an acidic aqueous environment bradykinin attains a secondary structure containing three



Scheme 4. Reactions yielding internal fragment ions observed in the PSD product ion spectrum of des-Arg^9-bradykinin $[M + H]^+$ ions. Relative abundances observed appear in parentheses and dominant reaction pathways in bold.

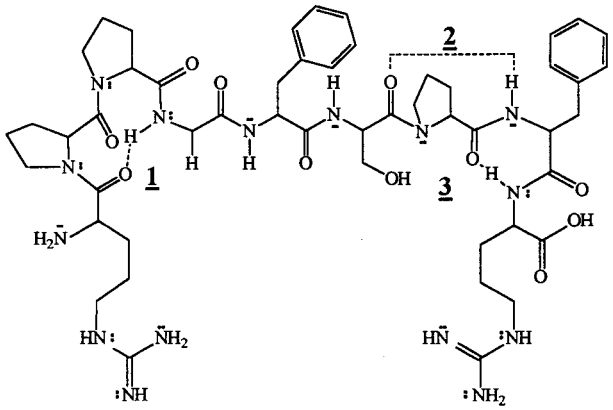
internal hydrogen bonds (**II**) that may be described as (i) a hydrogen bond between the carbonyl oxygen of Arg^1 and the amide hydrogen of Gly^4 ($3 \rightarrow 1$ hydrogen bond across Pro^3), (ii) a hydrogen bond between the carbonyl oxygen of Ser^6 and the amide hydrogen of Phe^8 (a $3 \rightarrow 1$ hydrogen bond across Pro^7) and (iii) a hydrogen bond between the carbonyl oxygen of Pro^7 and the amide hydrogen of Arg^9 (a $3 \rightarrow 1$ hydrogen bond across Phe^8). The hydrogen bond across Pro^3 forms a β-turn (type II) and is most stable when the Pro^2–Pro^3 bonds are in the *cis* conformation. The sequence of two hydrogen bonds bridging Pro^7–Pro^8 constitutes a 2.2_7 helix. The time bradykinin spends in this intramolecularly hydrogen-bonded conformation increases as a function of increasing temperature, the increasing concentration of counter ions such Na^+ and the decreasing polarity of the solvent.[29c] Each of these observations can be explained in terms of disruption of an ordered solvation system which causes the hydrophobic peptide to increase its intramolecular solvation.

Saulitis *et al.*[30] reported two-dimensional nuclear Overhauser effect (2D NOE) and conventional ^1H NMR measurements that give additional insight into the intramolecular interactions of the ionized bradykinin molecule. Studies of bradykinin lyophilized from acidified solutions and subsequently analyzed in dimethyl sulfoxide (DMSO) show that the charged peptide tends to form a quasi-cyclic structure in which the *C*-terminus intramolecularly solvates the *N*-terminus which carries the ionizing proton.

