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RESEARCH ARTICLE

Loss of 45 Da from a_2 Ions and Preferential Loss of 48 Da from a_2 Ions Containing Methionine in Peptide Ion Tandem Mass Spectra

Yamil Simón-Manso, Pedatsur Neta, Xiaoyu Yang, Stephen E. Stein

Chemical and Biochemical Reference Data Division, National Institute of Standards and Technology, Gaithersburg, MD, 20899, USA

Abstract

While analyzing tandem mass spectra of tryptic tripeptides, intense unassigned peaks were observed, corresponding to neutral loss of 45 Da from a_2 ions. This process was confirmed by MS^3 experiments. Based on exact mass analysis, the loss was ascribed to (NH_3 + CO) or formamide. The proposed mechanism involves a cyclic form of the a_2 ions. The structure of the a_2 – 45 ions was confirmed by their fragmentation in MS^3 experiments. Loss of (NH_3 + CO) from the a_2 ions occurs in competition with other paths, such as the loss of H_2O or the formation of immonium ions. However, if the a_2 ion contains methionine, a neutral loss of 48 Da (ascribed to CH_3SH) predominates, and is followed by the loss of (NH_3 + CO). These processes were confirmed by MS^3 experiments. The intensity of the a_2 – 48 peak formed from XaaMet has a maximum value of 42% (of the total intensity of all ions) for Xaa=Gly, varies between 15% and 40% for most other Xaa residues, is lower for residues that can undergo loss of water or ammonia, and is very low for Lys or Arg. When the order of the residues is reversed to MetXaa, the loss of 48 Da is much smaller. This effect can be used to determine the sequence of b_2 ions containing Met in proteomic studies. Considerable loss of CH_3SH is observed from doubly protonated tryptic tripeptides with N-terminal Met, but the loss is much less when they are singly protonated or when Met is in the center position.

Key words: Peptides, Peptide ions, Mass spectra, MS/MS spectra, Fragmentation, Neutral losses, Loss of formamide, Loss of methyl mercaptan, Collision energy dependence

Introduction

Dipeptides and tripeptides, formed in the digestion of proteins, are not pursued in proteomic studies because they do not provide sufficient specificity for protein identification. In metabolomic studies, however, it may be important to identify di- and tripeptides, as well as single amino acids and their metabolites. To this end, and as part of the continuing work of our group to establish and expand a library of metabolite MS/MS spectra, we are measuring such spectra for all dipeptides and all tryptic tripeptides. MS/MS spectra are measured in several mass spectrometers, mainly a linear ion trap and a quadrupole time-of-flight instrument. Most peaks in these spectra are assigned to y, b, and a ions, their water or ammonia loss ions, and the expected immonium ions. When significant peaks remain unassigned, attempts are made to determine and confirm the assignments. Two such cases are reported here.

One of the most prominent fragment ions in the MS/MS spectra of protonated peptide ions is the b_2 ion [1–3]. This is

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Correspondence to: Pedatsur Neta; *e-mail:* pedi@nist.gov

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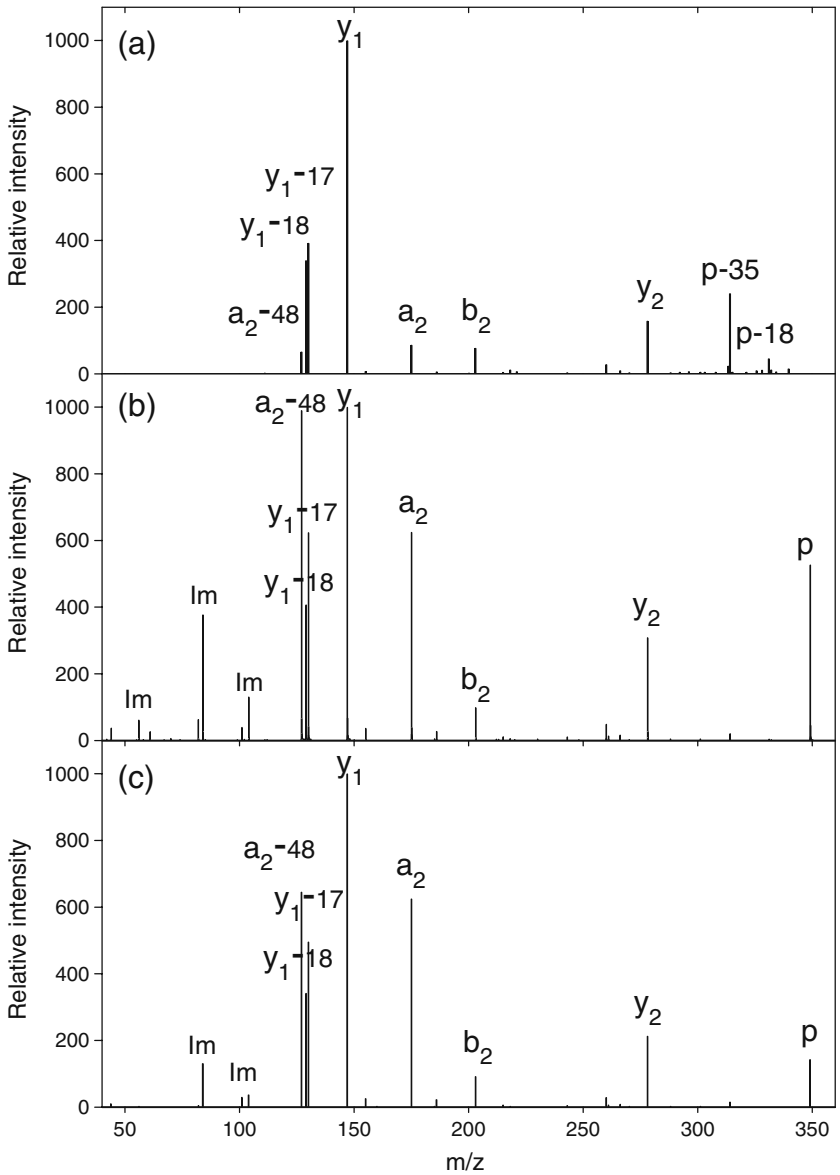


