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Novel Peptide Dissociation: Gas-Phase Intramolecular Rearrangement of Internal Amino Acid Residues

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Abstract: Unique intramolecular rearrangement product ions have been observed in the product ion spectra of a number of peptides. Multiple stages of mass analysis (MS^n), molecular modeling, and chemical modifications of peptides have been used to provide insight into the mechanism of this rearrangement reaction. The rearrangement process begins with a four-residue immonium ion that transfers a proton from the immonium nitrogen to the primary amine on the N-terminus. The proton transfer leads to the rearrangement of the peptide, exposing an internal amino acid on the terminus of the new ion. The internal amino acid that becomes the terminus of the new ion is then readily lost. The reaction seems to benefit from an extended experimental time frame available for reaction. The reaction is most prominent in quadrupole ion trap and Fourier-transform ion cyclotron resonance experiments, is observed under some conditions in a triple quadrupole, but is not seen in a sector instrument. Without previous knowledge of this process, the peptide sequence as determined by MS/MS could be misidentified.

Introduction

Tandem mass spectrometry (MS/MS) has been used extensively to determine the primary sequence of peptides.[1-4] Peptide ion dissociation provides important information about the primary structure of peptides, and much research has been devoted to understanding the dissociation patterns of peptides in mass spectrometers.[2,4] The vast majority of this research has focussed on the dissociation trends of peptide ions in beam instruments such as quadrupole[1,5] and sector[6-8] instruments with

product ions formed by collision-induced dissociation (CID). A great deal of information has been learned about the dissociation patterns of peptides under high and low energy collision conditions. High energy CID typically results in a broader range of fragmentation at the cost of increased spectrum complexity. Low energy CID usually affords simpler spectra, but amino acid side chain information is not available, and thus, for example, differentiation between leucine and isoleucine is not possible. Despite the extensive research on MS/MS of peptides, only a small number of studies have attempted to understand the dissociation patterns of peptide ions in a quadrupole ion trap.[9-11] It is expected that the MS/MS spectra

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(10) Doroshenko, V. M.; Cotter, R. J. *Anal. Chem.* **1995**, *67*, 2180.
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of peptide ions in a quadrupole ion trap should be similar to MS/MS spectra obtained on a triple quadrupole because both instruments operate in the low energy collision regime. However, the longer experimental time frames available in the quadrupole ion trap enable reactions not seen in other instruments to be observed. Also the ability to perform multiple stages of mass spectrometry (MS^n) in the quadrupole ion trap opens an entirely new area of investigation as the MS^n spectra of peptides are studied.

Of particular interest in studying the dissociation patterns of peptide ions in the quadrupole ion trap are dissociations, such as rearrangements, that might benefit from longer reaction times. Because collisions in the quadrupole ion trap typically occur at low kinetic energies with helium as the target gas, the internal energy gained by an ion during a collision is very low. To overcome the low internal energy deposition per collision, ion activation involves hundreds to thousands of collisions with the target gas(es) over the span of tens of milliseconds. This slow activation process may allow ions to probe numerous conformations at a given internal energy and increase the possibility of rearrangement reactions. Rearrangement reactions can often hinder interpretation of MS/MS spectra by providing confusing or contradictory information about the structure of the ion of interest. Therefore, any efforts to obtain sequence information from peptides with a quadrupole ion trap could potentially be hindered. In the course of trying to understand peptide ion dissociation patterns in the quadrupole ion trap, unusual product ions have been observed in a number of peptides. MS^n in the quadrupole ion trap along with molecular modeling and chemical modifications of these peptides have provided insight into the origin and mechanisms of what appears to be a common intramolecular rearrangement reaction. This rearrangement reaction is favored on the time scale of the quadrupole ion trap, but can be observed in appropriate triple quadrupole experiments. Under certain conditions in a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer the rearrangement reaction is the only reaction observed. The reaction is not observed, however, in corresponding experiments in a sector mass spectrometer.

Experimental Section

The quadrupole ion trap experiments were performed on a modified Finnigan MAT ITMS fitted for electrospray as previously described.[12] Peptides were dissolved in a mixture of 20:75:5 water−methanol−acetic acid to a concentration of 250 μM and infused into the electrospray needle at a flow rate of 0.5 μL/min. Ions were generated externally by electrospray and then directed by a series of lenses to an electronic gate that allowed ions to be injected through an endcap for 200−400 ms during the course of a scan function. Ions were then trapped, and the ions of the mass-to-charge ratio (m/z) of interest were isolated while all other ions were ejected from the ion trap prior to the MS/MS stage of the experiment. The selected ions were then resonantly excited by the application of a supplementary dipolar ac signal to the endcaps to effect CID and produce an MS/MS spectrum. MS^n experiments were performed by isolating product ions of the desired m/z from the previous stage of MS/MS, and then resonantly exciting those ions. Resonant excitation was done in the presence of helium or a mixture of helium and a small percentage of xenon for 20 ms.

Double resonance experiments were performed with a Textronix AWG 2020 Arbitrary Waveform Generator (Beaverton, OR). In these experiments parent ions of a chosen m/z were resonantly excited to effect CID using the frequency synthesizer on the ITMS electronics while the AWG 2020 supplied an ac signal to the endcaps corresponding to the secular frequency of selected product ions. The amplitude of the signal supplied by the AWG was sufficient (5 V$_{p-p}$) to resonantly eject the product ions as soon as they were formed. The double resonance experiments were performed to assess the possibility of consecutive versus competitive dissociation channels.

Sector experiments were performed on a Finnigan MAT900 double-focussing mass spectrometer with an EB geometry. Ions were generated by Fast Atom Bombardment (FAB) ionization and extracted from the source with a potential of 4750 V. Two microliters of a 1 mM solution of peptide dissolved in 20:75:5 water−methanol−acetic acid was dissolved in a glycerol matrix. Collision-induced dissociation (CID) experiments were performed with helium as the collision gas and by attenuating the parent ion beam by 65%. MS/MS experiments were carried out by using a constant B/E linked scan.

