Isotope Labeling and Theoretical Study of the Formation of a_3^* Ions from Protonated Tetraglycine

Travis Cooper, Erach Talaty, Jerod Grove, and Michael Van Stipdonk

Department of Chemistry, Wichita State University, Wichita, Kansas, USA

Sándor Suhai and Béla Paizs[‡]

Department of Molecular Biophysics, German Cancer Research Center, Heidelberg, Germany

Extensive ^{13}C , ^{15}N , and ^2H labeling of tetraglycine was used to investigate the $b_3^+ \rightarrow a_3^*$ reaction during low-energy collision-induced dissociation (CID) in a quadrupole ion-trap mass spectrometer. The patterns observed with respect to the retention or elimination of the isotope labels demonstrate that the reaction pathway involves elimination of CO and NH_3 . The ammonia molecule includes 2 H atoms from amide or amino positions, and one from an α -carbon position. The loss of NH_3 does not involve elimination of the N-terminal amino group but, instead, the N atom of the presumed oxazolone ring in the b_3^+ ion. The CO molecule eliminated is the carbonyl group of the same oxazolone ring, and the α -carbon H atom is transferred from the amino acid adjacent to the oxazolone ring. Quantum chemical calculations indicate a multistep reaction cascade involving CO loss on the $b_3 \rightarrow a_3$ pathway and loss of $\text{NH}=\text{CH}_2$ from the a_3 ion to form b_2 . In the postreaction complex of b_2 and $\text{NH}=\text{CH}_2$, the latter can be attacked by the N-terminal amino group of the former. The product of this attack, an isomerized a_3 ion, can eliminate NH_3 from its N-terminus to form a_3^* . Calculations suggest that the ammonia and a_3^* species can form various ion-molecule complexes, and NH_3 can initiate relay-type mobilization of the oxazolone H atoms from α -carbon positions to form a new oxazolone isomer. This multiple-step reaction scheme clearly explains the isotope labeling results, including unexpected scrambling of H atoms from α -carbon positions. (J Am Soc Mass Spectrom 2006, 17, 1654–1664) © 2006 American Society for Mass Spectrometry

CID and tandem mass spectrometry [1, 2] are now well established as effective tools for deriving sequence information from peptides and proteins. The various reaction pathways involved in the dissociation of protonated peptides have recently been extensively reviewed [3]. Fragmentation of protonated peptides under low-energy collision conditions is dominated by reactions that involve cleavage of the amide bonds along the peptide backbone [4, 5]. Qualitatively, protonated peptide dissociation can be described using the “mobile proton model” [6–19], for which a main tenet is the intra-molecular migration of protons from the most basic group on a peptide to the site of cleavage. The recently introduced “pathways in competition” (PIC) fragmentation model [3] provides a more general framework, taking into account specific features of

individual peptide fragmentation pathways (PFPs) and their interaction. PIC states that fragment ion abundances in the MS/MS spectra of peptides are determined by predissociation, bond-cleavage, and post-cleavage events. The predissociation phase involves proton transfers (mobile proton) and internal rotations necessary to populate fragmenting species. Besides the actual bond-cleavage mechanisms, energetics, and kinetics that determine competition of the PFPs, the postcleavage phase determines the fate of the added proton (e.g., which fragment will appear as charged species) based on the proton affinities (or gas-phase basicities) of the species involved. In the present article we will show that additional transitions like association and relay type exchange reactions can occur in post-cleavage ion-molecule complexes formed by peptide fragments.

The dominant product ions from the CID of protonated peptides are those of the b_n and y_m series, with the former representing ionized fragments containing the N-terminus and the latter containing the C-terminus of the original peptide. While originally proposed to be acylium ions [20–22], the b_n ions are now generally accepted to have a cyclic oxazolone structure [23–30].

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Address reprint requests to Professor M. J. Van Stipdonk, Department of Chemistry, Wichita State University, Wichita, KS 67260-0051, USA. E-mail: Mike.vanStipdonk@Wichita.edu, and Béla Paizs, Department of Molecular Biophysics, German Cancer Research Center, Im Neuenheimer Feld 580, Heidelberg, D-69120, Germany. E-mail: b.paizs@dkfz-heidelberg.de

Scheme 1. Pathway for generation of a_3^* from b_3 proposed by Harrison and Young.