Figure 1. MS/MS spectra of the AMK tripeptide ion in three types of mass spectrometers: (**a**) linear ion trap (LTQ) at 35% collision energy, (**b**) quadrupole time-of-flight (QTOF) at 15 V collision voltage setting, and (**c**) triple quadrupole (QQQ) at 20 V collision voltage setting

due to the relative stability of this ion and the facile loss of neutral amino acid residues from larger b ions. Upon increasing collision energy, b_2 ions lose CO to form a_2 ions, which can further fragment to form either a_1 ions or immonium ions [4–8]. Direct formation of a_1 from b_2 also has been observed, but only with certain peptides [4, 5]. The most stable structure of b_2 ions is a cyclic protonated oxazolone structure [2, 4]. Although direct formation of a_1 from b_2 was first suggested [4] to involve a different structure for b_2, that process was later [5] suggested to involve the same protonated oxazolone structure. Loss of CO from b_2 necessarily leads to the opening of the oxazolone ring, and may lead to a noncyclic structure for a_2 ions. However, it was recently suggested that a_2 ions are also cyclic; they form a protonated 4-imidazolidinone structure [8]. The present results provide further evidence in support of this cyclic structure, as it is a required intermediate in the subsequent loss of a 45 Da neutral species.

Experimental

Tryptic tripeptides were synthesized in mixtures using an AAPPTEC (Louisville, KY, USA) APEX 396 synthesizer with standard procedures. The C-terminal K or R were individually used in the form of Wang resins, the center residue was also introduced individually, and the N-terminal residues were introduced in three separate mixtures, aimed at minimizing mass overlap. The mixtures were (a) A, P, T, L, D, E, H, (b) G, S, I, K, F, Y, and (c) V, C, N, Q, M, R, W. The final 120 synthesized mixtures were analyzed by HPLC with electrospray ionization tandem mass spectrometry,

using an ion trap (LTQ, Thermo Electron Corp., Waltham, MA, USA) and a quadrupole time-of-flight (Agilent Model 6530 QTOF) instrument. Almost all tryptic tripeptides were detected and analyzed in these synthetic mixtures. Additional peptides were synthesized individually. The raw data from the mass spectrometers were processed as described before [9] in order to derive a consensus spectrum for each peptide ion at each of the collision energies used. To determine the sequence of fragmentation for selected peptide ions, tandem mass spectra were recorded at 20 collision voltages in a triple quadrupole mass spectrometer (QQQ, Micromass Quattro Micro, Waters Corp., Milford, MA, USA), and peak intensities were plotted as a function of collision voltage.

Results and Discussion

The MS/MS spectra of all tryptic tripeptide ions detected in the 120 synthesized mixtures were inspected individually in an attempt to assign structures to all significant peaks that were not assigned in the standard procedure. A large peak was identified as $a_2 - 48$ in the spectra of most tripeptides with methionine in the center position. Another large peak, identified as $a_2 - 45$, was first noticed with tripeptides that have threonine at the center, but was then found in other peptides, though at lower intensities. In the present study we examine the formation of these peaks and attempt to identify the structures of the ions that they represent. For this purpose, several of the tripeptides were studied in greater detail and additional peptides were synthesized for further studies. Results for the methionine peptides are discussed first.

Peptides Containing Methionine

The MS/MS spectra of all the peptides were measured in an ion trap and a QTOF mass spectrometer, and several peptides were also studied using a triple quadrupole (QQQ) instrument. The spectra for the tripeptide AMK in the three instruments are presented as representative examples (Figure 1). Spectrum a was obtained with the LTQ using a relative collision energy setting of 35% of the maximum. In this spectrum, the precursor ion peak (p) is depleted and the fragment ions in the low *m/z* range are not observed. Spectra b and c were obtained with the QTOF and the QQQ instruments at collision voltage settings of 15 and 20 V, respectively. Spectra were recorded with various collision voltages in these instruments, and these particular spectra were chosen for their similarity to the LTQ spectrum. All of the main peaks are observed in the spectra from all three mass spectrometers, although with some variations in their relative intensities. The peaks marked Im are from the immonium ions of the various amino acids, and are not observed in the LTQ because of their low *m/z* values. An intense peak at *m/z* 127 is observed, which corresponds to a neutral loss of 48 Da from the a_2 ion. The dependence of peak intensity on collision voltage (Figure 2) shows that the onset of formation of the $a_2 - 48$ ion occurs at a higher

