Backbone and Side-Chain Specific Dissociations of *z* Ions from Non-Tryptic Peptides

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Backbone *z*-type fragment ions formed by electron-transfer dissociation (ETD) of doubly protonated peptides AAHAL, AHDAL, and AHADL were subjected to collisional activation and their dissociation products were studied by ETD-CID-MS³ and MS⁴. Electron structure theory calculations were performed to elucidate ion structures and reaction mechanisms. All *z* ions showed competitive eliminations of C₃H₇ and C₄H₈ from the C-terminal Leu side chain. The energetics and kinetics of these dissociations were studied computationally for the *z*₄ ion from AAHAL, and optimized structures are reported for several intermediates and transition states. RRKM calculations on the combined B3LYP and PMP2/6-311++G(2d,p) potential energy surface provided unimolecular rate constants that closely reproduced the experimental branching ratios for C₃H₇ and C₄H₈ eliminations. Mechanisms were also studied for the loss of CO₂ from *z* ions generated by ETD of AHDAL and AHADL and for a specific radical-induced Asp-C_α-CO backbone cleavage. CID of the *z* ions under study did not produce any fragment ions that would indicate cascade backbone dissociations triggered by the radical sites. In contrast, the majority of backbone dissociations occurred at bonds that were remote from the radical sites (spin-remote dissociations) and were triggered by proton migrations that were analogous to those considered for standard peptide ion fragmentations. (J Am Soc Mass Spectrom 2010, 21, 1279–1295) © 2010 American Society for Mass Spectrometry

Dissociations of peptide ions induced by electron-transfer produce series of N-terminal fragments (*c* type ions) and C-terminal fragments (*z* type ions, Scheme 1) [1–3]. Distinguishing these two types of fragments is important for determining the peptide sequence and, in proteomics studies, identifying the protein of interest. The *c* and *z* type fragments are unequivocally distinguished by their electron parity, which is even for *c* ions and odd for *z* ions. This is reflected in the ion elemental composition according to the nitrogen rule [4]. This fundamental feature has been reiterated by Coon and coworkers [5] but its implementation requires high mass resolution measurements to establish the ion elemental composition. An alternative approach relies on MS^{*n*} analysis using collision induced dissociation (CID) or infrared multiphoton dissociation (IRMPD) of *c* and *z* ions produced by electron-transfer dissociation (ETD) and stored in an ion trap. Recently, McLuckey and coworkers reported a survey of collision induced dissociations of *z* ions produced by ETD of a series of peptides [6]. In particular, these authors identified several radical-induced side-chain dissociations that were specific for particular amino acid residues. Side-chain losses occurring in electron capture dissociation have also been studied by several

groups and shown to provide additional structure information [7–12].

The reaction mechanisms for side-chain dissociations remain largely tentative, mainly because the structures of *c* and *z* ions are mostly unknown. *c*-Type ions are as a rule represented as peptides C-terminated with an enolimine or the thermochemically more stable amide moiety [13]. A recent infrared multiphoton dissociation study using a tunable free-electron laser identified a small *c* ion from a charge-tagged peptide as an amide [14], possibly formed by exothermic isomerization of an enolimine [15]. *z*-Type ions are usually represented as C_α radicals, which are stable in the gas phase [16].

The goal of the present work is to analyze by experiment and electron structure theory the structures, mechanisms, and dissociation kinetics of *z* ions produced by ETD. We chose three model pentapeptides, AAHAL, AHDAL, and AHADL, which are of interest because they display specific dissociations involving the histidine and leucine residues and also show different reactivities of the C-terminal and aspartic acid carboxyl groups. Another interesting feature of these peptides is that they show dramatic differences between dissociations induced by electron capture and those induced by electron-transfer [17]. The nature of *z* ion dissociations is also of interest from the point of view of dissociation mechanisms in electron-based methods. In particular, O'Connor and coworkers pro-

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Scheme 1

posed a cascade scheme to explain electron capture dissociations of cyclic peptide ions in which a rupture of the peptide macrocyclic ring by an N- C_α bond cleavage was followed by subsequent rearrangements and dissociations producing backbone fragments [18]. Although the specific mechanistic nature of this scheme has not been studied, it can possibly operate in linear peptides where consecutive dissociations of larger primary z fragments would yield smaller, secondary, z ions to appear in the spectrum.