The dissociation/decomposition of b_n ions has also been the subject of both experimental and theoretical studies [24, 29, 31, 32]. A principal product derived from the dissociation of a b_n ion is the a_n species, which is formed through a ring-opening reaction and the elimination of CO [24, 33]. A second product, labeled a_n^* , has been observed in the metastable ion dissociation [24] and CID of small peptides [34, 35]. Formation of this particular product involves the net loss of 45 mass units (Da), which in earlier reports was proposed to involve elimination of CO and NH_3 .

In an investigation of the CID of protonated alanine oligomers, Harrison and Young proposed that a_3^* is generated by decomposition of the a_3 ion through the mechanism shown in Scheme 1. [34]. The pathway suggested involves proton transfer from the imine nitrogen atom to the N-terminal amino group, followed by nucleophilic attack (by the same imine nitrogen atom) upon the α -carbon position of the N-terminal amino acid and subsequent elimination of the amino terminus as NH_3 . The structure proposed for the product ion (a_3^*) is reminiscent of substituted diketopiperazines, which have been implicated in the formation of y_m type ions from protonated peptides [36].

Glish and coworkers used the relative reactivities of b_n^+ ions with dimethylamine in an ion-trap mass spectrometer to demonstrate an apparent unique behavior for b_3^+ ions [35]; in particular that the formation rate for a dimethylamine adduct was lowest for b_3 ions when compared to b_2 and b_4 ions generated from a range of peptides. The differences in reaction rate were used as evidence that the b_3 ion may be formed by a pathway similar to that shown in Scheme 1, and a similar process was proposed by Vachet et al. to describe formation of a_4 and a_5 ions from a series of leucine enkephalin analogues [37].

In this article, formation of a_3^* ions from a model peptide is discussed using experimental and theoretical strategies. The experimental work involved synthesis of tetraglycine (GGGG) with specific ^{13}C , ^{15}N , and 2H labeling of amino acid residues and elucidation of CID pathways using multiple-stage tandem mass spectrometry. The goal of the isotope labeling studies was to

identify the atoms within the b_3 ion that are eliminated upon formation of a_3^* . A comprehensive molecular dynamics and computation/theoretical investigation was then undertaken to provide mechanistic details of the underlying chemistry necessary to produce a_3^* along a pathway that accounts for the observations made in the isotope labeling study.

Experimental and Computational Details

Experimental work was performed at the Department of Chemistry, Wichita State University, Wichita, KS. The molecular dynamics simulations and the quantum chemical calculations were carried out at the German Cancer Research Center at Heidelberg. Both the experimental and theoretical strategies are briefly described below.

Mass Spectrometry

All peptides used in this study were generated by conventional solid-phase synthesis methods [38] using 9-fluorenylmethoxycarbonyl (Fmoc)-glycine loaded Wang resin (Sigma Chemical, St. Louis, MO) and a custom-built, multiple reaction vessel peptide synthesis apparatus. Glycine- ^{15}N ($H_2^{15}NCH_2COOH$, **G- ^{15}N**), glycine-1- ^{13}C ($H_2NCH_2^{13}COOH$, **G-1- ^{13}C**), α -d $_2$ -glycine (H_2NCD_2COOH , **α -d $_2$ -G**), and Fmoc-chloride were purchased from Sigma Chemical (St. Louis, MO) and used to generate N-terminus Fmoc-protected amino acids for incorporation into the model peptides. Fmoc protected glycine (**G**) were purchased from Sigma and used as received. Peptides, once cleaved from the resin, were used without subsequent purification in the CID studies. The sequence of each peptide, and in particular the position of isotope labels, was confirmed using multiple-stage CID of Na^+ and Ag^+ cationized versions. The positions of the isotope labels were also used to confirm that the major sequence ions generated by CID of protonated GGGG are b_3^+ arising by elimination of the C-terminal G residue, and y_2^+ arising by elimination of 2 G residues from the N-terminus. Solutions of each peptide were prepared by dissolving the appropriate amount of solid material in a 1:1 (vol:vol) mixture of HPLC grade MeOH (Aldrich Chemical, St. Louis, MO) and deionized H_2O to produce final concentrations of 10^{-5} to 10^{-4} M.