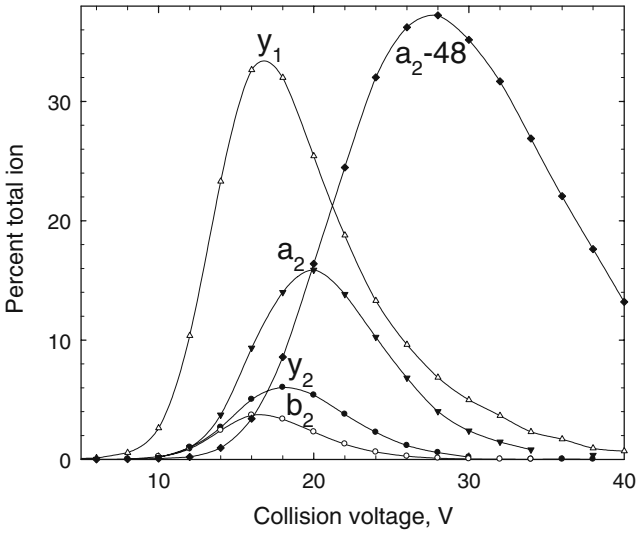


Figure 2. Dependence of peak intensity (percent total ion) on collision voltage in the MS/MS spectrum of the AMK tripeptide ion in the triple quadrupole mass spectrometer, showing only the main peaks

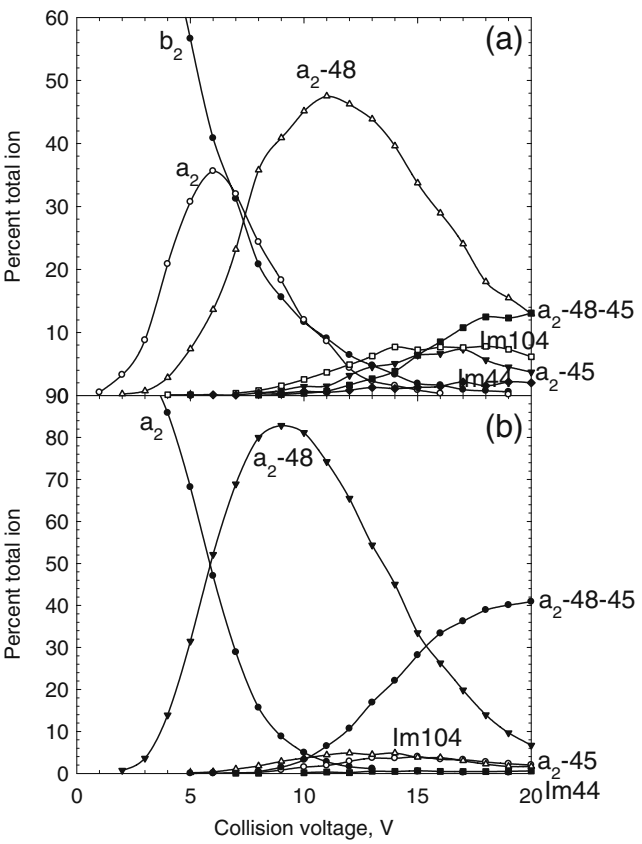


Figure 3. Quasi-MS^3 spectra of the b_2 (**a**) and a_2 (**b**) ions derived from AMK. The ions were produced in-source at high cone voltage (50 V), selected into the collision cell, and their fragmentations were followed at different collision voltages

voltage than that for the formation of a_2 ions, so it is likely that the 48 Da species is a neutral loss from the a_2 ion. Examination of other peptides revealed intense peaks corresponding to $a_2 - 48$ ions in most of the spectra of tripeptides with methionine at the central position. Since this fragment is specific to methionine, it must be due to the loss of CH_3SH from the side chain.

To examine the source of the $a_2 - 48$ peak more directly, we carried out quasi-MS^3 experiments. We increased the electrospray cone voltage to maximize the in-source intensities of the b_2 and a_2 fragment ions and then selected one of these ions into the collision cell and recorded its fragmentation products as a function of collision voltage. It is clear that the b_2 ion (Figure 3a) fragments predominantly to the a_2 ion, and that other fragment ions are formed only at higher collision energies. The a_2 ion (Figure 3b) fragments predominantly through the loss of a 48 Da neutral to give the $a_2 - 48$ ion. Immonium ions are formed at higher collision voltages.

The maximal intensity of the $a_2 - 48$ peak varies with the N-terminal residue (Table 1). With most tripeptides, this peak reaches a maximum value of 20–40% of the total ion intensity at a collision voltage of 24–29 V. Significantly lower values are observed for EMK and QMK because of competing neutral losses from these N-terminal residues. In fact, the MS^3 spectrum of the EMK-H_2O ion gives a high intensity for the $a_2 - 48$ peak because the competing water loss route is removed. The very low values for KMK and RMK are clearly due to the high basicities of the K and R residues and their alternate fragmentation route (ammonia loss). As is noted in the comments column of Table 1, when the position of M is changed from central to N-terminal, the intensities of the $a_2 - 48$ peaks are much smaller (see the discussion below).

Formation of the $a_2 - 48$ peak is not limited to tripeptides. Longer peptides with M in the second position also produce these ions, as demonstrated by the last group of peptides in Table 1. Increasing the peptide length from three to seven residues leads to a gradual decrease in the intensity of the $a_2 - 48$ peak by less than 40%, although the collision voltage at which the maximum intensity is observed increases by a factor of 2 (due to the increase in precursor *m/z*). When M is placed in the third position (last peptide in Table 1), the $a_2 - 48$ peak from AA is not observed, but a small peak due to $a_2 - 48$ from the internal fragment AM is observed.