The paper is organized as follows. First, we will present experimental data on ETD/ MS^n activation of z ions from doubly charged AAHAL, AHDAL, and AHADL ions. Then we analyze by ab initio and density functional theory computations the structures of selected z ions and transition states for their isomerizations and dissociations. Finally, we use RRKM theory to assess the kinetics of unimolecular rearrangements and dissociations of select z ions.

Experimental

Materials and Methods

The peptides AAHAL, AHDAL, and AHADL (>95% pure) were purchased from GenScript and their sequence was checked by electrospray MS/MS.

Electron-transfer dissociation mass spectra were obtained on a Thermo-Fisher (San Jose, CA, USA) LTQ linear ion trap mass spectrometer equipped with an electrospray ion source and a chemical ionization source for the production of fluoranthene anion radicals. Peptide ions were produced by electrospray from 50/50 methanol-water solutions containing 1% of acetic acid. Typically, 5–10 μ M peptide solutions were electrosprayed at 1.6–2.5 μ L/min flow rates to form abundant doubly charged ions, $(M + 2H)^{2+}$, which were accumulated in the ion trap and selected by their m/z ratios. The mass selection window was 2 mass units to include nearest ^{13}C isotopologues. Electron-transfer dis-

sociation was accomplished at 100 and 200 ms ion–ion interaction time. z -Type ions from ETD were mass-selected and collisionally dissociated. The ion excitation energy was set at 20% to 35% on the instrument scale.

Calculations

Standard ab initio and density functional theory calculations were performed using the Gaussian 03 suite of programs [19]. Geometries were optimized with the hybrid B3LYP functional [20] using the 6-31+G(d,p) basis set. Stationary points were characterized by harmonic frequency calculations to identify local energy minima (all real frequencies) and first-order saddle points (one imaginary frequency). Additional sets of energies were obtained by single-point calculations using B3LYP and the Møller-Plesset perturbational theory [21] (second-order, frozen core) with the larger 6-311++G(2d,p) basis set. For the pentapeptide ions under study (71–74 atoms) the basis set consisted of 1761–1878 primitive Gaussians and the MP2 calculations required >50 Gbyte scratch space. Calculations on all open-shell species (radicals and cation-radicals) used the spin-unrestricted formalism. Spin contamination was quite modest in most cases and was treated by Schlegel's spin annihilation protocol [22]. The B3LYP and spin-projected MP2 (PMP2) energies were averaged (B3-PMP2) to compensate for small errors inherent to both approximations according to the procedure reported previously [23]. Unimolecular rate constants were calculated using the Rice-Ramsperger-Kassel-Marcus (RRKM) theory [24] and employing a modified Hase's program [25], which was recompiled for Windows XP [26]. The RRKM rate constants were obtained by direct count of quantum states at internal energies that were increased in 2 kJ mol^{−1} steps from the transition-state up to 400 kJ mol^{−1} above the reactant. Rotations were treated adiabatically and the calculated microscopic rate constants $k(E, J, K)$ were then Boltzmann-averaged over the thermal distribution of rotational states at 298 K.

Results

ETD of doubly charged AAHAL, AHDAL, and AHADL peptide ions were found to produce c - and z -ion series from dissociations of all four amide N–C_α bonds (Figure 1). Note that the charge always remained in the histidine containing fragment. In addition, ETD resulted in abundant loss of ammonia from the charge-reduced ions, which most likely involved the N-terminal ammonium group [27]. Thus, these fragment ions were considered to be C_α radicals analogous to the other z ions and are denoted as z_5 (Figure 1). The relative intensities of the charge-reduced ions and backbone c and z fragments practically did not change ($\pm 10\%$) when the ion–ion interaction time was increased from 100 to 200 ms.