For experiments in which the CID of amino, amide, and acid deuterium labeled peptides was examined, the relevant peptides were incubated in a 50/50 mixture of D_2O and CH_3OD (Aldrich Chemical, St. Louis, MO) for 2 h. The peptide solutions in deuterated solvent were then injected into the ESI without further modification. In this case, ESI produced abundant $(M + D)^+$ ions.

ESI mass spectra were collected using a Finnigan LCQ-Deca ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Peptide solutions were infused into the ESI-MS instrument using the incorporated syringe pump and a flow rate of 5 $\mu l/min$. The atmospheric

pressure ionization stack settings for the LCQ (lens voltages, quadrupole, and octopole voltage offsets, etc.) were optimized for maximum $(M + H)^+$ transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5 kV, the N_2 sheath gas flow at 25 units (arbitrary for the Finnigan systems, corresponding to ~ 0.375 L/min) and the capillary (desolvation) temperature at 200 °C. Helium was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for CID experiments.

The CID studies (MS/MS and MS^n) were performed as follows. The $(M + H)^+$ ions were isolated for the initial CID stage (MS/MS) using an isolation width of 0.9 to 1.2 mass to charge (m/z). Product ions selected for subsequent CID (MS^n) experiments were isolated using widths of 1.0 to 1.3 m/z . The exact width was chosen empirically and reflected the best compromise between high precursor ion abundance and the isolation of a single isotopic peak. The (mass) normalized collision energy, which defines the amplitude of the R.F. energy applied to the end cap electrodes in the CID experiment, was set between 20 and 25%, which corresponds roughly to 0.55 to 0.68 V with the instrument calibration used in this study). The activation Q (as labeled by ThermoFinnigan, used to adjust the q_z value for the precursor ion) was set at 0.30. Subsequent CID stages were performed using similar activation parameter settings. The activation time employed at each CID stage was 30 msec.

Computational Details

The potential energy surface (PES) of the GGGG b_3 ion was investigated using the strategy developed recently [3, 28, 29] to deal with protonated peptides. These calculations involved molecular dynamics simulations using the InsightII program (Biosym Technologies, San Diego, CA) to produce starting geometries and quantum chemical calculations at the HF/3-21G, B3LYP/6-31G(d) and finally at the B3LYP/6-31 + G(d,p) levels.

Transition structures (TS) corresponding to various fragmentation pathways of the GGGG b_3 ion were determined at the B3LYP/6-31G(d) and B3LYP/6-31 + G(d,p) levels of theory. In most of the cases the transition structures were checked by using intrinsic reaction path calculations (IRC) to unambiguously define which minima are connected by the TS investigated. Relative energies were calculated by using the B3LYP/6-31 + G(d,p) total energies and zero-point energy corrections (ZPE) determined at the B3LYP/6-31G(d) level. For all ab initio calculations the Gaussian [39] program was used.

Results and Discussion

CID of Native and Isotope Labeled GGGG

Figure 1 shows the multiple-stage CID spectra generated from protonated GGGG. CID (MS/MS stage) of $(M$

Figure 1. Multiple-stage CID spectra derived from protonated GGGG: (a) CID (MS/MS) of $(M + H)^+$, (b) CID (MS^3) of b_3^+ , (c) CID (MS^4) of a_3^+ .

