Fragmentation of Protonated Oligoalanines: Amide Bond Cleavage and Beyond

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The fragmentation reactions of the singly-protonated oligoalanines trialanine to hexaalanine have been studied using energy-resolved mass spectrometry in MS^2 and MS^3 experiments. The primary fragmentation reactions are rationalized in terms of the b_x-y_z pathway of amide bond cleavage which results in formation of a proton-bound complex of an oxazolone and a peptide/amino acid; on decomposition of this complex the species of higher proton affinity preferentially retains the proton. For protonated pentaalanine and protonated hexaalanine the major primary fragmentation reaction involves cleavage of the C-terminal amide bond to form the appropriate b ion. The lower mass b ions originate largely, if not completely, by further fragmentation of the initially formed b ion. MS^3 energy-resolved experiments clearly show the fragmentation sequence $b_n \rightarrow b_{n-1} \rightarrow b_{n-2}$. A more minor pathway for the alanines involves the sequence $b_n \rightarrow a_n \rightarrow b_{n-1} \rightarrow b_{n-2}$. The a_5 ion formed from hexaalanine loses, in part, NH_3 to begin the sequence of fragmentation reactions $a_5 \rightarrow a_5^* \rightarrow a_4^* \rightarrow a_3^*$ where $a_n^* = a_n - NH_3$. The a_3^* ion also is formed from the b_3 ion by the sequence $b_3 \rightarrow a_3 \rightarrow a_3^*$ with the final step being sufficiently facile that the a_3 ion is not observed with significant intensity in CID mass spectra. A cyclic structure is proposed for the a_3^* ion. (J Am Soc Mass Spectrom 2004, 15, 1810–1819)

With the advent of soft ionization techniques such as electrospray ionization (ESI) [1–3] and matrix-assisted laser desorption-ionization (MALDI) [4, 5] which efficiently ionize by protonation a wide variety of peptides and proteins, tandem mass spectrometry [6, 7] has become a method of significant importance for the sequencing of peptides through collision-induced dissociation (CID) studies. As a result, the main types of fragmentation reactions occurring are well-established [8–11], at least in a phenomenological sense and are outlined in Scheme 1. A major mode of fragmentation in many cases involves cleavage of an amide bond in the protonated peptide. When the charge is retained by the C-terminus fragment migration of a labile hydrogen from the N-terminus neutral fragment occurs to form a protonated amino acid (y_1) or peptide (y_n) [12, 13]. When the charge remains on the N-terminus fragment, a neutral amino acid or peptide is eliminated and b_n ions are formed. Although these b_n ions were initially considered to be acylium ions [8, 10, 11], extensive experimental and theoretical studies [14–19] have shown that, in many cases, the b_n ions have protonated oxazolone structure as shown in Scheme 2 formed by cyclization involving the carbonyl function next-nearest to the amide bond being ruptured. In several cases where there are more reactive side-chain groups in the peptide, alternative cyclic structures are formed by interaction with side-chain functionality as the amide bond cleavage occurs [20, 21].

Although the fragmentation reactions of b_2 ions have been studied in some detail [14, 18, 22, 23], the fragmentation reactions of larger b ions have seen less study [15] and the fragmentation of a_n ions have seen even less study. A related question is whether, for larger protonated peptides, the lower mass b ions are formed directly by fragmentation of the protonated peptide or are secondary fragmentation products. In the present work we have undertaken a detailed study of protonated oligoalanines with two goals in mind, first, to determine as far as possible the relative importance of primary cleavage of the different amide bonds in the peptide and, second, to elucidate further the fragmentation pathways of the various b ions and a ions which are observed in the CID mass spectra. The fragmentation reactions of various protonated oligoalanines have seen extensive study [15, 24–29] although little attention has been paid to the question of the relative propensity for cleavage of the different amide bonds, and the secondary fragmentation reactions have seen only limited attention [15]. The oligoalanines are of particular interest in the present context because Paizs and Suhai [29] have recently reported ab initio calculations of the proton affinities of the oxazolones and amino acids/peptides involved in the fragmentation of protonated

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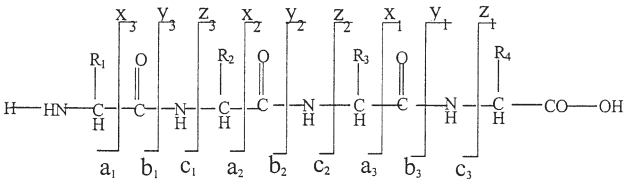


Scheme 1

pentaalanine which allows one to relate the fragmentation reactions observed for the various oligoalanines to the relative thermochemistry involved.