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The z ions from AAHAL (z_5 at m/z 466, z_4 at m/z 395, and z_3 at m/z 324) were further analyzed by CID spectra. CID of the z ions from AAHAL showed loss of water as a common dissociation. More interestingly, the z ions lost C₃H₇ radicals (43 Da loss) and C₄H₈ molecules (56 Da loss), which originated from the leucine side chain [11]. These major fragments appear at m/z 423 and 410 in the ETD-CID spectrum of the z_5 ion (Figure 2a), at m/z 352 and 339 from z_4 (Figure 2b), and at m/z 281 and 268 from z_3 (Figure 2c). The branching ratios for the C₄H₈ and C₃H₇ loss, $[z_n - C_4H_8]/[z_n - C_3H_7]$ depended on the z ion and increased by a factor of two from 0.26 for z_5 to 0.52 and 1.1 for z_4 , and z_3 , respectively. We will address the detailed mechanism and kinetics of these competitive eliminations later. Another series of z -ion dissociations was due to backbone cleavages. Thus, CID of the z_5 ion gave MS³ fragments at m/z 335 and 264 that corresponded to eliminations of Leu and Ala-Leu neutral molecules, respectively (Figure 2a). Likewise, CID of the z_4 ion gave m/z 264 and 193 fragments by elimination of Leu and Ala-Leu molecules, respectively (Figure 2b), and CID of the z_3 ion gave m/z 193 by elimination of the Leu molecule (Figure 2c). The eliminations of Leu and Ala-Leu from the z_5 and z_4 ions occur competitively, not consecutively. This was established by an ETD-CID-MS⁴ spectrum of the m/z 264 fragment prepared by loss of Leu from the z_4 ion that showed a dominant elimination of a 44 Da neutral fragment, presumably CO₂, to give m/z 220 but no formation of the m/z 193 fragment. Note that the elimination of Leu was always more facile than elimination of Ala-Leu.

The eliminations of neutral Leu and Ala-Leu molecules presumably proceed by mechanisms similar to those for the formation of b -type ions [28], which require proton migration to the departing amino acid or peptide molecule. The fact that the amide bond more remote from the His residue is more prone to dissociate indicates the formation of oxazolone structures as shown for the z_4 ion in Scheme 1 [29]. The subsequent elimination of CO₂ is compatible with the formation of a neutral oxazolone ring (PA = 877 kJ mol^{−1}) [30] while the charging proton is presumably transferred to the far more basic imidazole ring (PA = 946 kJ mol^{−1}). Alternative mechanisms, e.g., neighboring participation by the His imidazole ring in the amide backbone dissociation, as considered previously [31, 32], would be expected to favor cleavage closer to the His residue and loss of Ala-Leu to form a five-membered ring intermediate, whereas loss of Leu would require an eight- or nine-membered intermediate. An interesting feature of Scheme 1 dissociations is that the remote C_α radical site in the z ion is not engaged in the amide bond cleavage. Thus, these dissociations can be viewed as spin-remote ion fragmentations, a concept we introduced previously for metal-containing cation-radicals [33].

Figure 1. Electron-transfer dissociation mass spectra of doubly charged ions from (a) AAHAL, (b) AHDAL, and (c) AHADL.

AHDAL and AHADL

The z ions from AHDAL and AHADL (z_5 at m/z 510 and z_4 at m/z 439) were investigated by obtaining the ETD-CID-MS³ spectra (Figure 3a–d). CID of the z_5 ions showed a dominant loss of C_3H_7 (m/z 467), common eliminations of water (m/z 492), CO_2 (m/z 466), C_4H_8 (m/z 454), and His containing sequence fragments at m/z 379, 264, 193, and 166 (Figure 3a, b). No z_4 fragment formation from the z_5 ions was observed at m/z 439, indicating that the cascade mechanism was not competitive in this case. The main distinguishing feature of the CID spectra of the z_5 ions was the presence of fragments at m/z 352 from the AHADL sequence and at m/z 281 from the AHDAL sequence. The m/z 281 ion was further investigated by obtaining an ETD-CID-MS⁴ spectrum,