+ H) $^+$ at m/z 247 (Figure 1a) produced prominent $(M - H_2O + H)$, b_3 and y_2 ions at m/z 229, 172, and 133, respectively. Subsequent CID of b_3^+ (MS^3 stage, Figure 1b) produced a_3^+ and b_2^+ at m/z 144 and 115, respectively. The peak at m/z 127 in Figure 1b is 45 Da lower in mass than the b_3^+ ion and represents the a_3^* ion as identified in an earlier study of the CID of oligoalanine peptides [34]. Isolation and dissociation of a_3^+ at m/z 144 (MS^4 stage, Figure 1c) led primarily to the a_3^* species, with the b_2 ion as a minor product. The multiple-stage CID results suggest a reaction sequence for generation of a_3^* that involves the decomposition of b_3^+ to a_3^+ , and then a_3^+ to a_3^* . Identification of a pathway for direct formation of a_3^* from b_3^+ , without a_3^+ as an intermediate, would require double-resonance experiments [31], which are not possible with our unmodified LCQ.

Figure 2 shows the product ion spectrum generated by CID (MS^3 stage) of the b_3 ion at m/z 176, derived initially from D^+ cationized GGGG in which all amino, amide, and acid groups were labeled with D via solution-phase hydrogen/deuterium exchange before ESI. The a_3^* ion appeared as three peaks at m/z 128, 129, and 130, corresponding to net neutral losses (from the b_3^+ species) of 46, 47, and 48 Da and reflecting the elimination of 1, 2, and 3 D atoms, respectively. The elimination

Figure 2. CID (MS^3) of b_3^+ generated by dissociation (MS/MS) of $(M + D)^+$ derived from amino, amide, and acid D-exchanged GGGG.

of up to 3 D atoms supports the proposal that the MS^n CID pathway to generate a_3^+ involves the elimination of CO and NH_3 , and the isotopic distribution in Figure 2 suggests that the favored route is the elimination of CO and ND_2H for the amino, amide, and acid deuterium-exchanged peptide. An alternative reaction pathway that involved the loss of CO and OH (also a net loss of 45 Da for unlabeled GGGG) would produce a maximum shift in the H/D exchange experiment of only 46 Da.

In the spectrum shown in Figure 2, the b_2 species appears as discrete isotopic peaks at m/z 118 and 119. The peak at m/z 118 represents the formation of b_2 via the elimination of 58 Da, consistent with the elimination of CO and a D labeled imine ($ND=CH_2$). The peak at m/z 119, instead, reflects the loss of 57 Da during formation of the b_2 ion from the b_3 species. This observation suggests that H atoms from an α -carbon position may be transferred during the reactions to produce certain b ions by CID. Similar observations were made following CID of the peptides containing α - d_2 -G as described below.

The product ion spectra generated from CID (MS^3) of the b_3 ions initially derived from GGGG precursor peptides containing G-1- ^{13}C or G- ^{15}N are shown in Figure 3 and Figure 4, respectively. The loss of 45 Da to produce a_3^+ shifted to 46 Da when the precursor peptide was either GG(G-1- ^{13}C)G or GG(G- ^{15}N)G (^{13}C or ^{15}N label contained within the amino acid adjacent to the C-terminus) as shown in Figures 3a and 4a, respectively. No shift in mass of the neutral eliminated was observed when the glycine residue with the isotope label was positioned either adjacent to the N-terminus (Figures 3b and 4b) or at the N-terminus (Figures 3c and 4c).

The amino acid adjacent to the C-terminus in the precursor tetrapeptides makes up the oxazolone ring portion of the b_3^+ product ion. For GG(G-1- ^{13}C)G the neutral loss to generate the a_3 ion from b_3^+ (Figure 3a) shifted to 29 Da. The mass shift of neutral loss is consistent with the opening of the oxazolone ring and elimination of CO (^{13}CO for the peptide with G-1- ^{13}C adjacent to the C-terminus).

The product ion spectra generated from CID (MS^3

stage) of the b_3 ions initially derived from GGGG precursors containing α - d_2 -G are shown in Figure 5. For CID of b_3^+ generated from GG(α - d_2 -G)G (α - d_2 labeled residue positioned adjacent to the C-terminus), a_3^+ was formed through the net loss of 45 Da (Figure 5a). However, the neutral loss associated with formation of a_3^+ exhibited a shift to 46 and 47 Da, with a preference for the loss of 46 Da (one α -carbon position D-atom eliminated), when the precursor peptide was G(α - d_2 -G)GG (labeled residue positioned adjacent to the N-terminus) as shown in Figure 5b. The preferred loss of 1 D atom from the labeled G residue is consistent with the isotopic distribution in the neutral loss from the amino, amide, and acid deuterium-labeled peptides (Figure 2), in which the preference was for a loss of two amide or amino D atoms.