Triple quadrupole experiments were performed on a Finnigan MAT TSQ7000, Sciex API 3000, and a Micromass Quattro II. Ions were generated using electrospray by infusing 2−20 μM solutions of peptide at flow rates of 3−5 μL/min. To obtain MS/MS spectra of fragment ions, dissociation was induced in the skimmer region and the fragment ions of the mass-to-charge ratio of interest were mass selected with Q1 to undergo CID (15 eV collision energy) in the collision multipole.

FTICR experiments were performed on an instrument with a 9.4 T magnet constructed at the National High Magnetic Field Laboratory (Tallahassee, FL), which is described elsewhere.[13] Ions were generated using electrospray by infusing 250 μM solutions of peptide at a flow rate of 1 μL/min. MS^3 experiments were obtained by first isolating the parent ions of interest by a stored waveform (SWIFT)[14] and then subjecting the ions to sustained off-resonance irradiation (SORI)[15] for 0.5 s, causing them to undergo energetic collisions with N$_2$. The product ions of interest were then isolated by using a SWIFT waveform and dissociated by SORI (1-s irradiation).

The peptides (using the single letter symbols for the amino acids) YGGFL, YGGFLR, VGVAPG, 3,5-dibromo(Tyr^1)-leucine enkephalin, RKEVY, and RPPGFSPF were purchased from Sigma Chemical Company (St. Louis, MO). All other peptides were synthesized in house with the following procedure. Each peptide was assembled manually on a 0.10-mmol scale on a 9-fluorenylmethoxycarbonyl (Fmoc)-aminoacyl Wang resin (Advanced Chemtech) by solid phase methods. Each residue was coupled by using 0.40 mmol of the Fmoc-amino acid, the dehydrating agent O-benzotriazole-N,N,N′,N′-tetramethyluronium hexafluorophosphate (0.30 mmol), the base N-methylmorpholine (0.45 mmol), and 1-hydroxybenzotriazole (0.30 mmol). The glycine sequences were coupled as Fmoc-Gly-Gly. The Fmoc groups were removed with 1:4 (v/v) piperidine/N,N-dimethylformamide. Each peptide was cleaved from the resin with 19:1 (v/v) trifluoroacetic acid/water. The peptide/resin cleavage mixture was extracted with cold diethyl ether. The peptide was extracted with 5% acetic acid in water. Lyopholization of the acidic solution afforded the peptide as the acetate salt.

Peptides were acetylated by dissolving 1 mg of peptide into 1 mL of methylene chloride. Thirty microliters of acetic anhydride was added to the peptide/methylene chloride solution, and the mixture was allowed to react at room temperature for 30 min. The methylene chloride was then allowed to evaporate off, leaving an acetylated peptide with 50−75% yield.

Semiempirical molecular orbital calculations with the MOPAC package, version 6.0, were done in conjunction with the molecular modeling software program, PCMODEL (Serena Software). PCMODEL was first used to generate a structure based on molecular mechanics. This structure was then used as the starting structure for MOPAC, in which the AM1 (Austin Model 1) Hamiltonian was used.[16] MOPAC was used to provide insight into whether a proposed structure was reasonable with respect to a reference structure. In all cases the reference structure was the linear protonated peptide structure generated by PCMODEL and energy minimized in MOPAC.

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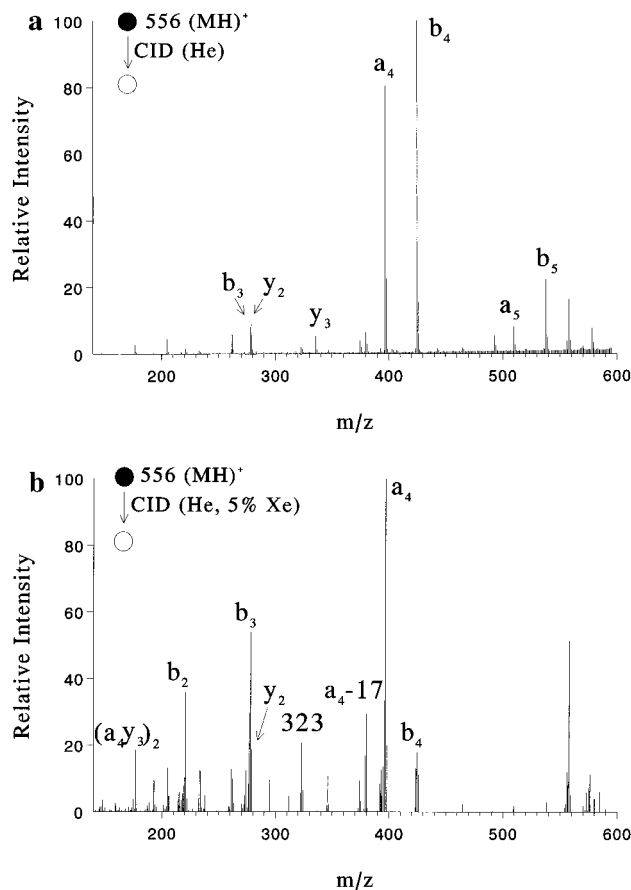
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Figure 1. (a) MS/MS spectrum of the protonated molecule of leucine enkephalin (*m/z* 556) in the presence of helium only. (b) MS/MS spectrum of the protonated molecule of leucine enkephalin in the presence of helium with 5% xenon.