Experimental

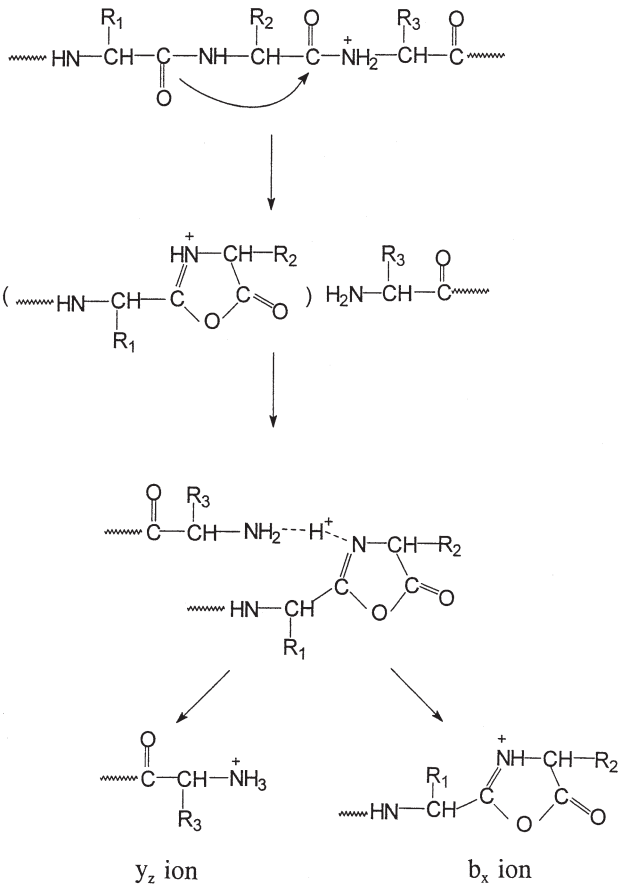
Initial CID studies were carried out using an electrospray/quadrupole mass spectrometer (VG Platform, Micromass, Manchester, UK) with CID in the interface between the atmospheric pressure source and the quadrupole mass analyzer, so-called cone-voltage CID. It has been clearly established [30–34] that, by varying the field in this interface region, energy-resolved mass spectra [35] similar to those obtained by variable low-energy CID in quadrupole cells can be obtained. The results of these cone-voltage CID experiments are presented in the following as breakdown graphs expressing the percentage of total ion signal as a function of the cone voltage. MS^2 and MS^3 experiments were also carried out using an electrospray/quadrupole/time-of-flight (QqTOF) mass spectrometer (QStar, MDS Sciex, Concord, Canada). In the MS^3 experiments, CID in the interface region produced fragment ions; those of interest being mass-selected by the first quadrupole mass analyzer (Q) to undergo collisional activation in the quadrupole collision cell (q) with the ionic fragmentation products being analyzed by the time-of-flight analyzer [36, 37]. By varying the collision energy in the quadrupole cell, breakdown graphs for selected fragment ions were obtained under multiple collision conditions.

Ionization was by electrospray on both instruments. For the single quadrupole the peptide was dissolved in 1:1 CH_3CN/1% formic acid and introduced into the source at a flow rate of 30 μL min^{-1} with nitrogen as both nebulizing and drying gas. For the QTOF instrument the peptide was dissolved in 1:1 CH_3OH/0.1% formic acid and introduced into the source at a flow rate of 80 μL min^{-1}. Nitrogen was used as nebulizing gas, drying gas and collision gas in the quadrupole cell.

All peptide samples were obtained from BACHEM Biosciences (King of Prussia, PA) and were used as received.

Results and Discussion

The detailed mechanism of amide bond cleavage in protonated peptides has not been definitively established although there is increasing evidence [29, 38–41] that fragmentation occurs from the amide N-protonated species to form, at low internal energies, a proton-bound complex of an oxazolone and a truncated peptide or amino acid as illustrated in Scheme 2, the so-called b_x-y_z pathway [41]. The fragmentation of such a proton-bound complex should be determined, largely by the relative proton affinities of the oxazolone and the peptide/amino acid, with that having the greater proton affinity being preferentially formed as the ionic species. The results of the present study will be interpreted in terms of the pathway of Scheme 2 and, indeed, provide support for the pathway.



Scheme 2

Trialanine

The CID mass spectra of protonated trialanine obtained at three collision energies in the quadrupole collision cell are presented in Table 1. At the lowest collision energy only the b_2 fragment ion is observed indicating preferred fragmentation of the C-terminal amide bond. That the b_2 ion should be formed rather than the y_1 ion is in agreement with the relative proton affinities, that for the neutral b_2 oxazolone being 222.3 kcal mol^{-1} compared with 214.6 kcal mol^{-1} for alanine [29]. With increasing collision energy fragmentation of the b_2 ion

Table 1. CID mass spectra of protonated trialanine

		Collision energy		
m/z	Ion	6 eV	10 eV	14 eV
232	MH^+	100	55.9	10.1
214	$-H_2O$		2.1	1.1
161	y_2		8.4	21.8
143	b_2	23.6	100	100
115	a_2		11.1	37.4
90	y_1			2.1

to the a_2 ion is observed as well as formation of the y_2 ion arising from cleavage of the N-terminal amide bond. Laskin and co-workers [26–28] have examined the fragmentation of protonated trialanine by surface-induced dissociation (SID) and by multiple-collision activation (SORI) using Fourier transform/ion cyclotron resonance (FT/ICR) mass spectrometry. Our present results are in best agreement with their SID spectrum, particularly with respect to the intensity of the y_2 ion. As they have noted [26], multiple-collision activation in FT/ICR discriminated against processes of higher critical reaction energy, in this case formation of the y_2 ion.

Tetraalanine

The CID mass spectra of protonated tetraalanine at three collision energies are given in Table 2 while the breakdown graph obtained by cone-voltage CID is presented in Figure 1. The breakdown graph for the b_3 ion obtained on the QTOF instrument is presented in Figure 2. Clearly, the b_3 and y_2 ions are the major primary fragmentation products. An earlier metastable ion study [15] of the fragmentation of protonated tetraalanine produced by fast atom bombardment (FAB) showed y_2(100), b_3(52), and $-H_2O$(14). It is clear that the majority, if not all, of the b_2 ion arises by further fragmentation of the b_3 ion; this is in agreement with the conclusions of Laskin et al. [27]. (We cannot preclude the possibility that some of the b_2 ion arises by direct fragmentation of the central amide bond at high

Table 2. CID mass spectra of protonated tetraalanine*

		Collision energy		
m/z	Ion	6 eV	15 eV	20 eV
303	MH^+	100	22.5	2.1
285	$-H_2O$	1.2	3.9	
232	y_3		5.6	6.6
214	b_3	6.4	43.0	25.2
169	a_3^*		11.8	19.3
161	y_2	11.4	100	100
143	b_2	1.5	38.4	85.0
115	a_2		8.3	29.7
44	a_1			3.5