Other Peptides

The spectra for the tripeptide ATK in the three mass spectrometers are presented as representative examples (Figure 4). The general patterns and the differences among the instruments are similar to those in Figure 1, except that the p-18 fragment peak is more intense in Figure 4 due to loss of water from threonine. An intense peak at *m/z* 100 is observed, which corresponds to a neutral loss of 45 Da from the a_2 ion. The dependence of peak intensity on collision voltage (Figure 5) shows that the onset of formation of the $a_2 - 45$ ion is at a higher voltage than that for the formation of a_2 ions, so it is likely to be produced by the loss of a 45 Da neutral species from the a_2 ion. This was confirmed by quasi-MS^3 experiments similar to those discussed above. The results show again that the b_2 ion fragments to give

Table 1. Maximal intensities of the $a_2 - 48$ peaks in the triple quadrupole (QQQ) spectra of peptides containing methionine

Peptide	*m/z* for singly protonated	$a_2 - 48$ peak		Comments
		Intensity (max) %	*V* (max)	
GMK	335	42	28	MGK, 0.6% (32 V)
SMK	365	38	28	
AMK	349	37	28	MAK, 1.6% (34 V)
TMK	379	33	28	
EMK-18	389	31	29	
LMK	391	30	27	MLK, 1% (33 V)
PMK	375	29	27	
VMK	377	26	27	MVK, 1.2% (32 V)
HMK	415	26	26	4% for $a_2 - 17 - 48$
FMK	425	23	28	MFK, 0%
CMK	381	22	28	
IMK	391	22	27	
YMK	441	21	28	MYK, 0%
NMK	392	19	24	2% for $a_2 - 17$
DMK	393	18	28	MDK, 0%
MMK	409	15	24	
EMK	407	10	28	21% for $a_2 - 18 - 48$
QMK	406	5	25	13% for $a_2 - 18$, 9% for $a_2 - 18 - 48$
KMK	406	2	28	3% for $a_2 - 17$
RMK	434	0		
AMAK	420	32	36	21% from doubly protonated peptide
AMAAK	491	28	46	24% from doubly protonated peptide
AMAAAK	562	27	59	27% from doubly protonated peptide
AMAAAAK	633	23	70	27% from doubly protonated peptide
AAMK	420	0		7% for a_2(AM) − 48

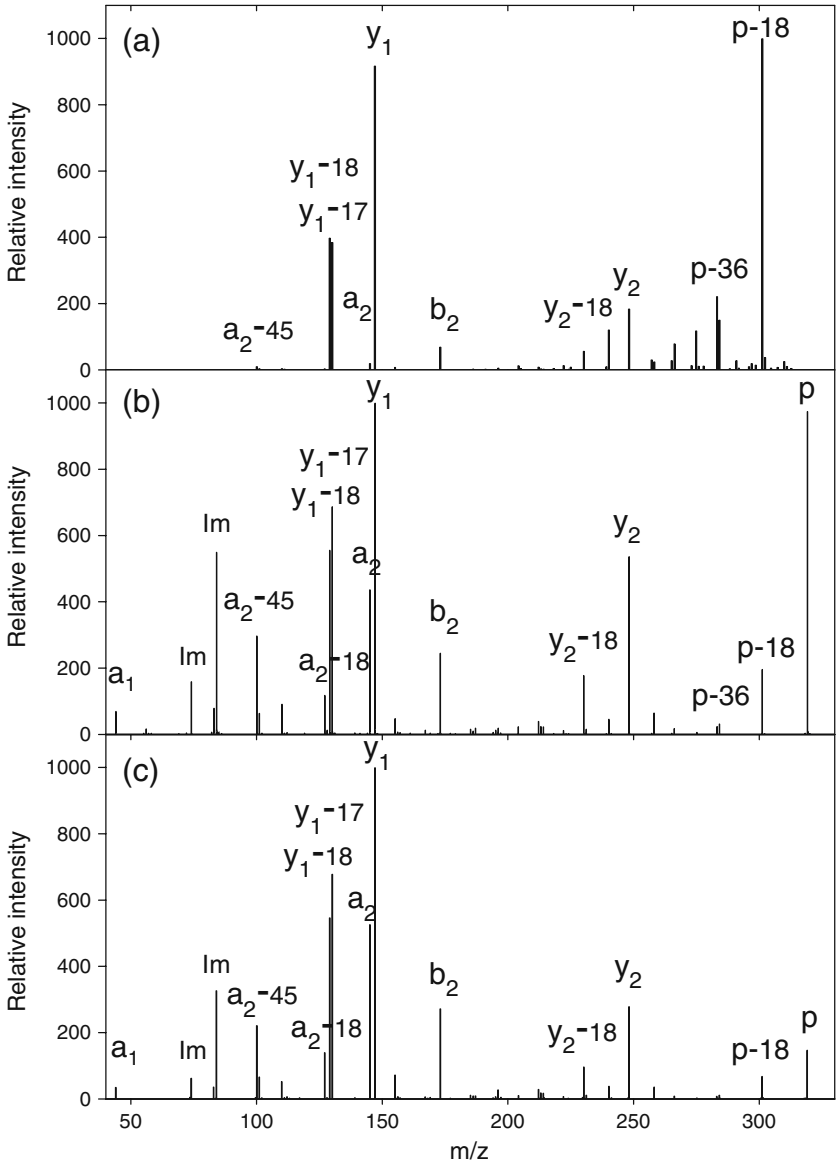


Figure 4. MS/MS spectra of the ATK tripeptide ion in three types of mass spectrometers: (**a**) linear ion trap (LTQ) at 35% collision energy setting, (**b**) quadrupole time-of-flight (QTOF) at 15 V collision voltage setting, and (**c**) triple quadrupole (QQQ) at 20 V collision voltage setting

predominantly the a_2 ion (Figure 6a), and that higher collision energies lead to the formation of smaller fragments. The a_2 ion fragments (Figure 6b) to give predominantly $a_2 - 45$ and a smaller amount of $a_2 - 18$, the latter due to loss of water from threonine. Immonium ions are formed at lower rates; in other words they become apparent at higher collision voltages.