Results and Discussion

The dissociation pattern of leucine enkephalin (YGGFL) has been well-studied in beam instruments under both low energy and high energy collision conditions.[17−21] A CID MS/MS spectrum of YGGFL in the quadrupole ion trap at a helium pressure of 8.6×10^{-4} Torr results in product ions that correspond to expected cleavages along the peptide backbone (Figure 1a). However, when the same experiment is done in the presence of 5% xenon (Figure 1b) a peak at *m/z* 323 appears along with a number of other new peaks. An ion at *m/z* 323 had not previously been reported in the MS/MS spectra of leucine enkephalin under either low or high energy collision conditions. Furthermore, the ion does not correspond to any expected product ion of leucine enkephalin. Upon further experimentation, it was found that the ion at *m/z* 323 was the most intense product ion in the MS[3] spectrum of the a_4[22] (*m/z* 397) ion (MH$^+ \rightarrow a_4 \rightarrow$?) of YGGFL in the presence of helium alone (Figure 2). (Peptide product ion nomenclature is based

(17) Katakuse, I.; Desiderio, D. M. *Int. J. Mass Spectrom. Ion Processes* **1983**, *54*, 1.
(18) Alexander, A. J.; Boyd, R. K. *Int. J. Mass Spectrom. Ion Processes* **1989**, *90*, 211.
(19) Ballard, K. D.; Gaskell, S. J. *Int. J. Mass Spectrom. Ion Processes* **1991**, *111*, 173.
(20) Thibault, P.; Alexander, A. J.; Boyd, R. K.; Tomer, K. B. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 845.
(21) Yalcin, T.; Csizmadia, I. G.; Peterson, M. R.; Harrison, A. G. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 233.
(22) The structure of the a_4 ion is formally presumed to be an immonium ion in which the leucine residue on the C-terminus and the carbonyl group on the phenylalanine residue have been lost. The charge can formally be assigned to the nitrogen in the phenylalanine residue having four covalent bonds (pept-CO-NH=CHR).
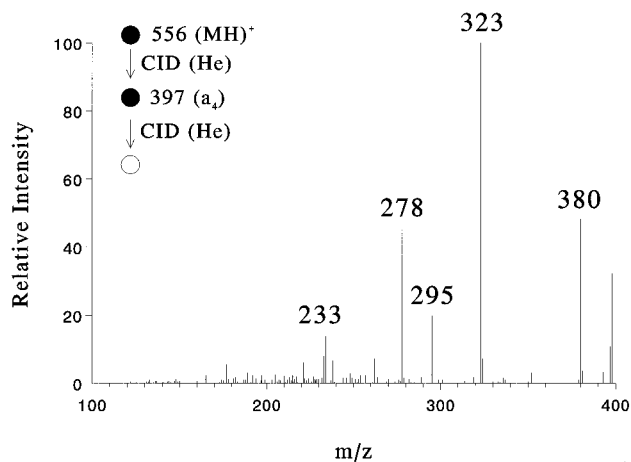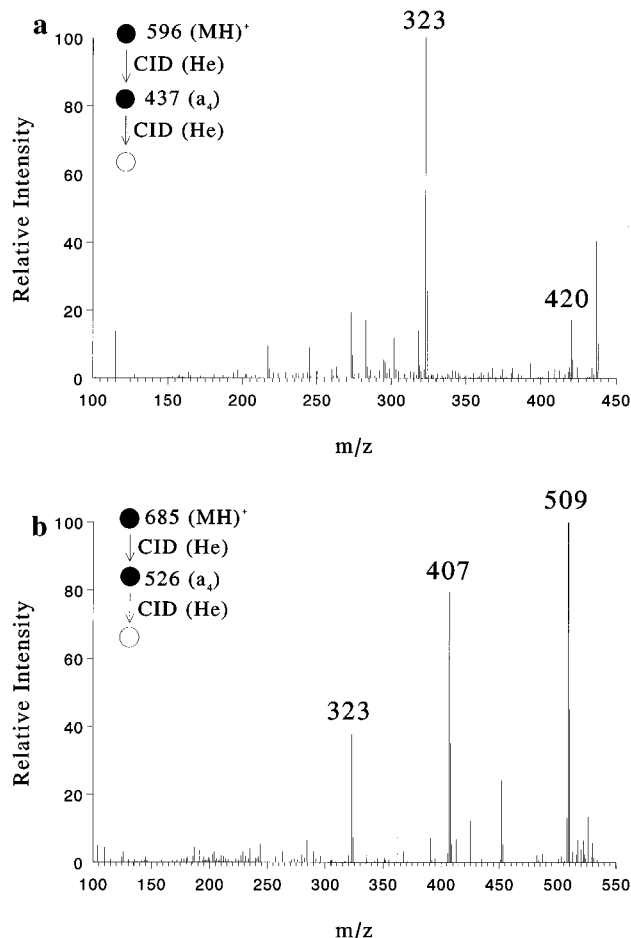

Figure 2. MS[3] spectrum in the presence of helium only of the protonated molecule of leucine enkephalin showing the product ions of the a_4 ion (*m/z* 397).

upon Biemann's modification[23] of Roepstorff's[24] original nomenclature. The a_4 ion is an immonium ion with four amino acid residues in which the original N-terminus of the peptide is retained.) No obvious structural feature would lead to a loss of 74 Da from *m/z* 397 to form *m/z* 323. One possibility is the loss of 17 Da (*m/z* 380), which is observed in the MS[3] spectrum, combined with a loss of one of the glycine residues (57 Da). This loss, however, seemed unlikely because the two glycines are internal residues. An MS[4] experiment on the product ion at *m/z* 380 (MH$^+ \rightarrow a_4 \rightarrow 380 \rightarrow$?) revealed a product ion at *m/z* 323, suggesting the possibility of a loss of a glycine residue (57 Da) is feasible. To determine if one of the two glycine residues in YGGFL was lost as a neutral, peptides in which the glycine residue in the third position was replaced by serine, proline, tryptophan, and leucine were synthesized. In all four cases the a_4 ion dissociated to form an ion at *m/z* 323. Figure 3 shows examples of the MS[3] spectra obtained for the peptides ((a) YGPFL and (b) YGWFL). In each of the four leucine enkephalin analogues in which the glycine in the third position had been replaced, the a_4 ion somehow rearranges to facilitate a loss of 17 Da and a loss of the third residue in the four residue sequence, leaving product ions that contain tyrosine, glycine, and phenylalanine residues at *m/z* 323. MS[4] experiments on the ion at *m/z* 323 result in identical spectra for all five peptides (YGGFL, YGLFL, YGPFL, YGSFL, YGWFL) (Figure 4). MS[5] experiments carried out on the major product ion (*m/z* 295) from the MS[4] spectra also result in identical spectra (Figure 5). These experiments confirm that the formation of the ion at *m/z* 323 from the a_4 ion occurs by a loss of 17 Da along with a loss of the third residue. Without previous knowledge of the peptide structure or the possibility of this reaction, the rearrangement ion could easily be interpreted as an a_3-17 ion, which would lead to incorrect assignment of the primary sequence (a_n-17 ions are common in CID MS/MS spectra obtained in a quadrupole ion trap[10]).