*$a_3^* = a_3 - NH_3$

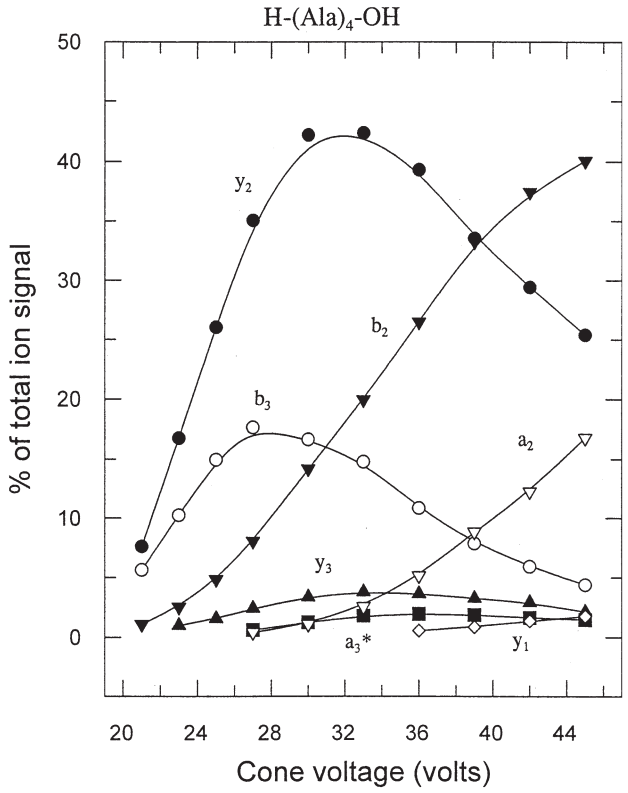


Figure 1. Breakdown graph for protonated tetraalanine obtained by cone-voltage CID. Ion signal for MH^+ not shown.

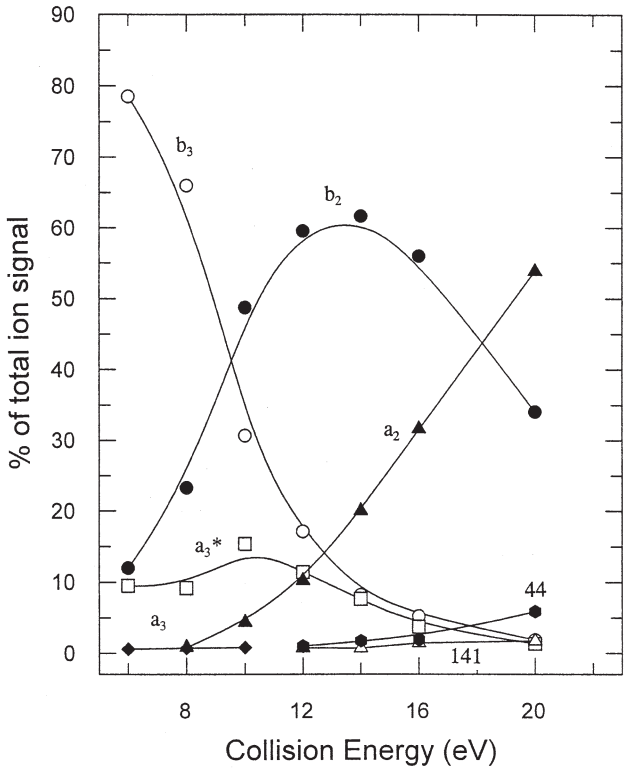


Figure 2. Breakdown graph for the b_3 ion derived from tetraalanine.