Examination of other peptides revealed intense peaks corresponding to $a_2 - 45$ ions in most of the spectra of tripeptides with threonine at the central position (Table 2). The intensities of the $a_2 - 45$ peaks are generally lower than those of the $a_2 - 48$ peaks from methionine peptides discussed above. This difference may be partially due to competing processes, such as loss of water from threonine. In Table 2, only TTK is listed with a high intensity for the $a_2 - 45$ peak, but this is due to the fact that part of this peak intensity is due to the $y_1 - 17$ ion, which happens to have the same *m/z* value as $a_2 - 45$ for this peptide. Longer peptides with N-terminal AT show the same trend as those with AM, but the results are more scattered. What is clearly different between peptides with central T as compared with M is that placing the T in the N-terminal instead of the central position sometimes lowers the intensity of the $a_2 - 45$ peak but also sometimes increases it (Table 2). This finding led us to examine whether the $a_2 - 45$ peak is observed in peptides that do not contain T, and indeed it is, although with mostly lower intensities.

The relative intensity of the $a_2 - 45$ peak was highest when the central amino acid was T or V. It was lower for I, L, H, F, S, Y, still lower for other amino acids, and lowest

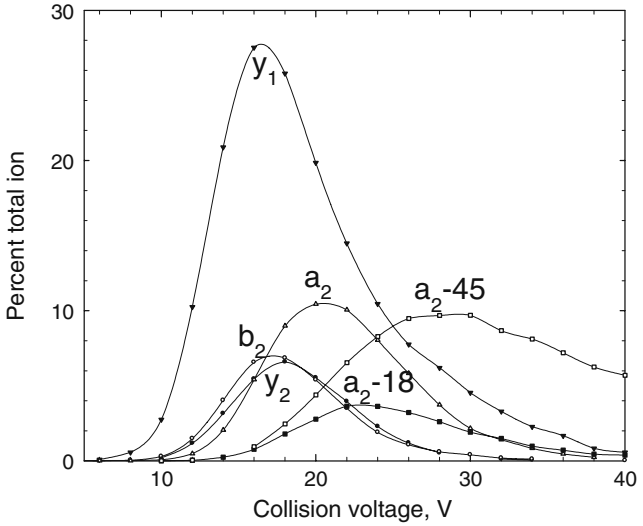


Figure 5. Dependence of peak intensity (percent total ion) on collision voltage in the MS/MS spectrum of ATK tripeptide ion in the triple quadrupole mass spectrometer, showing only the main peaks

for P, R, and K. This approximate average order varied considerably for different N-terminal residues. The only consistent result is that P, R, and K are the lowest and T and V are among the highest. When the central residue was kept constant and the N-terminal residue was varied, we found again that P, R, and K give the lowest intensities for the $a_2 - 45$ ions. The highest intensities were observed with M, S, and C in the N-terminal position, although the order varied considerably for different sets of peptides. The finding that R and K in the central or N-terminal positions decrease the relative intensities of the $a_2 - 45$ ions to near zero must be due to the high basicities of these residues; they bind the proton strongly on their side chains and prevent it from reaching the expected fragmentation site. Proline and histidine are also slightly basic, but only proline has a strong inhibitory effect on the formation of the $a_2 - 45$ ion, probably due to its cyclic structure. The reasons for the varying intensities with the various other residues are unclear. On the one hand, the amino acid residues of the a_2 ions can affect the rate of loss of the 45 Da species by their relative basicities or steric effects; on the other hand, they can affect the rates of competing processes, such as other neutral losses, side chain fragmentations, or the formation of immonium ions. Other neutral losses that are clearly observable include the loss of water from D, E, S, T, the loss of ammonia from Q, H, K, R, and the loss of CH_3SH from M (discussed above). However, variations exist even without these competing reactions. It should be noted in this regard that the loss of 45 Da was reported [7] for the a_2 ion derived from the sequence AG, but that it was not detected for the a_2 ion derived from the sequence GA.