The most likely explanation for the formation of the ion at *m/z* 323 involves the occurrence of a rearrangement reaction. CID MS/MS of (M + H)$^+$ and an ion source generated a_4 ion of YGGFL in a sector instrument do not result in the formation of this rearranged product ion at *m/z* 323. However, MS/MS on the a_4 ion of YGGFL produced by skimmer dissociation in triple quadrupole instruments results in the product ion at *m/z* 323 present at an intensity of 35% relative to the b_3 product ion (*m/z* 278), which is the base peak in the spectrum. The b_3

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(24) Roepstorff, P.; Fohlman, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601.

Figure 3. (a) MS[3] spectrum of the peptide YGPFL (*m/z* 596) showing the product ions of the a_4 ion (*m/z* 437). (b) MS[3] spectrum of the peptide YGWFL (*m/z* 685) showing the product ions of the a_4 ion (*m/z* 526).
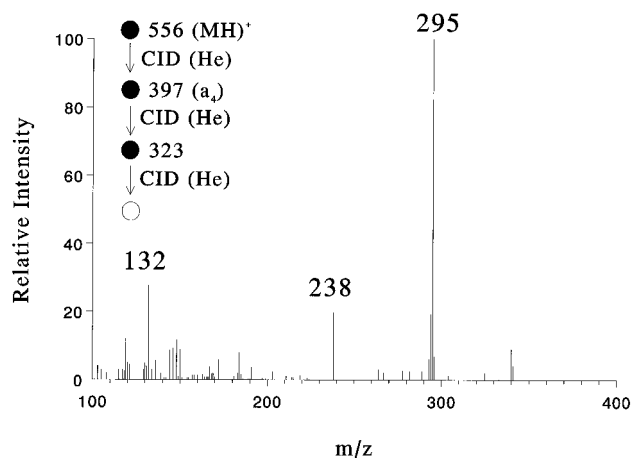
Figure 4. MS[4] spectrum of the protonated molecule of leucine enkephalin showing the product ions of the rearrangement ion (*m/z* 323).

product ion is expected to be formed if the peptide ion does not rearrange. The spectrum in Figure 2 shows that the product ion at *m/z* 323 is twice the intensity of the b_3 product ion (*m/z* 278) in the quadrupole ion trap. In the MS[3] experiment on the FTICR (MH$^+$ → a_4 → ?), the product ion at *m/z* 380 is the dominant ion and the product ion at *m/z* 323 is present at an intensity of 50% relative to *m/z* 380. Also, in the FTICR experiment the b_3 product ion is not present at all, suggesting that all the ions have rearranged. The results on each of the instruments suggest that the formation of the product ion at *m/z* 323 becomes more competitive as the experimental time frame
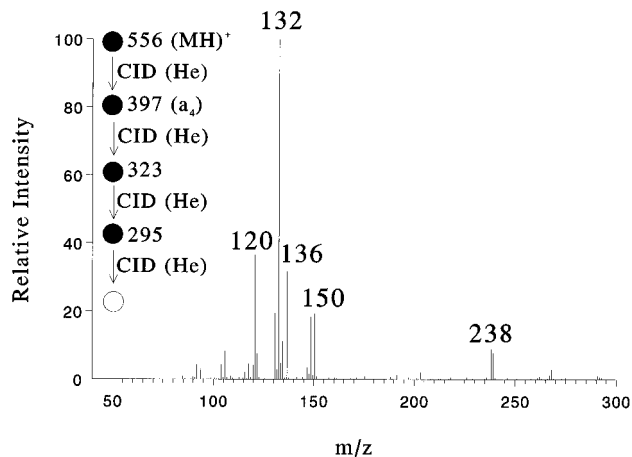
Figure 5. MS[5] spectrum of the protonated molecule of leucine enkephalin showing the product ions of the ion at *m/z* 295.

Table 1. Approximate Time Frames for Reactions in Various Instruments and Results for the Observed Rearrangement Reaction[a]

instrument	approx time frame	323/278 ratio
sector	0.01 ms	0
triple quadrupole	0.1 ms	0.35
quadrupole ion trap	20–800 ms	2.5–11.0
FTICR	30 s	∞

[a] The approximate times given correspond to the time from the beginning of activation until mass analysis.

available for the reaction increases, which is consistent with the proposition that the formation of the product ion at *m/z* 323 occurs by a rearrangement reaction. Table 1 shows a summary of the results obtained on each of the instrument types, including the approximate time frame for the experiment and the ratio of the intensities of the product ion at *m/z* 323 and the b_3 product ion (*m/z* 278) of YGGFL. The approximate times given in Table 1 correspond to the time from the beginning of ion activation until mass analysis. In the quadrupole ion trap, experiments were performed in which the activation time was varied over a wide range, and the resulting spectra differed as a result. The ratio of the intensity of the product ion at *m/z* 323 to the intensity of the product ion at *m/z* 278 reflects the main difference in the spectra at various reaction times.

The loss of 17 Da in YGGFL could occur by either loss of NH$_3$ from the N-terminus of the peptide or OH loss from the side chain of tyrosine. The loss of OH seems unlikely, and this possibility is completely eliminated by the observation of the rearrangement product ion in the spectra of peptides like FGGFL that do not contain a tyrosine residue. Further proof in the case of leucine enkephalin is provided by H/D exchange experiments that show that the loss of 17 Da shifts to a loss of 20 Da, which is ND$_3$ (data not shown).