which showed losses of water and CO_2 and a dominant formation of an m/z 194 fragment ion by loss of 87 Da. The CO_2 loss indicated that the m/z 281 ion probably retained a carboxyl group, and its charge by protonation indicated retention of the basic histidine residue. The formation of the m/z 281 fragment ion can be rationalized by a common mechanism, which is shown for the latter ion in Scheme 2. This mechanism presumes a radical-induced migration of an Asp β -hydrogen atom (shown in blue in Scheme 2) onto the N-terminal C_α radical site. The intermediate then undergoes a radical-induced β -scission of the Asp C_α -CO bond to eliminate a C-terminal radical and forming the even-electron m/z 281 fragment ion. The dominant loss of an 87 Da neutral fragment upon further collisional activa-

Figure 2. ETD-CID-MS³ mass spectra of (a) z_5 , (b) z_4 , and (c) z_3 fragment ions from AAHAL.

tion of the m/z 281 ion can be explained by elimination of β -aminoacrylic acid from the modified aspartic acid residue (Scheme 2). The m/z 194 ion is shown as an imidazole protonated tautomer, which is expected to be more stable than the oxazolone protonated isomer (vide supra). The formation of the homologous m/z 352 fragment ion from the AHADL z_5 precursor can be depicted by an analogous scheme that involves transfer of an Asp β -hydrogen atom followed by β -scission of the Asp C $_{\alpha}$ -CO bond. The energetics of this mechanism as well as the structures of relevant intermediates were analyzed in more detail for z_4 ions from ETD-CID-MS³ of AHDAL (vide infra).

CID of the isomeric z_4 ions, HADL, and HDAL (m/z 439) showed common losses of water (m/z 421), C₃H₇ (m/z 396), CO₂, (m/z 395), C₄H₈ (m/z 383), and their

combinations (m/z 352, 339). In addition, CID formation of specific homologous sequence ions at m/z 281 and 210 for HADL and HDAL, respectively, was observed (Figure 3c, d). According to the Scheme 2 mechanism, in both cases the backbone cleavage occurs at the Asp C $_{\alpha}$ -CO bond, and the m/z 281 ion retains a truncated HAD sequence whereas the m/z 210 ion retains a truncated HD sequence in which the Asp residues have been modified into β -aminoacrylic acid. Accordingly, CID of the m/z 281 fragment ion shows a dominant loss of 87 Da, presumably β -aminoacrylic acid from the modified Asp residue.

We note that the eliminations of C₃H₇ and CO₂ from the z_4 ions are competitive but do not show much dependence on the peptide sequence. Compared to CID of the z_5 ions, the loss of CO₂ is substantially enhanced

Figure 3. ETD-CID-MS³ mass spectra of z_5 fragment ions from (a) AHDAL and (b) AHADL, and z_4 fragment ions from (c) AHDAL and (d) AHADL.

in the shorter z_4 fragments. The largest difference in the CID spectra of the isomeric z_4 ions is the elimination of water, which is much more abundant from the HADL ion. The facile loss of water from the latter ion may be due to an interaction of the Asp and C-terminal carboxyl groups, but it was not studied here in detail.

Energetics of AAHAL z_4 Ion Dissociations

To recapitulate the ETD-CID-MS³ spectra, the major dissociations observed upon collisional activation of z ions were loss of C₃H₇ and elimination of C₄H₈, which occurred in all ions studied here regardless of their length and sequence. The z ions containing the Asp residue competitively underwent elimination of CO₂ and specific backbone cleavages of the Asp C_α-CO

bonds. We now analyze the energetics and kinetics of these dissociations for z_4 ions from AAHAL and AHDAL using electron structure theory and RRKM calculations of unimolecular rate constants.