In Figure 5b, the b_2 species generated by CID of the b_3 ion derived from protonated G(α - d_2 -G)GG was also split into discrete isotopic peaks at m/z 116 and 117. The latter represents the elimination of CO and an imine (as a net loss of 57 u) from the oxazolone ring to generate b_2^+ . The former, however, reflects the elimination of 58 Da. The loss of 58 Da suggests the exchange of an H atom from the oxazolone ring (or portions of the ring) for a D atom at the α -carbon position of the amino acid

Figure 3. CID (MS^3) of b_3^+ species derived for dissociation (MS/MS) of protonated, ^{13}C labeled forms of GGGG: (a) GG(^{13}C - G)G, (b) G(^{13}C - G)GG, (c) (^{13}C - G)GG.

Figure 4. CID (MS^3) of b_3^+ species derived for dissociation (MS/MS) of protonated, ^{13}C labeled forms of GGGG: (a) $GG(^{15}N-G)G$, (b) $G(^{15}N-G)GG$, (c) $(^{15}N-G)GGG$.

adjacent to the N-terminus during the formation of b_2^+ . The labeling data suggest that the splitting of the b_2 ion in Figure 5b is induced by exchange that involves α -carbon H atoms of the G residue adjacent to the N-terminus. The exchange/transfer of the H and D atoms during formation of b_2^+ from the peptide containing α - d_2 -G is consistent with the splitting of the b_2 ion peak observed following CID of GGGG with D exchanged onto the amino, amide, and acid positions.

Mechanism of Formation of a_3^* Ions

The consensus based on a number of experimental and theoretical studies is that the b_n ions of peptides with aliphatic side chains have a cyclic, oxazolone structure [23–31]. With this in mind, the ^{13}C and ^{15}N isotope labeling experiments, particularly the results obtained for protonated $GG(G-1-^{13}C)G$ and $GG(G-1-^{15}N)G$ (substitution with $G-1-^{13}C$ or $G-1-^{15}N$ adjacent to the C-terminus of GGGG), demonstrate that formation of a_3^* involves elimination of the carbonyl group (as CO) and the N atom, as part of NH_3 , from the presumed oxazolone ring of the b_3 ion. It is interesting to note that N-terminal acetylation completely suppressed the formation of a_3^* (spectra not shown), a result that suggests

that the reaction pathway involves an attack by the N-terminal amino group. However, the fact that the mass of the neutral species eliminated during formation of a_3^* did not change when the precursor peptide was $(G-^{15}N)GGG$ clearly shows that the formation of a_3^* does not involve elimination of the protonated N-terminal amino group as suggested in Scheme 1.

It is important to recognize that the a_3^* isomer of Scheme 1 is a diketopiperazine derivative having two *cis* amide bonds. The N-terminal amide bonds of GGGG and that of the related b_3 and a_3 ions are in the *trans* isomerization state, and formation of the diketopiperazine derivative a_3^* of Scheme 1 requires their *trans-cis* isomerization. In other words, the attacking imine N can approach the protonated N-terminal amino group only if the N-terminal amide bond is in the *cis* isomerization state. This means that the two structures depicted in Scheme 1 for the a_3 ion are *two distinct* isomers that are connected by transition structures with significant barriers [40] and cannot simply be equated as shown in Scheme 1. Gas-phase *trans-cis* isomerization of amide bonds involves amide N protonated species as described in detail in a recent study of protonated GGG [40]. The major conclusion of the work with GGG is that *trans-cis* isomerization of amide bonds is energetically

Figure 5. CID (MS^3) of b_3^+ species derived for dissociation (MS/MS) of protonated GGGG containing α - d_2 glycine: (a) $GG(\alpha-d_2-G)G$, (b) $G(\alpha-d_2-G)GG$, (c) $(\alpha-d_2-G)GGG$.