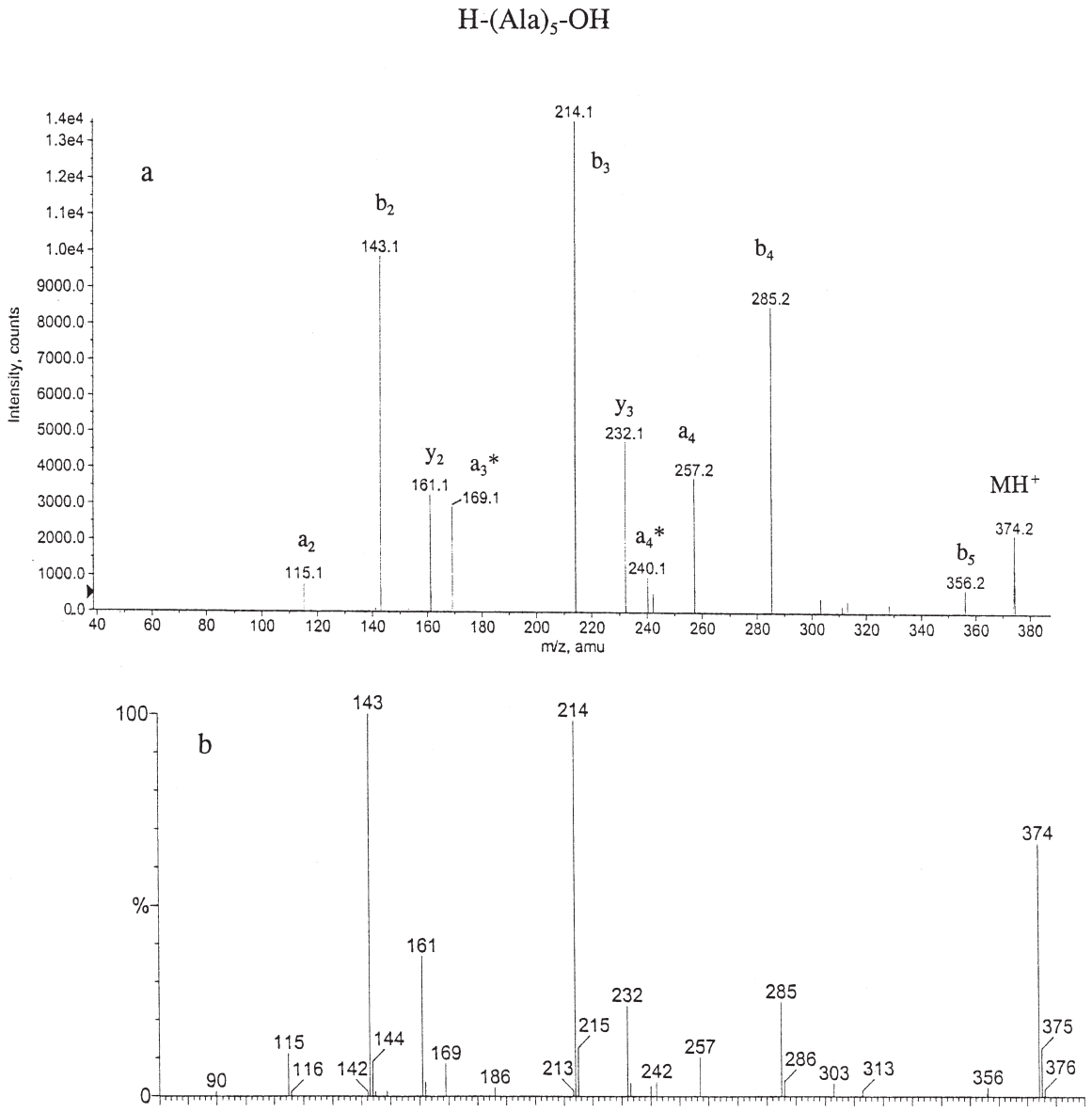


Figure 3. (**a**) CID mass spectrum of protonated pentaalanine obtained on QTOF at 20 eV collision energy. $a_x^* = a_x - NH_3$. (**b**) CID mass spectrum of protonated pentaalanine obtained by cone-voltage CID at 40 V cone voltage.

collision energies even though this is the thermochemically disfavored product; see below.) Figures 1 and 2 clearly indicate the reaction sequence $b_3 \rightarrow b_2 \rightarrow a_2$; this sequence is also evident from the breakdown graph obtained earlier [15] for protonated tetraalanine. Cleavage of the C-terminal amide bond leads to formation of the b_3 ion rather than the y_1 ion since the proton affinity of the corresponding oxazolone is 226.0 kcal mol^{-1} compared with 214.6 kcal mol^{-1} for alanine [29]. Similarly, cleavage of the central amide bond leads primarily to formation of the y_2 ion since PA(AA) = 225.4 kcal mol^{-1} compared with 222.3 kcal mol^{-1} for the neutral oxazolone corresponding to the b_2 ion. An earlier metastable ion study of the fragmentation of the b_3 ion produced from pentaalanine showed formation of a_3(81), a_3^*(100) and b_2(71), where $a_3^* = a_3\text{-}NH_3$; as shown in Figure 2, the a_3 ion is barely observed in the CID mass spectrum of the b_3 ion while the a_3^* ion is observed in relatively low abundance. The formation of a_n^* ions will be discussed in detail below.

Pentaalanine

Figure 3 compares the CID mass spectrum for protonated pentaalanine obtained with the QTOF instrument with that obtained by cone-voltage CID on the single quadrupole instrument. The spectra are quite similar and are in agreement with the spectra reported by Laskin and Futrell [27], particularly those obtained by surface-induced dissociation. The breakdown graph for

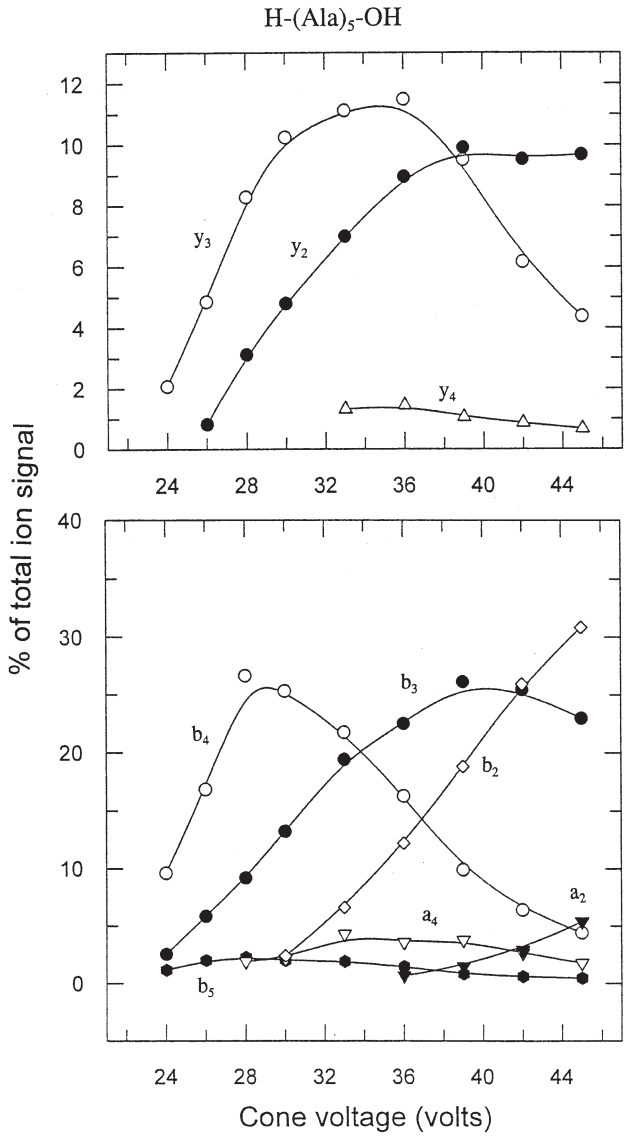
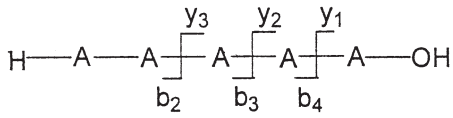


Figure 4. Breakdown graph for protonated pentaalanine obtained by cone-voltage CID. MH^+ ion signal not shown.