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The eliminations of C₃H₇ and C₄H₈ occur from the Leu side chain [7–12]. Since strong C–C bonds are to be broken, it is presumed that these dissociations are preceded by H atom migrations that activate the C_β–C_γ bond for the loss of C₃H₇ and the C_α–C_β bond for the loss of C₄H₈ [11]. The optimized geometry of the z_4 ion (1, Scheme 3, Scheme S1, Supplementary Material, which can be found in the electronic version of this article), indicates that the Ala-C_α radical center is re-

Scheme 2

mote from both the Leu C_α -H bond and the side-chain C-H bonds. Conformational changes in **1** are necessary to bring the C_α radical center and the Leu C_α -H bond within a bonding distance. However, these are hampered by the rigid *trans*-amide backbone, so that reaching the transition-state for the Leu- C_α -H $\rightarrow$ Ala- C_α transfer is accompanied by substantial distortions of the dihedral angles (Scheme S1) and requires 132 kJ mol^{-1} in the transition-state (TS1, Table 1). The Leu- C_α -H $\rightarrow$ Ala- C_α transfer is 34 kJ mol^{-1} exothermic to form the Leu- C_α radical isomer **2** (Table 1, Scheme S1). Dissociation of the Leu C_β - C_γ bond in **2** proceeds through a late transition-state (TS2 at 132 kJ mol^{-1} above **2**) and results in the loss of a C_3H_7 radical and formation of the m/z 352 fragment ion (**3**). Overall, the loss of C_3H_7 from **1** is 99 kJ mol^{-1} endothermic.

With regard to the relatively high energy for the strained TS1 we sought alternative pathways for the loss of C_3H_7 . For example, *cis-trans* isomerization of the His amide bond introduces a backbone turn, which might allow for facile approach of the Ala- C_α radical center and the Leu- C_α -H bond. The *cis-trans* isomerization was examined in quite a detail and several transition states were found that differed in their intramolecular H-bonding patterns. The lowest-energy transition-state (TS3 at 79 kJ mol^{-1} above **1**, Table 1) developed a stabilizing hydrogen bond between the Ala₂ amide N-H and the carboxyl group (Scheme 3). The *cis*-amide isomer **4** was 35 kJ mol^{-1} less stable than **1** (Table 1) but had an arrangement of the Ala and Leu C_α atoms that

favored subsequent hydrogen atom transfer. The corresponding TS4 was only 66 kJ mol^{-1} above **4** and the hydrogen migration was 20 kJ mol^{-1} exothermic to form the Leu- C_α radical **5** (Scheme 3). Cleavage of the Leu C_β - C_γ bond in **5** can proceed through TS5, which is analogous to TS2 and requires 115 kJ mol^{-1} from **5**. The dissociation energy from **4** to the *cis*-isomer of the m/z 352 fragment ion (**6**) was 97 kJ mol^{-1} . Note that this threshold energy, when referred to **1** (132 kJ mol^{-1}), was higher than that for the *trans* isomer **3**, reflecting the lower stability of the His *cis*-amide group in **6**.

The competitive elimination of C_4H_8 from **1** can proceed by a hydrogen atom transfer from the C_γ position of the Leu side chain (Scheme 4). Because of the practically unimpeded rotations about the single C-C bonds in the Leu isobutyl group, the TS for the H_γ -atom transfer (TS6) is substantially less strained than TS1 and requires a lower energy (103 kJ mol^{-1} relative to **1**, Table 1). However, the isomerization is endothermic forming C_γ radical **7**, which is 46 kJ mol^{-1} above **1**. Cleavage of the C_α - C_β bond in **7** is facile and requires only 53 kJ mol^{-1} in the pertinent transition-state (TS7). Analysis of the potential energy surface after C_α - C_β bond cleavage revealed a local minimum corresponding to an ion-molecule complex (**8**). However, the combined B3LYP and MP2 energies placed complex **8** at 59 kJ mol^{-1} above **1**, which was slightly higher than the dissociation threshold for the elimination of C_4H_8 and formation of the m/z 339 ion (**9**, 52 kJ mol^{-1} relative to **1**). We did not pursue this minor topic

Scheme 3

any further to establish if there were any more stable complexes of the same type. Finally, 1,3-H atom migration between the Leu C_α and C_γ positions in **2** and **7** has a high TS energy (TS8, 139 kJ mol⁻¹ relative to **7**) and cannot compete with the loss of C_3H_7 and C_4H_8 . Thus,

radicals **2** and **7** are unlikely to interconvert by 1,3-H atom migration.