The loss of 45 Da from a_2 ions was similar for peptides with C-terminal K or R, although the average relative intensities of the $a_2 - 45$ ion peaks were generally higher

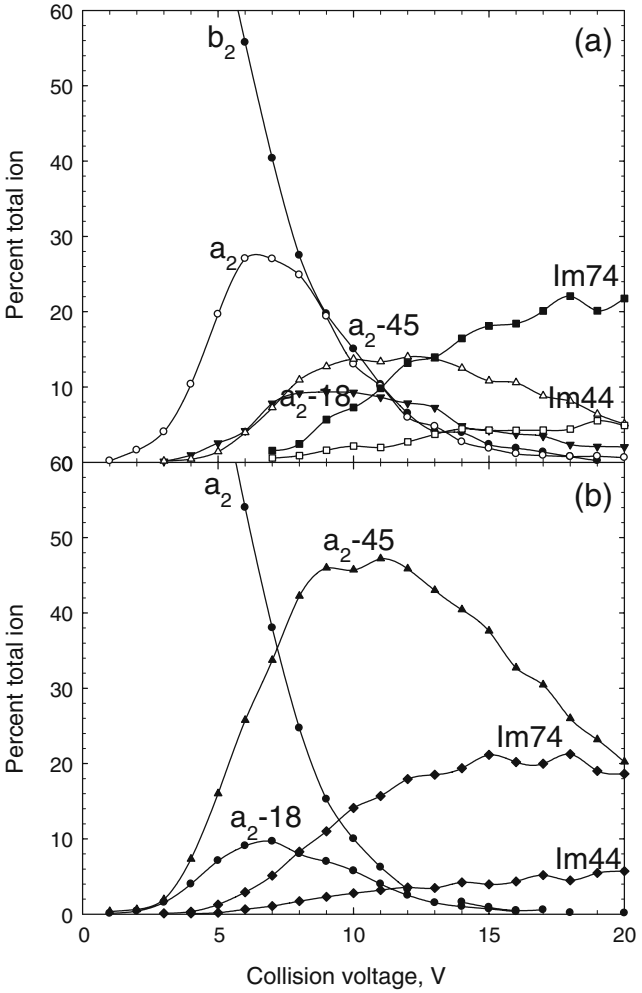


Figure 6. Quasi-MS^3 spectra of the b_2 (**a**) and a_2 (**b**) ions derived from ATK. The ions were produced in-source at high cone voltage (50 V), selected into the collision cell, and then their fragmentations were followed at different collision voltages

with K than with R (by 30–50%). The reason for this difference may be the higher basicity of R, which decreases the likelihood of forming b_2 ions and consequently a_2 ions as compared with y ions. $a_2 - 45$ ions were also observed upon the fragmentation of the diprotonated ions of the same peptides, although with generally lower relative intensities.

Fragmentation Pathways

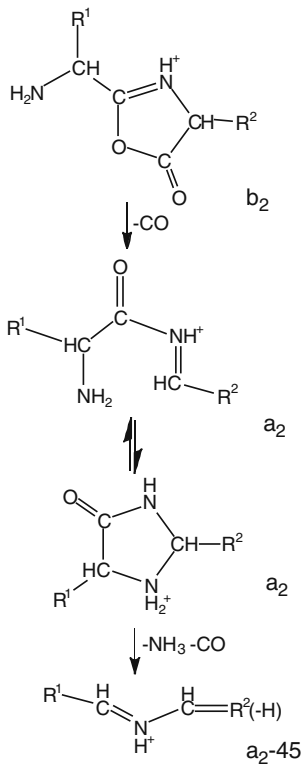
Loss of the 45 Da neutral species takes place from a_2 ions with various sequences and with varying contributions. The exact mass of this 45 Da species corresponds to (NH_3 + CO), eliminated in one step (as $HCONH_2$), or, more likely, eliminated in rapid sequential steps. No evidence was found for the loss of CO from the a_2 ions, and only scattered evidence for the loss of NH_3. Nevertheless, this does not necessarily indicate the loss of $HCONH_2$ in one step; it is possible that the loss of NH_3 is followed very rapidly by the

Table 2. Maximal intensities of the $a_2 - 45$ peaks in the triple quadrupole (QQQ) spectra of peptides containing threonine

Peptide	*m/z* for singly protonated	$a_2 - 45$ peak		$a_2 - 18$ ($a_2 - 17$)	Comments
		Intensity (max) %	*V* (max)		
TTK	349	31	28	6	$m/z(a_2 - 45)=m/z(y_1 - 17)$
STK	335	20	32	9	
MTK	379	18	28		
ETK	377	17	34	5	TEK, 2% (32 V)
DTK	363	14	30	2	TDK, 1% (36 V)
HTK	385	14	34	2 (2)	
FTK	395	14	28		TFK, 5% (32 V)
QTK-17	359	13	32	3	
YTK	411	11	29		TYK, 5% (32 V)
ATK	319	10	29	4	TAK, 3% (38 V)
GTK	305	9	30	9	TGK, 1% (40 V)
VTK	347	8	28	2	TVK, 9% (34 V)
LTK	361	7	26	2	TLK, 15% (38 V)
NTK	362	7	26	1	
ITK	361	6	26	1	
CTK	351	4	32	12	
PTK	345	2	29	1	
QTK	376	1	28	11 (4)	
KTK	376	0		(2)	
RTK	404	0		(5)	
ATAK	390	8	38		4% from doubly protonated peptide
ATAAK	461	6	54		7% from doubly protonated peptide
ATAAAK	532	7	68		5% from doubly protonated peptide
ATAAAAK	603	5	76		7% from doubly protonated peptide
AATK	390	0			2% for a_2(AT) − 45

loss of CO at the high collision energies at which these processes are taking place. To eliminate (NH_3 + CO) and leave the remaining parts of the a_2 ion intact, the structure of the a_2 ion must be cyclic, or at least in equilibrium with a cyclic form. Evidence for a cyclic structure for a_2 ions has been reported recently [8]. Therefore, we propose the pathway outlined in Scheme 1 to account for the loss of (NH_3 + CO) from a_2 ions (following the loss of CO from b_2 ions). MS^3 experiments with several a_2 − 45 ions show neutral losses that can be attributed to the side chains of each of the two residues, indicating that R^1 and R^2 in Scheme 1 remain intact through the 45 Da loss process.