MH^+ obtained by cone-voltage CID is presented in Figure 4. Clearly, formation of b_4 is the most prominent fragmentation reaction with minor formation of y_3, b_3, b_5 (-H_2O), and y_2. We believe that the apparent higher onset for the y_2 product in Figure 4 is not real but reflects the detection limit for low-intensity ions. Metastable ion fragmentation of protonated pentaalanine showed b_5(34), b_4(100), b_3(22), y_3(28), and y_2(8) as fragmentation products [15]. If there was an equal propensity for cleavage of the various amide bonds, one would have expected the yields of b_3, y_3, and y_2 to be greater in both the metastable ion and lowest energy CID mass spectra. The dominance of the b_4 ion in both indicates that the C-terminal amide bond is preferentially cleaved. The calculations of Paizs and Suhai [29] have shown that the energy requirement for transfer of the proton from the most-favored amino position to the C-terminal amide nitrogen is lower than for transfer to other amide nitrogens and that the energy requirement for cleavage of the C-terminal amide bond is lower than for cleavage of other amide bonds.

Table 3. Thermochemistry for fragmentation of protonated pentaalanine



N-Terminus		C-Terminus	
Ion	PA(Neut)[a]	Ion	PA(Neut)[a]
b_4	232.6	y_1	214.6
b_3	226.0	y_2	225.4
b_2	222.3	y_3	233.1

[a]kcal mol^{-1}

The primary fragmentation reactions of protonated pentaalanine can be rationalized in terms of the relative proton affinities of the respective products expected by the b_x-y_z mechanism outlined in Scheme 2 and, indeed, provides support for the fragmentation mechanism. The proton affinities of the relevant oxazolones and amino acid/peptides, calculated by Paizs and Suhai [29], are presented in Table 3. Clearly, cleavage of the C-terminal amide bond will lead to formation of the b_4 ion since the b_4 oxazolone has a much higher proton affinity than that of alanine (y_1); this is what is observed in both metastable ion and CID mass spectra. On the other hand, cleavage of the next amide bond should lead to formation of both b_3 and y_2 ions since the proton affinities of the corresponding neutral are very similar; thus, both b_3 and y_2 are observed in metastable ion fragmentation. Cleavage of the b_2–y_3 bond should result in formation of the y_3 ion rather than the b_2 ion since the proton affinity of trialanine is considerably greater than that of the b_2 oxazolone. The dominance of the b_3 ion (with respect to the y_2 ion) and the abundant ion signal for the b_2 ion (Figure 3) arise from the dominant primary cleavage of the C-terminal amide bond to form the b_4 ion which fragments, in part, by the sequence $b_4 \rightarrow b_3 \rightarrow b_2$. This sequence is shown by the breakdown graph for the b_4 ion presented in Figure 5. Formation of the a_4 ion (CO loss) and the b_3 ion are the major low-energy fragmentation routes of the b_4 ion; a metastable ion study [15] of the b_4 ion showed a_4(100), b_3(38), and b_2(7). It is evident that under CID conditions the b_3 ion is more readily formed. In the earlier study it was proposed that the neutral accompanying the $b_n \rightarrow b_{n-1}$ fragmentation was a cyclic aziridinone, however, subsequent ab initio calculations [42, 43] have shown that this is a very energy-demanding route which cannot compete with the stepwise process $b_n \rightarrow a_n \rightarrow b_{n-1}$. Thus, the detailed pathway for the direct $b_n \rightarrow b_{n-1}$ reaction is not known; a possibility is outlined in Scheme 3. That the reaction sequence $b_4 \rightarrow a_4 \rightarrow b_3$ does occur is shown by the formation of the a_4 ion from b_4

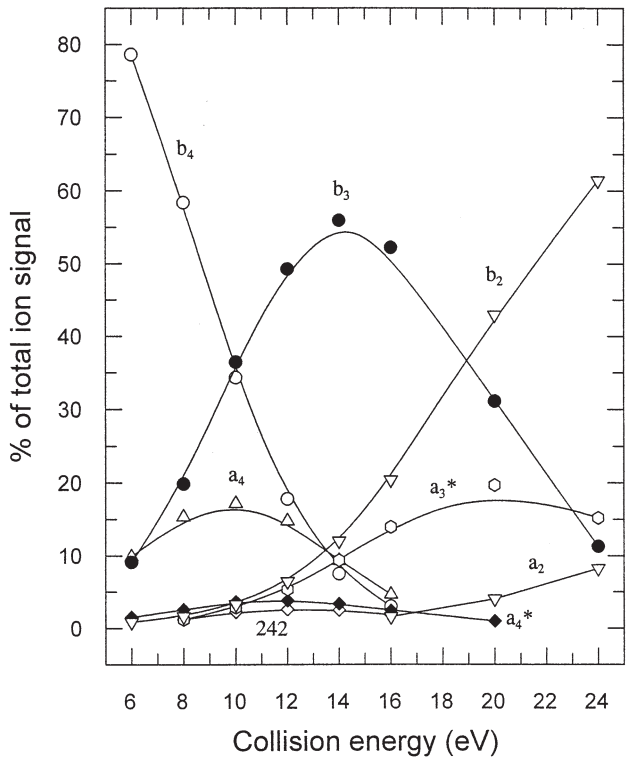


Figure 5. Breakdown graph for the b_4 ion derived from pentaalanine.