A number of peptides were studied in an attempt to determine the structural features that might facilitate the formation of a rearranged product ion. Table 2 lists all the peptides studied. In leucine enkephalin (YGGFL) the formation of the product ion at *m/z* 323 occurred by a loss of 17 Da and a loss of a glycine residue (57 Da). It is possible that the loss of 17 Da occurs simultaneously with the loss of the glycine residue. However, a product ion at *m/z* 380 in the MS[3] spectrum of the a_4 ion of leucine enkephalin suggests that the reaction could occur stepwise. Two pieces of data support a stepwise reaction mechanism. First, MS[3] experiments on the a_4 ion done at the threshold of dissociation indicate that formation of the a_4-17 product ion is the lowest energy pathway. Secondly, MS[4] experiments performed on a_4-17 product ions result in the rearrangement ion being one of the most intense peaks.

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Table 2. List of Peptides Studied To Determine the Nature of the Rearrangement Reaction

peptide	four-residue immonium ion	rearranged product ion
YGGFL	YGGF	FYG
YGSFL	YGSF	FYG
YGPFL	YGPF	FYG
YGWFL	YGWF	FYG
YGLFL	YGLF	FYG
FGGYL	FGGY	YFG
FGGFL	FGGF	FFG
YGGLF	YGGL	LYG
YGGFLR	GGFL	LGG
YGGLL	YGGL	LYG
LGGFL	LGGF	FLG
LGGAL	LGGA	ALG
VGVAPG	VGVA	AVG
RKEVY	RKEV	VRK
RPPGFSPF	PPGF	not observed
Ac-FGGFL	Ac-FGGF	not observed

While the above experiments indicate a stepwise mechanism, double resonance experiments suggest that the reaction can occur both stepwise and as a concerted reaction. Double resonance experiments were performed in which m/z 397 (a_4) was resonantly excited in an MS^3 experiment while the product ions at m/z 380 (a_4-17) were simultaneously resonantly ejected. In this experiment the intensity of the product ion at m/z 323 (rearrangement ion) is reduced by only 25%. This result implies that the formation of the product ion at m/z 323 occurs by two mechanisms: one in which m/z 323 can be formed with m/z 380 as a stable ion intermediate, and another in which the rearrangement ion is formed directly from the ion at m/z 397.

From all the cases shown in Table 2, the results suggest that the rearrangement occurs with the loss of NH$_3$ along with a loss of the third residue in the four-residue immonium ion. To determine the possible mechanism of this rearrangement, molecular modeling studies were performed to provide reasonable conformations of the a_4 ion of YGGFL that might reveal intramolecular interactions that initiate this reaction. A possible conformation is displayed in the top of Scheme 1. Other researchers have observed peptide rearrangements similar to the one proposed here.[5,25,26] Hunt and co-workers described the interaction of the C-terminus of **b**-type ions with the N-terminal amino group resulting in an apparent cyclic intermediate.[25] Tang and Boyd also reported a rearrangement involving doubly charged **b**-type ions that involved the interaction of the acylium group with the primary amine group on lysine and ornithine residues.[26]

The salient feature of the structure reported in our work is the intramolecular proton bridge between the immonium nitrogen and the nitrogen on the primary amine of the N-terminus. This type of interaction in the gas phase has been noted in a number of recent papers.[11,27,28] The interaction is facilitated by the presence of glycine residues. The absence of side chains in the glycine residues reduces steric interferences which afford greater flexibility for the peptide backbone. The ability of the peptide backbone to bend seems necessary for this interaction to occur. In fact the rearrangement product ion is most abundant in the peptide YGPFL (Figure 3a) where the proline residue helps to "twist" the peptide backbone into a

conformation favorable for the proposed intramolecular proton bridge interaction. The rearrangement product ion is not the most abundant peak in the MS^3 spectrum of the peptide YGWFL (Figure 3b), in which the tryptophan residue perhaps hinders the necessary conformation to some extent. From this interaction a possible mechanism can be proposed (Scheme 1). Scheme 1 shows the proposed structures in the rearrangement reaction and the heats of formation (ΔH_f) for each ion from semiempirical calculations.

Part of the driving force for this reaction is the transfer of a proton from the immonium nitrogen to the more basic primary amine on the N-terminus. This proton transfer leads to a loss of NH$_3$ and subsequent cyclization of the remaining peptide. The cyclized peptide can then reopen, exposing an internal residue on the terminus of the new ion. This a_4-17 product ion would then have a structure similar to a **b**-type ion. It is unclear whether this ion is an acylium ion or has a protonated oxazolone structure proposed by Harrison et al.[21,29] Whatever its structure, this ion can readily lose the terminal residue (in this case glycine) and form the aforementioned rearrangement product ion. The driving force for the specificity of the ring opening is most likely the fixed charge located on the immonium nitrogen. This ring opening contrasts with the ring openings of cyclic peptides, which occur at a number of different sites leading to isomeric linear peptides.[30] In the case of cyclic peptides, the ring openings are initiated under low energy collision conditions by a mobile proton that is distributed among many sites along the backbone.

As an alternative to the stepwise mechanism, the rearrangement ion can form via a concerted mechanism (Scheme 2) as the double resonance experiments imply. The concerted reaction is defined here as any reaction that can occur within 1 μs. This is approximately the time for one rf cycle, or the minimum time in which ions could be resonantly ejected during the double resonance experiments.[31] In this mechanism the reaction is again driven by the transfer of a proton which facilitates the loss of NH$_3$, but the loss of ammonia is accompanied by a loss of a glycine residue. There are two possibilities for this mechanism (Scheme 2). One possibility would result in the formation of a cyclic neutral product which is similar to the neutral product proposed in the formation of **y**-type ions.[32,33] The second possibility involves the formation of a neutral product that is a linear isomer of the cyclic neutral product.

To help verify these mechanisms, the N-terminus of the peptide FGGFL was acetylated. Acetylation of the N-terminal amine would act to reduce the basicity of the N-terminus and provide some steric interference. MS^3 on the a_4 ion of acetylated FGGFL fails to show the rearrangement ion. Also, an internal product ion PPGF (a_5y_7)$_4$ from RPPGFSPF, which

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(30) Eckart, K. *Mass Spectrom Rev.* **1994**, *13*, 23.