Interpretation of the PSD product ions in terms of arising from a single $[M + H]^+$ ion structure (conformation) is probably impractical because several stable conformations are energetically accessible to the gas-phase ion.[15] Although a single structure may comprise a large volume of phase space for the population of vibrationally cold ions, ions that possess sufficient internal energy to dissociate on the microsecond time-scale can sample numerous conformations prior to dissociation. On the other hand, intramolecular solvation, e.g. proton bridges, salt bridge interactions and/or β-turns may be energetically favorable and thereby serve to catalyze specific fragmentation reaction channels of activated ions. On the basis of the fragmentation chemistry presented in the paper, we propose that bradykinin $[M + H]^+$ ions with sufficient energy to dissociate on the microsecond time-scale sample at least two conformations. In both conformations, the *N*- and *C*-terminal arginine residues play important roles. For example, loss of m/z 60 (e.g. H_2O and HN=C=NH) from the *C*-terminal arginine residue side-chain is observed in both Lys^1-bradykinin and des-Arg^1-bradykinin PSD product ion mass spectra. This fragmentation reaction is absent in the PSD product ion mass spectrum of bradykinin analogues which contain an *N*-terminal arginine residue. In other words, the presence of the *N*-terminal arginine residue eliminates the *C*-terminal rearrangement product ion. We interpret this observation as an indication of a strong intramolecular hydrogen bond (proton bridge) between the *N*-and *C*-terminal arginine residues (**III**). This type of interaction would effectively bind the *C*-terminal arginine side-chain and prevent it from cyclizing and subsequently eliminating H_2O and HN=C=NH. Note that proton bridges between bases of equal strength such as $—NH_3^+\cdots H_2N—$ or $—NH_2\cdots H^+\cdots H_2N—$ form the strongest intramolecular hydrogen bonds known to exist.[31,32] Binding energies of 30–60 kcal mol^{-1} have been estimated by *ab initio* calculations.[33] Unlike ordinary hydrogen bonds, the proton in the proton bridge is equally shared and is equally likely to transfer to either base.[34] Proton bridging between the arginine and lysine residue side-chains would be less stable owing to the lower basicity of the lysine residue side-chain[35] thus resulting in unequal sharing of the bridging proton. Consequently, in Lys^1-bradykinin and des-Arg^1-bradykinin, the (protonated) *C*-terminal arginine residue side-chain is free to cyclize and eliminate H_2O and HN=C=NH. Our proposed intramolecular proton bridge is also consistent with the report of Thorne *et al.*[12] in which they suggested that the *N*-terminal arginine residue may assist the loss of the *C*-terminal arginine residue in the rearrangement reaction to form $[b_8 + H_2O]^+$ ions from the bradykinin $[M + H]^+$ ions. Two bradykinin $[M + H]^+$ ion conformations that involve a putative proton bridge between the two arginines (60–62% electrostatic charge density) were also predicted by Bowers and co-workers[15] based on molecular mechanics calculations.

An additional conformational feature of the bradykinin $[M + H]^+$ ion must have an influence on cleavage reactions that occur in the vicinity of the Ser^6–Pro^7 residue. These reactions are dramatically reduced in the PSD product ion mass spectrum of des-Arg^9-bradykinin. It is unlikely that hydrogen bonding between serine and the *C*-terminal carboxylic acid group (either as —COOH or $—COO^-$) or the guanidino group of Arg^1 could be sufficiently strong to influence the dissociation reaction of the bradykinin $[M + H]^+$ ion. Conversely, in bradykinin, an intramolecular interaction between the protonated guanidino group of Arg^9 and the Ser^6–Pro^7 residues could afford considerable stabilization to the $[M + H]^+$ ions and influence dissociative chemistry. The high frequency of occurrence for Ser–Pro at type I β-turns is well known, and this is explained in terms of specific hydrogen bonds to functionalized side-chains.[36] We rationalize the increased probability of cleavage at Ser^6–Pro^7 in terms of an intramolecular solvation of the protonated guanidino group of Arg^9 by the polar Ser^6–Pro^7 portion of the peptide (**IV**). The putative interaction may not be possible when Pro^7 is changed to Phe^7. More detailed studies on the effects of the Ser–Pro residues on the fragmentation reactions of peptide $[M + H]^+$ ions are under way.

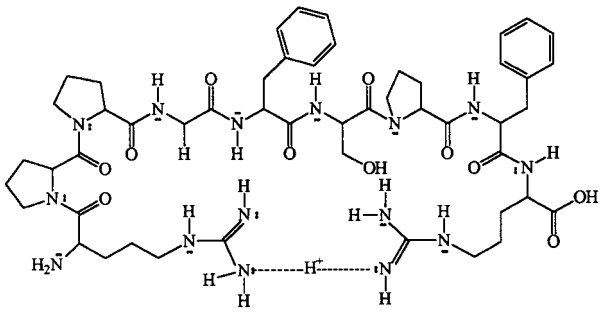
The overall conclusions of our studies (i.e. a strong interaction exists between the *N*- and *C*-terminal arginines in bradykinin $[M + H]^+$) are in general agreement with those of Glish and co-workers,[4] Williams and co-workers[10a] and Bowers and co-workers.[15] Recent studies by Gaskell and co-workers[37] on related arginine-containing peptides are also explained in terms of intramolecular acid–base interactions. In Bowers and co-workers' extensive modeling studies they did not find any evidence of a β-turn in the Ser^6—Pro^7—Phe^8—Arg^9 region of the molecule. It is important to note, however, that the molecular mechanics examined low internal energy forms of the molecule, whereas our



II

studies examined ions with appreciable amounts of internal energy near the dissociation threshold for reactions occurring on the microsecond time-scale. Hence it may be that **IV** represents a higher energy conformer of the molecule.