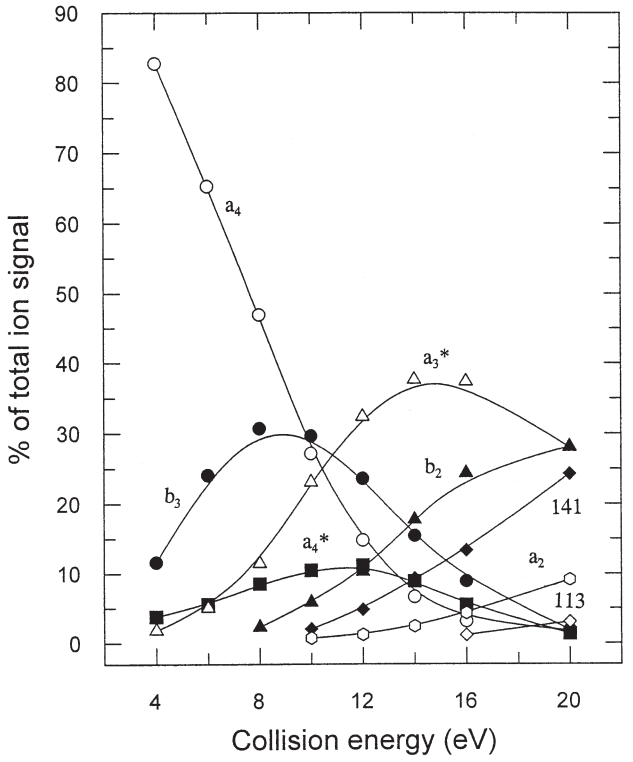


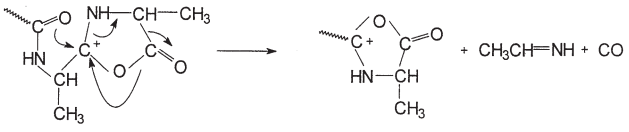
Figure 6. Breakdown graph for the a_4 ion derived from pentaalanine.

(Figure 5) and by the breakdown graph for the a_4 ion (Figure 6) which clearly shows the sequence $a_4 \rightarrow b_3 \rightarrow b_2 \rightarrow a_2$. The likely pathway for the $a_n \rightarrow b_{n-1}$ reaction is shown in Scheme 4. The other fragmentation pathway for the a_4 ion is loss of NH_3 to form the a_4^* ion. A prominent ion in the breakdown graph of Figure 6 is the a_3^* ion which appears to be formed both by loss of an alanine residue from the a_4^* ion and by loss of CO + NH_3 from the b_3 ion (see above). The a_3^* fragments further by loss of CO to give *m/z* 141 with further minor loss of CO to give *m/z* 113. Possible routes to and structures of the a_n^* ions are discussed later.

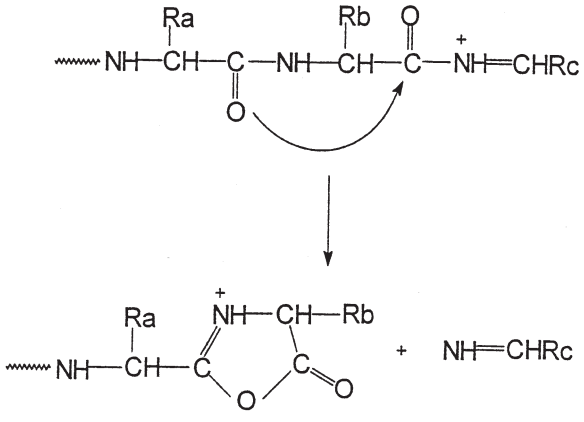
Hexaalanine

The CID mass spectrum of protonated hexaalanine, obtained on the QTOF instrument, is shown in Figure 7. The b_n ion series dominates the mass spectrum with more minor y_n ion series and a_n ion series. Of particular note is the absence of the a_3 ion and the significant intensities for the a_n^* series of ions. The breakdown graph for the MH^+ ion, obtained by cone-voltage CID, is shown in Figure 8. Clearly, the b_5 ion is the most abundant fragment ion at low collision energies with more minor yields of b_4, y_4, and y_3; the y_2, b_3, and b_2 ions are secondary products. Thermochemical data for fragmentation of protonated hexaalanine are presented in Table 4. (The proton affinities of the b_5 oxazolone and the y_4 peptide have not been calculated and are assumed to be equal to or greater than those for the

$$a_n \text{ -------> } b_{n-1}$$



Scheme 3



Scheme 4

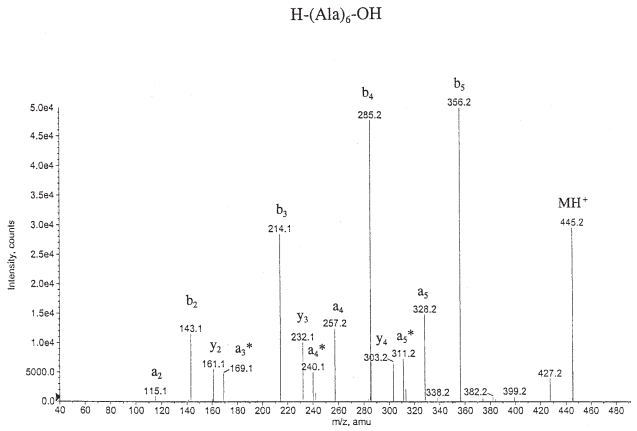


Figure 7. CID mass spectrum of protonated hexaalanine obtained on QTOF at 20 eV collision energy.

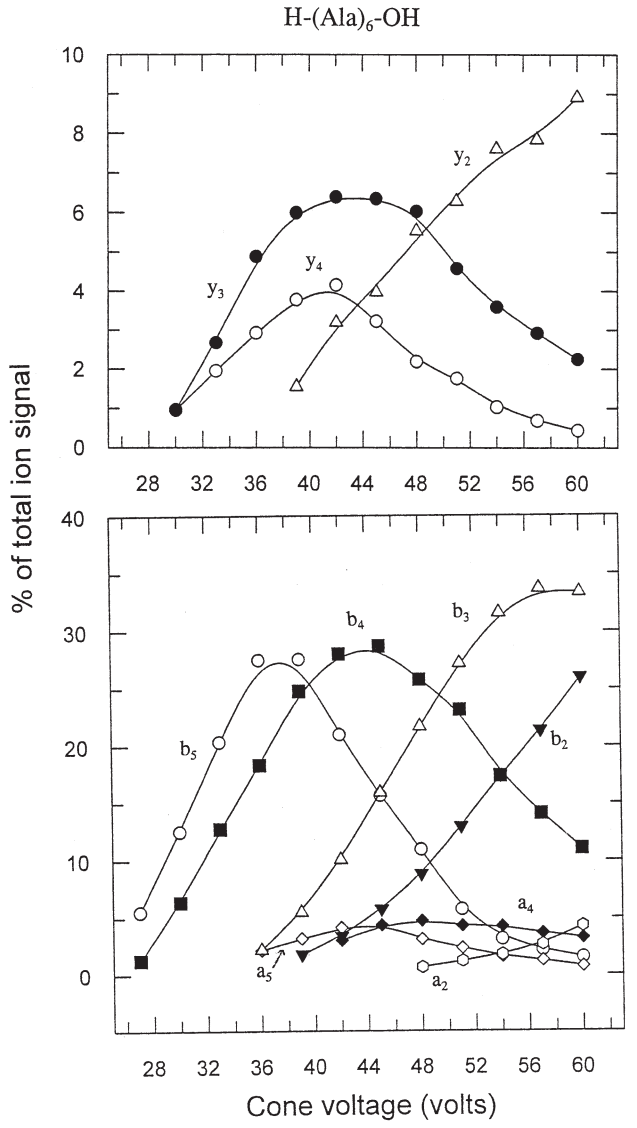
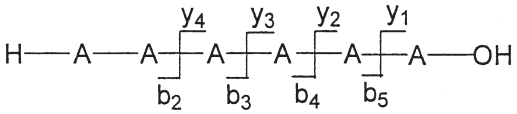