(31) At the pressures used in these experiments the ion at m/z 380 is expected to undergo on average about 0.01 collisions during one rf cycle. As a result it is unlikely that the ion at m/z 380 would dissociate to form m/z 323 as a result of collisional activation during the time when it is being resonantly ejected. It is also possible that m/z 380 could be formed from m/z 397 with sufficient excess internal energy to dissociate further to the ion at m/z 323 before it could be resonantly ejected. From a simple RRKM calculation, assuming a tight transition state and a critical energy of 1.2 eV, at least 5 eV of internal energy would be required to observe the dissociation of m/z 380 to m/z 323 in less than 1 μs. This amount of excess internal energy seems unlikely; therefore, it is reasonable to conclude that the double resonance experiments in this case are able to distinguish between a concerted reaction and two rapid consecutive reactions.

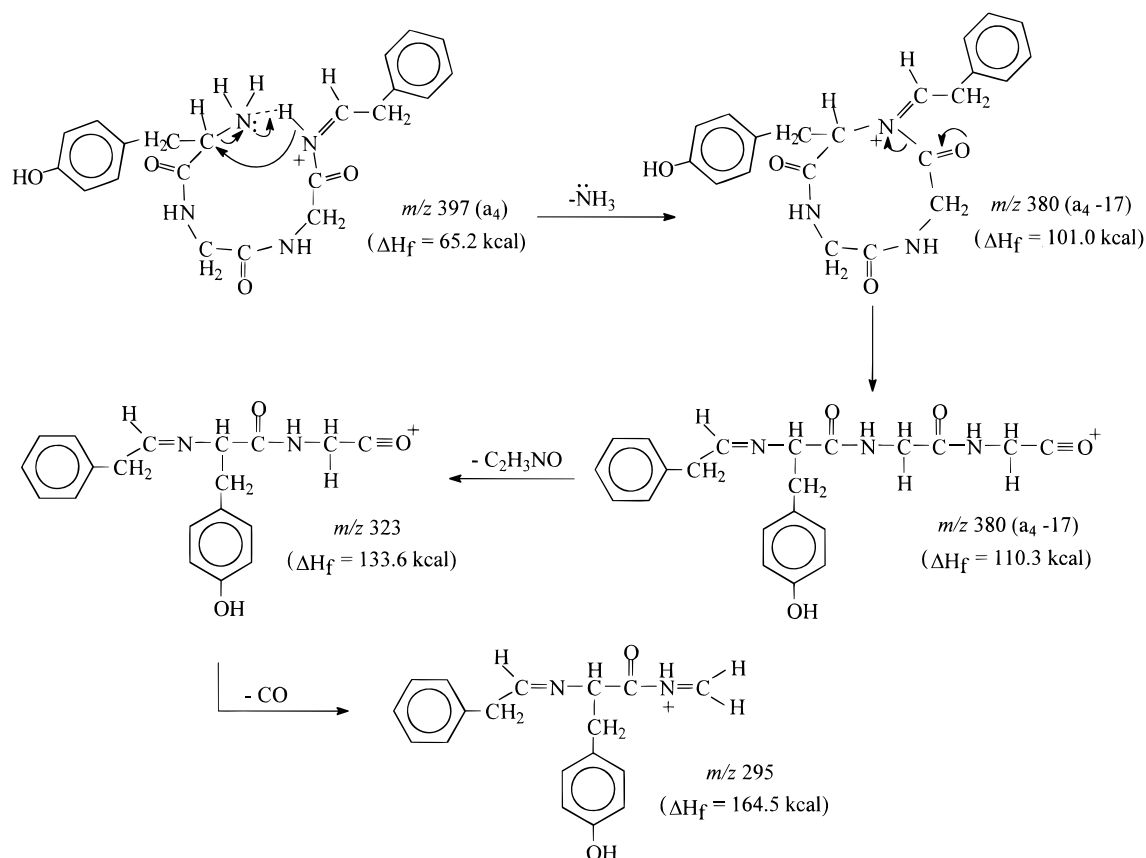(32) Mueller, D. R.; Eckersley, M.; Richter, W. J. *Org. Mass Spectrom* **1988**, *23*, 217.

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Scheme 1. Proposed Stepwise Mechanism for the Formation of the Rearrangement Ion at *m/z* 323 and *m/z* 295 from the **a₄** Ion of Leucine Enkephalin

Scheme 2. Two Possible Concerted Mechanisms for the Formation of the Rearrangement Ion at *m/z* 323 from the **a₄** Ion of Leucine Enkephalin
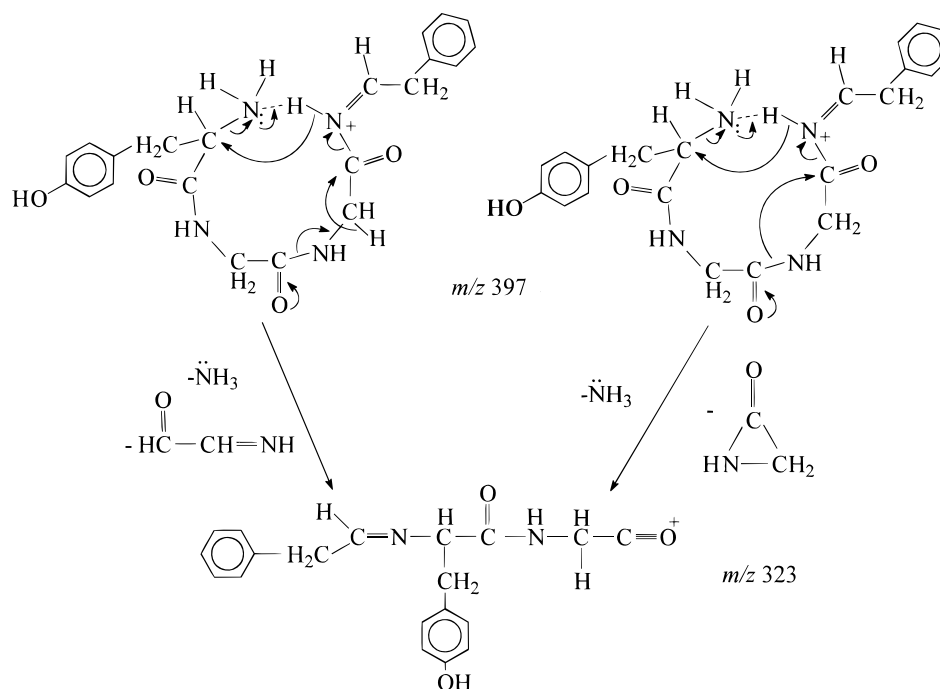
is assumed to have an immonium ion structure (**a**-type) at the phenylalanine residue and **y**-type structure at the proline residue, fails to dissociate to the expected rearrangement ion. In both cases the N-terminus is no longer a primary amine, and the rearrangement reaction seems to be prevented by steric interferences. In addition, the reduced basicity of the acetylated peptide could also decrease its propensity for rearrangement. It should