Competitive Kinetics of AAHAL z_4 Ion Dissociations

The ETD-CID-MS³ spectrum of the m/z 395 z_4 ion shows a 0.52:1 branching ratio for the eliminations of C_4H_8 and C_3H_7 . We used the B3-PMP2/6-311++G(2d,p) potential energy surface to calculate unimolecular rate constants for the isomerizations and dissociations leading to the eliminations of C_3H_7 and C_4H_8 . Since we placed no experiment-related constraints on the calculated rate constants, the comparison of the calculated and experimental branching ratios can provide a stringent test of the quality of the theoretical potential energy surface. The potential energy surface is visualized in Figure 4, which also shows the rate constants we considered in the kinetic scheme. The loss of C_3H_7 was first considered to occur via the reaction sequence: **1** → TS1 → **2** → TS2 → **3**. The calculated rate constants (k_1 and k_2 , Figure 5a) indicate that the Leu- C_α -H migration is the rate determining step. However, the pertinent rate constant (k_1) shows a very shallow rise with the ion internal energy and does not reach the value needed for the reaction to proceed on the experimental time scale of 200 ms (broken line in Figure 5). The second pathway that we considered included reversible *trans-cis* isomerization (k_3 , k_{-3}), followed by reversible Leu- C_α -H mi-

Table 1. Relative energies of [•]AHAL z_4 ions

Ion/reaction	Relative energy ^{a,b}			
	B3LYP	B3LYP	PMP2	B3-PMP2
	6-31+ G(d,p)	6-311++ G(2d,p)		6-311++ G(2d,p)
1 → TS1	154	154	111	132
1 → 2	-32	-33	-35	-34
2 → TS2	121	117	147	132
1 → 3 + $C_3H_7^\bullet$	83	77	120	99
1 → 4	44	44	26	35
1 → TS3	88	89	70	79
4 → TS4	76	77	54	66
4 → 5	-29	-29	-11	-20
5 → TS5	107	103	128	115
4 → 6 + $C_3H_7^\bullet$	73	68	126	97
1 → TS6	118	119	87	103
1 → 7	50	48	44	46
7 → TS7	46	44	62	53
1 → 8	48	41	76	59
1 → 9 + C_4H_8	35	27	77	52
7 → TS8	136	137	140	139

^aIn units of kJ mol⁻¹.

^bIncluding B3LYP/6-31+G(d,p) zero-point energies and referring to 0 K.

Scheme 4

gration in the *cis* isomer 4 (k_4 , k_{-4}) followed by C_3H_7 dissociation in 5 (k_5). The calculated rate constants (Figure 5b) indicate a rapid $1 \leftrightarrow 4$ equilibration through TS3, so that the population of 4 is given by the k_3/k_{-3} ratio. The Leu- C_α -H migration in 4 is also reversible, but

the rate constants favor the formation of 5 by >25-fold. The competition between the loss of C_3H_7 from 5 (k_5) and reverse isomerization to 4 (k_{-4}) is in favor of the dissociation at internal energies >260 kJ mol $^{-1}$. Under these circumstances, the overall rate constant for the

Figure 4. B3-PMP2/6-311++G(2d,p) potential energy diagram (kJ mol $^{-1}$) for dissociations and rearrangements of z_4 ions from AAHAL. The relative and TS energies include B3LYP/6-31+G(d,p) zero-point vibrational energies.