Scheme 2. Multi-step pathway for generation of a_3^* from b_3^+ of protonated GGGG.

possible but kinetically disfavored compared with other pathways like $b_2\text{-y}_1$.

The major steps of an alternative pathway to formation of a_3^* , one that explains the isotope labeling results (including those of the scrambling of H atoms from α -carbon positions), are depicted in (Scheme 2), and (Scheme 3). The corresponding relative energetics are graphically shown in Figure 6. (The most important structures occurring on this multi-step pathway are

presented in the Supplementary Material section along with their total electronic energies. The Supplementary Material section can be found in the online version of this article.) The first step is loss of CO on the $b_3 \rightarrow a_3$ PFP from b_3 (**I** in Scheme 2), the corresponding TS (**I_II**) is of 31.9 kcal/mol relative energy (E_{rel}) (Scheme 2). The a_3 ion (**II**) formed (Scheme 2) is protonated at the C-terminal imine group and the adjacent carbonyl C becomes a likely target of nucleophilic attack by the

Scheme 3. Relay-type H/D exchange pathway to explain scrambling of H atoms from α -carbon positions of a_3^* ions. Labile H atoms are denoted by D.

N-terminal amide oxygen. The corresponding $a_3 \rightarrow b_2$ PFP involves a TS (**II_III**) of 35.1 kcal/mol internal energy and leads to a complex of the oxazole and the imine fragments (**III**, E_{rel} at 30.9 kcal/mol). Under low-energy conditions this complex likely has a short but finite life-time, which may allow reisomerization to form various proton-bound dimers like **IV** (E_{rel} at 25.3 kcal/mol) and **V** (E_{rel} at 25.3 kcal/mol). For the latter, the imine fragment is protonated and the amino group of the oxazolone fragment is close to the carbon of the imine. The next step in Scheme 2 is association of the imine and the oxazolone fragments via attack by the amino group of the latter on the imine carbon, a process

that involves a TS (**V_VI**) of 30.0 kcal/mol relative energy. After some exothermic proton transfer reactions, species **VII** (E_{rel} at 17.6 kcal/mol) is formed that eliminates ammonia by passing TS **VII_VIII** of 33.7 kcal/mol relative energy. Note that this multi-step mechanism is energetically feasible: the highest energy point is the $a_3 \rightarrow b_2$ TS on the pathway at 35.1 kcal/mol relative energy. Furthermore, Scheme 2 clearly explains the ^{15}N labeling data presented above and predicts that the nitrogen of the oxazolone ring of b_3 is lost upon formation of a_3^* .

While the reaction pathway proposed in Scheme 2 accounts for the elimination of isotope labels from specific amide positions in the ^{15}N and ^{13}C labeled GGGG peptides, it does not explain why H atoms are transferred and eliminated from specific α -carbon positions (namely, those in the amino acid adjacent to the amino terminus) upon formation of a_3^* and b_2 . It is worth noting here that scrambling of α -carbon H atoms is usually not observed upon CID [11]. A plausible mechanism that accounts for the α -carbon H scrambling of the a_3^* ion is depicted in Scheme 3 where all labile H atoms are replaced by D atoms to reflect the experiment on D^+ cationized GGGG in which all amino, amide, and acid groups were labeled with D via solution-phase H/D exchange before ESI.

Species passing the **VII_VIII** ammonia-loss TS (Figure 7) can form various complexes and proton-bound dimers (for example, **VIIIa...NH₃**, Supplementary Material section) of a_3^* and NH_3 under low-energy CID conditions (the four a_3^* isomers (**VIIIa-d**) considered in the text are depicted in Figure

Figure 6. Schematics of the potential energy surface of the b_3 ion of protonated GGGG showing relevant minima, TSs and their relative energetics.

Figure 7. a_3^* - NH_3 complexes and TS HDX relay TS.