be pointed out that another internal product ion (GGFL from YGGFLR) undergoes the rearrangement, indicating the internal product ions have the assumed structure.

The most obvious experiments to verify the structures of the ions proposed in Schemes 1 and 2 are MS^n experiments on each ion. MS^n experiments were performed on the **a₄**-17 ions and the rearrangement product ions of all the ions listed in Table 2.

Table 3. Relative Product Ion Intensities in MS^3 and MS^4 Spectra of Selected Peptides

Product Ions of **a$_4$** Ion

peptide	**a$_4$**-17	rearrangement ion	**b$_3$**
YGGFL	48	100	40
YGLFL	100	20	32
FGGFL	63	100	35
YGGLL	69	100	42
FGGYL	51	100	16
VGVAPG	100	31	0

Product Ions of **a$_4$**-17 Ion

peptide	loss of 28	rearrangement ion (Re.)	*Re.* −57,-28	**a$_3$**-17
YGGFL	58	92	100	17
YGLFL	80	100	5	35
FGGFL	47	100	58	52
YGGLL	37	100	80	41
FGGYL	52	100	44	20
VGVAPG	38	100	9	91

Product ions of Rearrangement ion

peptide	loss of 28	other
YGGFL	100	*238*: 17 *132*: 23
YGLFL	100	*132*: 8
FGGFL	100	*132*: 5
YGGLL	100	no product ions above 5%
FGGYL	100	*148*: 8
VGVAPG	100	no product ions above 5%

In most of the cases in Table 2, the types of product ions observed were the same, but usually intensities were different due to the specific amino acids present in each peptide. Table 3 shows the product ion intensities from MS^3 and MS^4 experiments for selected peptides from Table 2. For the sake of simplicity, the following discussion will outline, as the illustrative case, the results obtained for MS^n experiments with YGGFL.

The first studies involved observing the product ions of **a$_4$**-17 (*m/z* 380). The **a$_4$**-17 product ions were formed in the MS^3 spectrum of the **a$_4$** ion, isolated, and then subjected to resonant excitation (MH$^+$ → **a$_4$** → **a$_4$**-17 → ?). The MS^4 spectrum that was generated contained predominantly three product ions at *m/z* 323, 238, and 352, but also contained a small intensity product ion at *m/z* 266. These product ions seem consistent with the proposed structure of the **a$_4$**-17 ion. The product ion at *m/z* 323 is the rearrangement product ion initially observed and would be formed by a loss of the glycine residue on the terminus of the **a$_4$**-17 product ion (−C$_2$H$_3$NO). The product ion at *m/z* 352 corresponds to a neutral loss of 28 Da, which is most likely CO. Carbon monoxide (CO) is a common loss from **b**- type ions, lending further proof that the **a$_4$**-17 ion has a structure similar to other **b**- type ions. The product ion at *m/z* 238 presumably occurs by the loss of CO and two glycine residues (−C$_5$H$_6$N$_2$O$_3$, or −CO, −C$_2$H$_3$NO, −C$_2$H$_3$NO). The remaining product ion at *m/z* 266 probably corresponds to a loss of two glycine residues (−C$_4$H$_6$N$_2$O$_2$, or −C$_2$H$_3$NO, −C$_2$H$_3$NO).

MS^4 experiments were also performed in which the product ions of *m/z* 323 were analyzed to help provide insight into the structure of this rearrangement ion (MH$^+$ → **a$_4$** → 323 → ?). Figure 4 shows the spectrum obtained for YGGFL during these experiments. The main product ions are observed at *m/z* 295, 238, and 132. In other experiments in which the resonant excitation voltage is reduced, the only product ion present is *m/z* 295. Again a loss of CO (28 Da) is consistent with peptide ions that have **b**-type ion structures. The product ion peak at

m/z 238 is presumably the same ion observed in the product ion spectrum of the **a$_4$**-17 ion. As a product ion from the parent ion at *m/z* 323, *m/z* 238 would be formed by a loss of CO and a glycine residue (−C$_3$H$_3$NO$_2$, or −CO, −C$_2$H$_3$NO). The means by which the product ion at *m/z* 132 is formed is not as clear, but an MS^5 spectrum (MH$^+$ → **a$_4$** → 323 → 295 → ?) of *m/z* 295 (Figure 5) shows the ion at *m/z* 132 to be the major product ion. It is possible that this ion forms by the loss of the glycine residue, a loss of CO, and a loss of the tyrosine side chain with a retention of an extra hydrogen in the product ion. Another possible origin of the ion at *m/z* 132 is from *m/z* 295 that has undergone rearrangement (see below).