Figure 5. RRM rate constants. (a) Leu C α hydrogen migration: $1 \rightarrow \text{TS1} \rightarrow 2$ (k_1) and loss of C $_3$ H $_7$: $2 \rightarrow \text{TS2} \rightarrow 3$. (k_2) (b) Reversible *trans-cis* amide rotation: $1 \leftrightarrow 4$ (k_3 , k_{-3}), reversible Leu C α hydrogen migration: $4 \rightarrow \text{TS4} \rightarrow 5$ (k_4 , k_{-4}), and loss of C $_3$ H $_7$: $5 \rightarrow \text{TS5} \rightarrow 6$. (k_5) (c) Reversible Leu C γ hydrogen migration: $1 \rightarrow \text{TS6} \rightarrow 7$ (k_6 , k_{-6}), and loss of C $_4$ H $_8$: $7 \rightarrow \text{TS7} \rightarrow 10$. (k_7), and overall rate constant for the loss of C $_3$ H $_7$ (k_8). The broken lines in all three panels indicate the rate constants necessary for 90% reaction on the 200 ms experimental time scale.

loss of C $_3$ H $_7$ can be written as $k_8 = \min(k_4 k_3 / k_{-3}$, $k_5 k_3 / k_{-3}$), which is $k_8 = k_5 k_3 / k_{-3}$ for internal energies < 430 kJ mol $^{-1}$ and $k_8 = k_4 k_3 / k_{-3}$ for internal energies > 430 kJ mol $^{-1}$ (Figure 5b). The pertinent overall rate constant (k_8) is plotted in Figure 5c, which also shows the rate constants for the reversible migration of the Leu C γ hydrogen (k_6 , k_{-6}) and the loss of C $_4$ H $_8$ from 7 (k_7). Note that all rate constants in Figure 5a–c are plotted on a common energy scale referring to 1. The Figure 5c data indicate that the rate determining step in the elimination of C $_4$ H $_8$ is the Leu-C γ hydrogen migration in 1 (TS6, k_6), which is followed by fast bond cleavage in

7 (TS7, k_7), which outcompetes the reverse H-atom migration (TS6, k_{-6}) by nearly four orders of magnitude. Hence, the branching ratio for the C $_4$ H $_8$ and C $_3$ H $_7$ eliminations can be expressed as k_6 / k_8 . The broken line drawn at $\log k = 1.06$ in Figure 5c indicates rate constants for dissociations occurring on the experimental time scale (200 ms) with 90% efficiency, which for k_6 and k_8 appears at internal energies around 362 kJ mol $^{-1}$.

The calculated branching ratio is plotted as the black curve (a) in Figure 6. At 362 kJ mol $^{-1}$ excitation, 1 is calculated to give a branching ratio of $[\text{C}_4\text{H}_8] / [\text{C}_3\text{H}_7] \approx$

Figure 6. RRKM calculated branching ratios for the C_3H_7 and C_4H_8 eliminations from **1**. The black curve is for rate constants calculated on the B3-PMP2/6-311++G(2d,p) + ZPVE potential energy surface. The blue curve is for rate constants from TS4 and TS6 energies that were adjusted by ± 2 kJ mol $^{-1}$.

1.0, which is higher than the experimental value (0.52). The above-described kinetic analysis indicated that the branching ratio is most affected by the rate constants in the rate-determining steps, k_4 , k_5 , and k_6 , which in turn strongly depend on the respective TS energies.

Energy calculations at high levels of theory aim at what is called the “chemical accuracy,” which was defined by Pople and coworkers as ± 1 kcal mol $^{-1}$ (± 4.184 kJ mol $^{-1}$) of the actual value [34]. We now apply this criterion to examine how the TS energies calculated within the chemical accuracy affect the branching ratio in Figure 6. The blue curve (b) shows the branching ratio calculated for RRKM rate constants that were obtained on an adjusted potential energy surface where the TS6 energy was increased by 2 kJ mol $^{-1}$ and the TS5 energy was decreased by 2 kJ mol $^{-1}$. These adjusted TS energies are within the Pople’s chemical accuracy limit and yield a branching ratio of $[C_4H_8]/[C_3H_7] = 0.42$. This points out that the calculated branching ratio is very sensitive to the TS energies, and an adjustment within ± 2 kJ mol $^{-1}$, which certainly is within the accuracy of the computational methods used here, brings the calculated branching ratio in a complete agreement with experiment. Of course, an opposite adjustment of the pertinent TS energies would change the ratio in favor of the C_4H_8 elimination. Nevertheless, one can conclude that the kinetic analysis on the suggested potential energy surface provides a very realistic representation of the competitive and consecutive reactions leading to eliminations of C_4H_8 and C_3H_7 .