Figure 8. Breakdown graph for protonated hexaalanine obtained by cone-voltage CID.

Table 4. Thermochemistry for fragmentation of protonated hexaalanine



N-Terminus		C-Terminus	
Ion	PA(Neut)[a]	Ion	PA(Neut)[a]
b_5	≥232.6	y_1	214.6
b_4	232.6	y_2	225.4
b_3	226.0	y_3	233.1
b_2	222.3	y_4	≥233.1

[a]kcal mol^{-1}

homologous b_4 and y_3 species.) The thermochemical data indicate that formation of b_5 should be favored over formation of y_1, formation of b_4 favored over formation of y_2 but formation of y_3 and y_4 should be favored over formation of the complimentary b_3 and b_2 ions; this is what is observed experimentally (Figure 8), thus providing support for the b_x–y_z pathway outlined in Scheme 2. If there were an equal propensity for cleavage of the various amide bonds, one would expect to see more pronounced ion signals for b_4, y_4, and y_3 at the lowest cone voltages; the relative dominance of the b_5 ion indicates that there is a preference for cleavage of the C-terminal amide bond as was observed for protonated pentaalanine.

The preferential cleavage of the C-terminal amide bond to form b_5 and the fragmentation sequence $b_5 \rightarrow b_4 \rightarrow b_3 \rightarrow b_2$, which is clearly shown by the breakdown graph for the b_5 ion (Figure 9), accounts for the dominance of the b ion series in the CID mass spectrum of Figure 7. A second, more minor, fragmentation route for the b_5 ion forms the a_5 ion (CO loss) which initiates the fragmentation $a_5 \rightarrow a_4$ and the sequence $a_5 \rightarrow a_5^* \rightarrow a_4^* \rightarrow a_3^*$, with the latter becoming a prominent product at higher collision energies. This fragmentation sequence is shown clearly by the breakdown graph for the a_5 ion (Figure 10) where the a_n^* series of ions dominate the breakdown graph along with more minor formation of the b_4 ion as a primary fragmentation product. The origin of and possible structures of the a_n^* ions are discussed below.

Formation of a_n^* Ions

The CID mass spectra of protonated tetraalanine to hexaalanine show significant ion signals for a_n^* ($a_n - NH_3$) ions. The only free amino group available to be lost as NH_3 is the N-terminal amino function. Loss of this group in the absence of any rearrangement would lead to an electron-deficient carbocation [44] which is destabilized by the α-carboxy group; such species are known [45–48] to have limited stability in the gas-phase and we suggest that cyclization has occurred upon loss

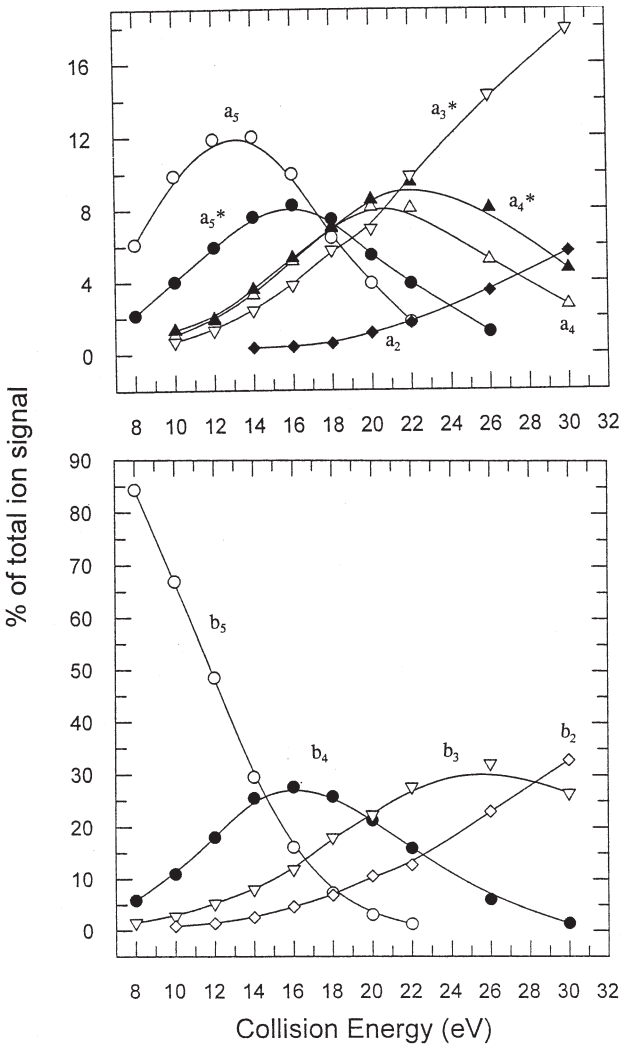


Figure 9. Breakdown graph for the b_5 ion derived from hexaalanine.