Some of the other product ions besides *m/z* 132 in the MS^5 spectrum of *m/z* 295 are difficult to rationalize based on the structure in Scheme 1. The other predominant product ions appear at *m/z* 120, 136, 148, and 150. The ions at *m/z* 120 and 136 are very likely the immonium ions of phenylalanine and tyrosine, respectively, and these ions are reasonable product ions from the structure proposed in Scheme 1 for *m/z* 295. The origin of the product ions at *m/z* 148 and 150 is not as clear. To determine the nature of these product ions, a leucine enkephalin analogue with the tyrosine side chain brominated in the third and fifth positions of the phenyl ring was studied. An MS^5 experiment was performed on the ions analogous to the ions at *m/z* 295 in leucine enkephalin. The product ion spectrum of these ions with two ^{79}Br (*m/z* 453) revealed product ions at *m/z* 132, 294, 306, 308, and 396. The appearance of ions at *m/z* 306 and 308 reveals the fact that ions at *m/z* 148 and 150 in unlabeled leucine enkephalin retain the tyrosine residue. The product ion at *m/z* 132 does not contain the tyrosine residue (or at least the side chain of tyrosine). The exact mechanism for the formation of these ions is not known, but the possible structures that can be drawn are limited and some of these structures could be derived from the parent ion at *m/z* 295.

Scheme 3 shows a possible rearrangement that the ion at *m/z* 295 could undergo to facilitate the formation of the product ion at *m/z* 132. This rearrangement is similar to a Cope rearrangement that has been observed for even-electron anions in the gas phase.[34,35] This aza-Cope rearrangement would presumably be prompted by the heating of the ion at *m/z* 295 by collisional activation during resonant excitation. The product of the aza-Cope rearrangement (Scheme 3) would likely have resonance structures—one as shown in Scheme 3 and another in which the ion has the charge more localized on the oxygen. The former of the two resonance structures is the more stable species.

The presence of two isomers at *m/z* 295 is feasible (i.e. the top and bottom structures in Scheme 3), but cannot be substantiated. Assuming the ion at *m/z* 295 can rearrange (aza-Cope rearrangement) and form an isomer, the ion at *m/z* 132 would be formed by a loss of 163 Da (−HNCO, −C$_8$H$_8$O). An MS^6 experiment observing the product ions of *m/z* 132 resulted in product ions at *m/z* 130, 117, and 105. These product ions suggest that the structure of the ion at *m/z* 132 derived from the rearranged ion at *m/z* 295 is reasonable.

The MS^3 spectra of three and five-residue immonium ions (i.e. **a$_3$** and **a$_5$**) of the same peptides mentioned above were examined to determine if a similar rearrangement reaction occurs with these product ions. It was found that the three-residue immonium ions did not rearrange in any manner similar to the four-residue immonium ions. However, the five-residue immonium ions reacted similarly to the four-residue immonium

(34) Rozeboom, M. D.; Kiplinger, J. P.; Bartmess, J. E. *J. Am. Chem. Soc.* **1984**, *106*, 1025.
(35) Eichinger, P. C. H.; Dua, S.; Bowie, J. H. *Int. J. Mass Spectrom. Ion Processes* **1994**, *133*, 1.

Scheme 3. Proposed Aza-Cope Rearrangement Undergone by *m/z* 295 of Leucine Enkephalin
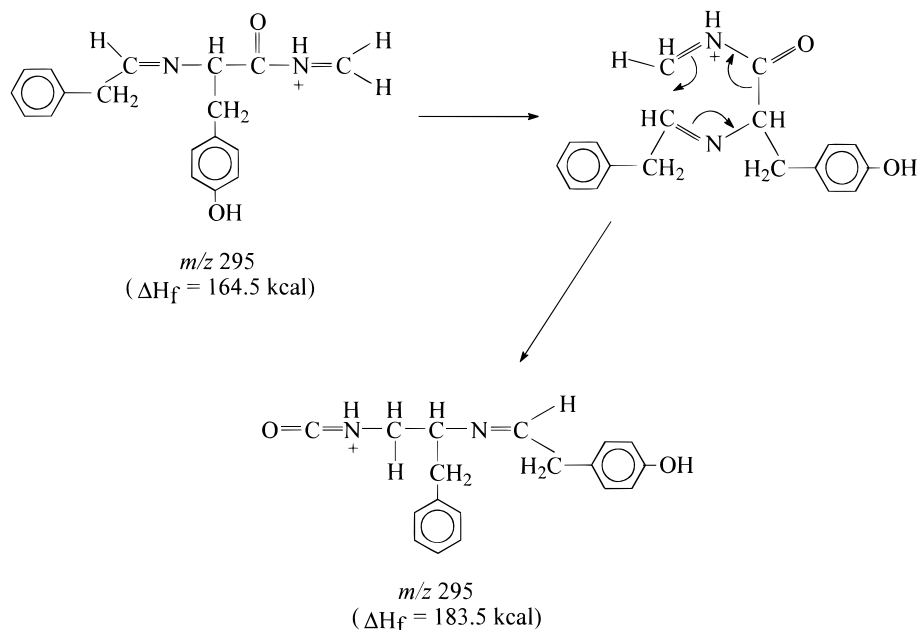
ions but to a much lesser extent. The rearranged product ion from the five-residue immonium ion never reached a relative intensity greater than 1% for any of the peptides studied. The reaction proceeded with a loss of 17 Da and a loss of the fourth residue in the five-residue sequence. It is not clear what factors facilitate the formation of the rearrangement product ion from the four-residue immonium ion relative to the three- or five-residue immonium ions. Apparently, there must be a trade-off between enthalpy and entropy that maximizes the probability of reaction for the four-residue species.

Conclusions

MS/MS experiments with longer time frames for reaction allow ions to undergo intramolecular reactions that result in unique CID product ions. In the cases outlined here, the incipient ion in the rearrangement reaction is a four-residue immonium ion, either an $\mathbf{a_4}$ or an internal fragment $(\mathbf{a}_m\mathbf{y}_n)_4$ ion. The reaction seems to be driven by the transfer of a proton from the immonium nitrogen to the more basic primary amine on the N-terminus of the peptides. This proton transfer leads to a loss of NH_3 and a loss of an internal residue by means of an intramolecular rearrangement that transposes the internal residue to the terminus of the peptide ion. Without prior knowledge it is possible that the resulting product ion would be misidentified, leading to inaccurate determination of the structure of the parent ion. Further stages of MS suggest that unique ions are formed from this rearranged ion including ones from a possible aza-Cope rearrangement. These results indicate that not only the energy of collisional activation but also the time scale of collisional activation is important in the dissociation of peptides.