8). The lifetime of these species can be long enough to allow proton transfer reactions and reisomerizations to form complex **VIIIc**. . NH_3 (Figure 7) in which the oxazolone fragment is protonated at the oxazolone N and ammonia is bonded by the C-H. . ND_3 bridge. A relay-type H/D exchange TS (relay **HDX TS**, Figure 7), as invoked for gas-phase H/D exchange reactions [41, 42], would lead to a complex of another a_3^* isomer (**VIIIId**) and NHD_2 . It is worth noting here that complex **VIIIId**. . NH_3 is the global minimum on the PES of a_3^* and NH_3 while species **VIIIa**. . NH_3 is only slightly less favored (Supplementary Material section). To explain the observed high H/D scrambling reaction rates of the oxazolone α -carbon Hs, we note that the relay **HDX TS** (37.1 kcal/mol relative energy) is energetically more favored than separated a_3^* and ammonia (37.9 kcal/mol relative energy, Figure 6).

Spatial separation of **VIIIId** and NHD_2 in **VIIId**. . NHD_2 (Figure 7) leads to a singly H/D exchanged a_3^* species. Exchange of the other α -carbon H can occur after rotation of NHD_2 around the O-D. . N axis in **VIIIId**. . NHD_2 and reversal of the reaction of Scheme 3 and an additional relay step to form finally **VIIIId**. . NH_2D where **VIIIId** bears two Ds at the α -C and OH positions.

In **VIIIId**, the 5-membered ring features a stabilizing aromatic character because of two ring double bonds and a lone pair on the ring oxygen atom, and the former carbonyl O becomes an alcohol group. It is worth noting here that **VIIIc** is the energetically most favored a_3^* isomer, the relative energies of **VIIIa**, **VIIIb**, and **VIIIId** (Figure 8) compared with **VIIIc** are 6.6, 3.6, and 7.9 kcal/mol, respectively. Isomers such as species **VIIIId** are possible for various b ions as

Figure 8. Various isomers of the GGGG a_3^* ion.

well and their structure and reactivity will be subject of a forthcoming study.

The computational/theoretical results suggest that the scrambling of the α -carbon H atoms observed in the experimental study does not occur before the first fragmentation step ($b_3 \rightarrow a_3$ PFP) as usual for labile H atoms of protonated peptides but happens in the postdissociation complexes of the fragments. It is worth noting here that this observation is difficult to explain using the “mobile proton” model, which deals strictly with predissociation events of peptide fragmentation [3]. On the other hand, the recent PIC fragmentation model [3] provides a flexible framework to understand such complex chemistries like formation of a_3^* ions and related scrambling of α -carbon H atoms of protonated peptides. Likely a similar

mechanism can be invoked to explain α -carbon H scrambling of the b_2 ion observed in Figure 2 and Figure 5.

Conclusions

A comparison of the CID of native and amino, amide and acid D-exchanged GGGG demonstrates that the formation of a_3^* from b_3^+ involves the elimination of CO and NH_3 , with the latter neutral species carrying two H atoms originating from amino or amide sites and one H atom from an α -carbon position. The use of ^{15}N labeled glycine residues within the GGGG sequence showed that the reaction to produce a_3^* does not result in the elimination of the N-terminal amino group, as suggested in Scheme 1, but instead involves the amide N

atom from the presumed oxazolone ring of the b_3 ion, two H atoms from amide positions, and one H atom from the α -carbon of the amino acid adjacent to the oxazolone ring.

A multi-step reaction pathway involving elimination of CO from b_3^+ to form a_3^+ and loss of $\text{NH}=\text{CH}_2$ from a_3^+ to form b_2^+ is proposed. The $\text{NH}=\text{CH}_2$ and b_2 products can form various complexes and proton-bound dimers allowing nucleophilic attack of the oxazolone amine N atom on the imine C atom. The attack leads to another a_3 isomer that can easily eliminate an ammonia molecule that includes the former C-terminal imine N atom of a_3^+ . Scrambling of H atoms from α -carbon positions is explained by interaction of ND_3 and various a_3^+ isomers on energetically feasible relay-type H/D exchange pathways. These results indicate that the MS/MS spectra of protonated peptides can be a result of rather complicated chemistries involving reisomerization, fragment association and exchange reactions. While the complicated chemistry is not readily explained using the “mobile proton” model of peptide fragmentation, the alternative PIC [3] model can easily account for the observed phenomena.

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