Examination of the bradykinin $[M + H]^+$ ions that fragment to give internal fragment ions does not suggest a dominant bradykinin $[M + H]^+$ ion conformation. For ions that have sufficient internal energy to dissociate to form internal fragment ions, the ionizing proton appears to be fairly mobile despite the fact that the amino acid residue sequence contains an arginine residue. Note that the formation of an internal fragment ion requires that (at least) two bonds be broken. Therefore, the peptide $[M + H]^+$ ions which yield internal fragment ions must have high internal energies. It is likely that such high internal energy ions would sample all available gas-phase conformations.



III

CONCLUSIONS

We examined the PSD of MALDI-generated $[M + H]^+$ ions of bradykinin and structural analogues to determine the influence of the *N*- and *C*-terminal arginine residues in gas-phase ion dissociation. Both sequence-specific and internal fragment ions are reported. The majority of the internal fragment ions appearing in the PSD product ion mass spectra of bradykinin, Lys^1-bradykinin, and des Arg^1-bradykinin $[M + H]^+$ ions appear to be derived from the (putative) y_7 sequence ion. In order to test the proposed fragmentation pathways which yield internal fragment ions, one would need to isolate each alleged precursor ion and cause it to dissociate, look at the fragment ion(s) and revise the proposed scheme accordingly. Studies such as these could be performed on a standard tandem sector mass spectrometer (e.g. EBE configuration) and would verify the identities of the internal fragment ions having identical masses but different amino acid sequences.

We also evaluated the PSD product ions in terms of the gas-phase bradykinin $[M + H]^+$ ion conformation(s) and the influence on peptide ion dissociation. The influence of gas-phase ion conformation on two specific dissociation reactions was examined in detail. A cyclization reaction of the *C*-terminal arginine side-chain was observed only in bradykinin analogues without *N*-terminal arginine residues. Cleavage reactions in the vicinity of serine decreased dramatically when the *C*-terminal arginine was removed or the Pro^7 changed to Phe^7. Based on these observations, we suggest that non-covalent interactions, specifically intramolecular hydrogen bonding (proton bridges), affect peptide ion dissociation in the gas phase. We propose two gross conformational features of the gas-phase bradykinin $[M + H]^+$ ions: (i) a intramolecular strong hydrogen bond (proton bridge) between the *N*- and *C*-terminal arginine residue side-chains and (ii) intramolecular hydrogen bond(s) between the hydroxyl group of the serine residue and the protonated guanidino group of the *C*-terminal arginine.

Acknowledgements

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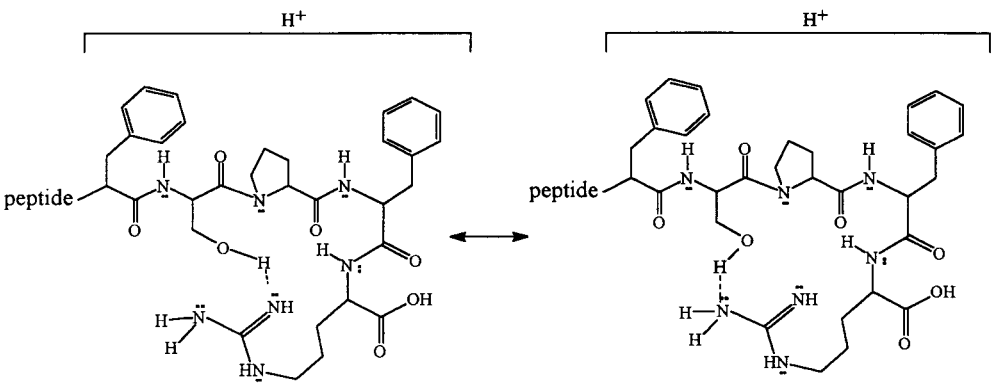

IV

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