Further support for the mechanism was obtained from theoretical calculations performed using the hybrid density functional method b3lyp [10] in conjunction with Pople's basis set (6–311+g(d,p)), as implemented in Gaussian 03 [11]. For all of the optimized structures, frequency analysis at the same level of theory was used to identify them as real minima and transition structures on the potential energy surface. Some intrinsic reaction coordinate calculations were performed to confirm the proposed mechanism. We have explored the potential energy surface for the formation of a_2 ions from the tryptic tripeptide ATK (or ATR) using DFT calculations. As shown in Table 3, the noncyclic structure is 84 kJ/mol less stable than the most stable cyclic structure (a 4-imidazolidinone ring) (Scheme 2). A second minimum is found for a cyclic structure protonated on N3 (39 kJ/mol higher than that protonated on N1). The most accessible pathway for reaction from the noncyclic structure is the formation of immonium ions (not shown in Scheme 2), but



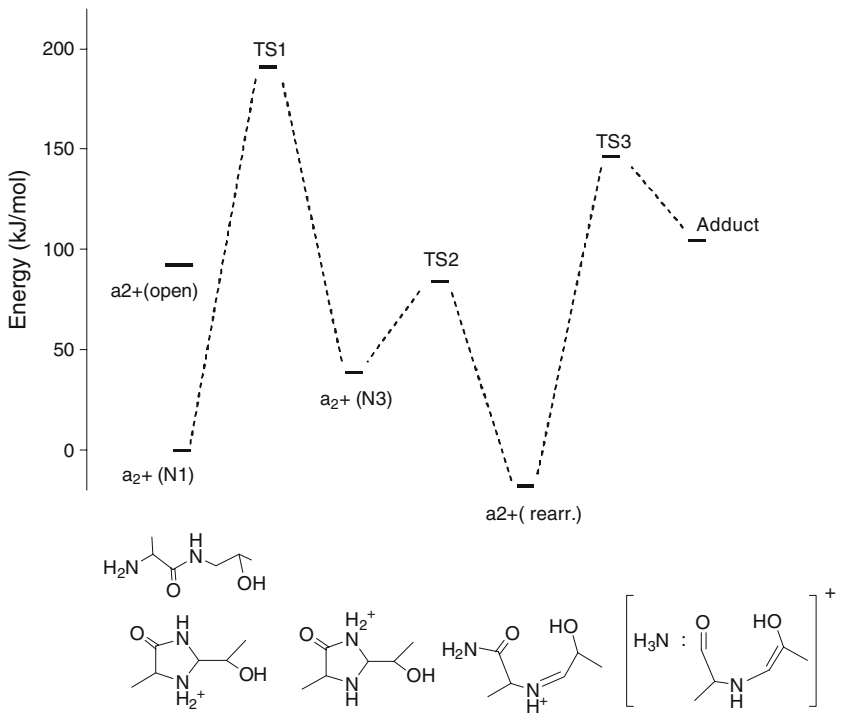
Scheme **1**. Proposed pathway to account for the loss of (NH_3 + CO) from a_2 ions (following the loss of CO from b_2 ions)

Table 3. DFT total energies and energy differences relative to the most stable a_2 ion derived from AT (protonated on N1)

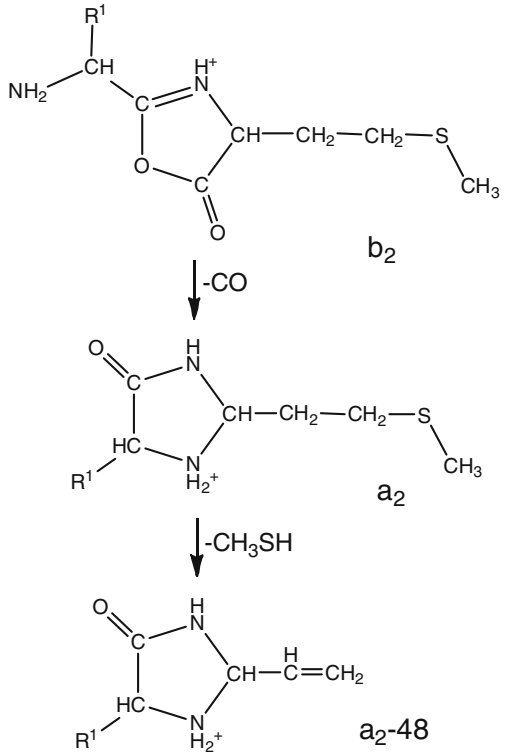
Molecular species	Energy (hartree)	Relative energies (kJ/mol)
a_2^+(open)	−496.298602	84.37
a_2^+(N1)	−496.330738	0.00
TS1	−496.258075	190.78
a_2^+(N3)	−496.315986	38.73
TS2	−496.298624	84.32
a_2^+(rearr.)	−496.337614	−18.05
TS3	−496.275039	146.24
Adduct (a_2 − 45 + NH_3)	−496.291073	104.14