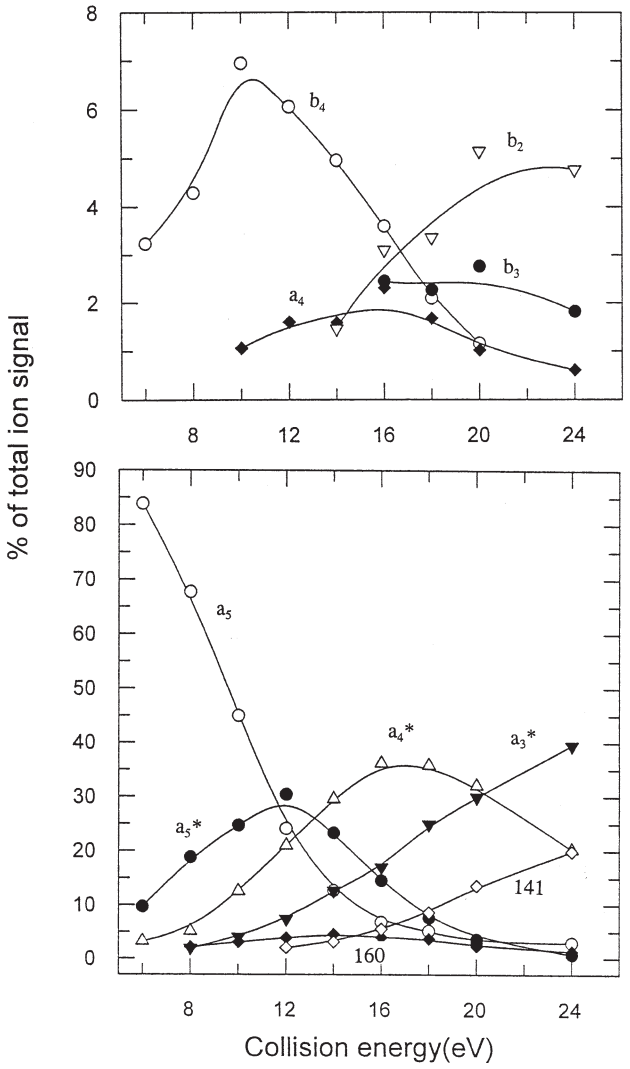


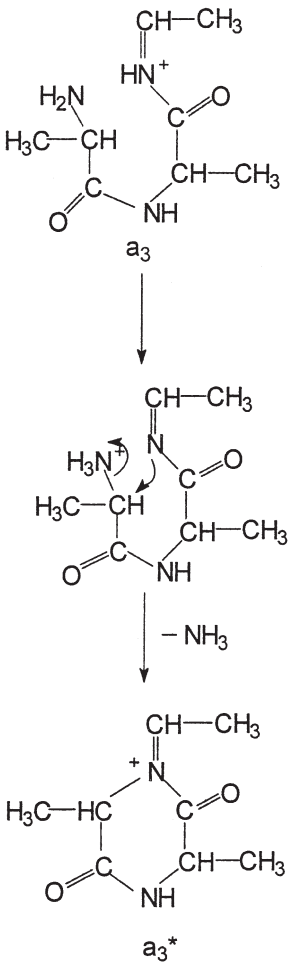
Figure 10. Breakdown graph for the b_5 ion derived from hexaalanine.

of NH_3. The most straightforward case is the formation of the a_3^* ion in the mass spectrum of protonated tetraalanine. Earlier metastable ion studies [15] indicate the reaction sequence $MH^+ \rightarrow b_3 \rightarrow a_3 \rightarrow a_3^*$; under CID conditions, the last step appears to be particularly facile since the a_3 ion abundance is very low in the CID mass spectrum. In Scheme 5 we propose a pathway for the $a_3 \rightarrow a_3^*$ reaction leading to a cyclic structure which might be expected to lose CO on further fragmentation, as observed experimentally.

The breakdown graph for the a_5 ion derived from hexaalanine (Figure 10) clearly shows the reaction sequence $a_5 \rightarrow a_5^* \rightarrow a_4^* \rightarrow a_3^*$, i.e., the initial loss of NH_3 is followed by sequential loss of two alanine residues resulting in formation of the stable a_3^* ion. It is not clear whether the a_5^* and a_4^* species are cyclic or remain in the acyclic form; certainly, if they are larger cyclic ions they must open to accommodate the loss of the alanine residues.

Conclusions

The primary fragmentation reactions of the protonated oligoalanines involve cleavage of an amide bond to produce b_x or y_z ions. The present work has shown that the primary fragmentation reactions can be adequately rationalized in terms of the b_x–y_z pathway outlined in Scheme 2, with the relative abundances of the b_x and y_z ions being determined by the relative proton affinities of the corresponding neutral oxazolones and peptides/amino acid. The results thus provide strong support for the fragmentation mechanism outlined in Scheme 2. For protonated pentaalanine and protonated hexaalanine there is a distinct preference for primary fragmentation by cleavage of the C-terminal amide bond to form the respective b ion. The majority, if not all, of the lower mass b ions originate by sequential fragmentation of the b_n ion formed by cleavage of the C-terminal amide bond. The major fragmentation pathway for the oligoalanines is the sequence $b_n \rightarrow b_{n-1} \rightarrow b_{n-2}$ with a



Scheme 5

minor contribution from the sequence $b_n \rightarrow a_n \rightarrow b_{n-1}$. The fact that most of the lower mass b ions are secondary products has significant implications for the conditions used to record CID mass spectra, if one wishes to observe the complete series of b ions, which are often of major importance in peptide sequencing. The behavior observed for the oligoalanines will undoubtedly change when amino acids with different functionalities are present in the peptide, since they will affect not only the favored site of amide nitrogen protonation but also the relative proton affinities of the oxazolones and peptides which will be reflected in the products formed by amide bond cleavage. In addition, they likely will have a distinct influence on the relative importance of the $b_n \rightarrow b_{n-1} \rightarrow b_{n-2}$ and $b_n \rightarrow a_n \rightarrow b_{n-1}$ pathways. These aspects are under study in our laboratory.

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