by assuming a Boltzmann distribution at the effective temperature it is easy to verify that the population of the open structure is very low. Relaxed scans, performed at the same level of calculation, of the various distances of the 4-imidazolidinone ring reveal a possible pathway for the fragmentation of the a_2 ion (Scheme 2). First the imidazolidinone ring is protonated on N1, structure a_2^+(N1) in Scheme 2. Then the proton migrates to N3 to form structure a_2^+(N3), causing a small stretching of the N3–C2 bond distance (from 1.44 Å to 1.54 Å), followed by ring opening at the N3–C2 bond to form structure a_2^+(rearr.), which is 18 kJ/mol more stable than a_2^+(N1). The protonation step is necessary because the a_2 ion protonated on N1 is very stable, resulting in very high energy barriers for the ring opening. The final step in Scheme 2 involves hydrogen abstraction from the threonine to form an adduct of ammonia with the open structure of the a_2 − 45 ion. The actual mechanism is more complicated because of several competing pathways, for example hydrogen abstraction could occur also from the side chain of alanine or N1. An alternative pathway for the loss of formamide is also possible from a_2^+(rearr.) in Scheme 2, but the energy barrier is 88 kJ/mol higher than that for the consecutive loss of ammonia and carbon monoxide. The adduct in Scheme 2 undergoes the loss of CO to form the a_2 − 45 ion, which further rearranges to a more stable structure that is dependent on the amino acids involved. For the case being calculated (i.e., the a_2 ion derived from AT), it is likely that the enolic structure of the a_2 − 45 ion (Scheme 1) will convert into a more stable keto form. It should be pointed out that the immonium pathway is accessible from a_2^+(N3) with the ring opening at N1–C2, but the energy barrier is higher than that to form a_2^+(rearr.): 46 kJ/mol vs. 91 kJ/mol for the amino acids used in this calculation. Our results in Table 2 indicate, however, that the extent of formation of the a_2 − 45 ion depends greatly on the amino acid residues present in the a_2 ion, and that in many cases the abundance of this ion is low relative to that of the immonium ion.

Loss of CH_3SH from methionine in XaaMet a_2 ions (Scheme 3) makes a significant contribution in most cases, but the reaction is much less important in MetXaa a_2 ions (Table 1). This was also confirmed by quasi-MS^3 experiments comparing the fragmentations of the a_2 ions derived from AMK and MAK. The reason for this difference may be due to the fact that the side chain of Met, when in position 2, is located between two amine groups, and thus the proton located on either of these groups may be transferred to the sulfur atom and lead to the loss of CH_3SH. On the other hand, when Met is at position 1, its side chain is located between an amine group and a carbonyl group (Scheme 3), and is thus less likely to be activated by proton migration.



Scheme **2**. Potential energy profile for the unimolecular decomposition of the a_2^+ ion derived from AT



Scheme **3**. Loss of CH_3SH from methionine in XaaMet a_2 ions

Moreover, the sulfur of Met at position 1—but not at position 2—may form a weak bond with the carbonyl group [12], which may inhibit its protonation.

Loss of CH_3SH from methionine in a_2 ions occurs preferentially to the loss of (NH_3 + CO), which is common to most a_2 ions. From several MS^3 experiments with various a_2 ions, we estimate that loss of the 48 Da species from the XaaMet a_2 ions is at least 20 times more favorable than the loss of the 45 Da species. Therefore, it is expected that XaaMet a_2 ions undergo the two losses sequentially. Indeed, MS^3 experiments with the a_2 − 48 ions derived from seven XaaMet a_2 ions all exhibit a substantial loss of 45 Da and additional losses specific to the side chain of the first amino acid. The 45 Da neutral loss is dominant in many cases, but is sometimes accompanied by the loss of water or ammonia from the side chain of Xaa (e.g., Asp, Glu, His). The sequential loss of 48 Da and then 45 Da is also visible in Figure 3.

Since CH_3SH is lost from the side chain of methionine in a_2 ions, we examined whether such a loss occurs also from b_2 or y_2 ions, or from the precursor peptide ions. No loss of CH_3SH was detected from b_2 ions containing Met, indicating that the loss of CO to form a_2 ions is energetically more favorable. This is in line with the previous finding [13] that the b_1 ion of Met undergoes loss of CO rather than loss of CH_3SH. Significant loss of CH_3SH from precursor ions was observed only for diprotonated tripeptides with N-terminal Met. Diprotonated tripeptides with central Met exhibited only minor (about ten times less) losses of CH_3SH, and singly protonated tripeptides exhibited none at all. Losses from y_2 ions also were very minor. In contrast with the present findings, dissociation of protonated peptides at much higher collision energies was found to involve loss of a $CH_3S\cdot$ radical in certain cases [14].

In summary, a_2 ions undergo neutral loss of (NH_3 + CO), which indicates that these ions have cyclic structures (4-imidazolidinone). The extent of this reaction is highly dependent on the specific amino acid residues in each of the two positions of the a_2 ion. In certain peptides, the a_2 − 45 ion has one of the most intense peaks in the MS/MS spectrum, but in other cases the a_2 − 45 ion is negligible compared with the immonium ions. In the case of the a_2 ions derived from XaaMet, loss of CH_3SH takes place preferentially and is followed by the loss of (NH_3 + CO) at higher energies. Because the loss of CH_3SH depends on the location of Met within the a_2 ion, this process can be utilized to assign amino acid sequences to b_2 ions containing Met when the order of the residues is uncertain. In addition, the loss of CH_3SH from the precursor peptide ion with multiple charges is much more favorable for N-terminal Met than for central Met. These results are important, because assigning all of the peaks in a spectrum increases confidence in the peptide/metabolite identification and thus in library spectra.

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