Energetics of AHDAL z_4 Ion Dissociations

z -Type ions from the AHDAL and AHADL peptides undergo competitive losses of water, C_3H_7 , C_4H_8 , CO_2 , and the Asp-specific backbone cleavage. Whereas the first three dissociations are common to z ions from the AAHAL, AHDAL, and AHADL sequences, the latter two dissociations are particular to the Asp-containing peptides. We now address the energetics of loss of CO_2 and Asp-specific backbone cleavage for the z_4 ion from AHDAL as a case in point. We first briefly describe the reaction pathways and then compare their energetics and competitiveness.

CO_2 Loss

The CO_2 molecule can originate from the Asp or Leu C-terminal carboxyl groups. The loss is presumably triggered by radical-induced rearrangements in the z ions. By comparison, Asp-containing even-electron fragment ions from AHDAL and AHADL were found not to decarboxylate upon CID. We first considered four different pathways (*a–d*) for CO_2 elimination from the Asp residue, each starting from a different z_4 HDAL conformer representing the m/z 439 ion. All conformers are protonated in the imidazole ring. The most stable conformer **10** has a hydrogen bond between the N-1’-H group of the protonated imidazole ring and the Asp amide carbonyl (Scheme 5, Scheme S2). Interestingly, the acidic imidazole C-2’-H bond is hydrogen bonded to the C-terminal carboxyl group. The Asp carboxyl group can be activated for CO_2 elimination in the following reaction sequences. Ion **10** first has to undergo a *trans-cis* amide rotation via TS9 (68 kJ mol $^{-1}$ relative to **10**) to form the *cis*-isomer **11**. In path *a* (Scheme S2), the latter isomerizes by COOH hydrogen atom migration to the His- C_α radical site (TS10, 169 kJ mol $^{-1}$ relative to **10**) to produce a high-energy Asp-COO radical (**12**), which exothermically loses CO_2 to form fragment ion **13**. Note that the overall loss of CO_2 from **10** is practically thermoneutral (Table 2).

Another possible pathway (*b*) for the loss of CO_2 from **11** commences with an Asp- H_α migration to the His- C_α radical site, which proceeds through a five-membered cyclic transition-state (TS11, 58 kJ mol $^{-1}$ above **11**) to form the Asp- C_α radical **14** at -56 kJ mol $^{-1}$ relative to **11** (Scheme S3, Table 2). Migration of the Asp-COOH hydrogen atom to the Asp- C_α radical center in **14** requires 177 kJ mol $^{-1}$ in TS12 forming an Asp-COO radical (**12**), which is followed by an exothermic loss of CO_2 .

A yet different but energetically similar pathway (*c*) starts from another z_4 ion conformer (**15**) and involves amide *cis-trans* isomerization to **16**, Asp- H_α migration to **17**, COOH migration (TS13) to **18**, and exothermic CO_2 loss via TS14 to form fragment ion **19** (Scheme S4, Table 2).

A common feature of pathways *a–c* for Asp CO_2 loss from **10** and **15** is the existence of relatively high-energy

Scheme 5

transition states (TS10, TS12, and TS13) for the carboxyl H-atom transfer to the pertinent C_α radical sites. We therefore examined a fourth pathway (*d*) where the carboxyl H-atom was transferred to the Asp amide oxygen (Scheme 5). Pathway *d* starts from Asp- C_α radical 14, which undergoes internal rotations to form a new conformer 20 in which the COOH proton is H-bonded to the Asp amide while the protonated His ring is H-bonded to the Ala₄ amide (Scheme 5). Conformer 20 is 38 kJ mol⁻¹ less stable than 14 but has a favorable arrangement of the COOH and amide groups for H transfer. This H-atom transfer proceeds through a seven-membered cyclic transition-state (TS15, 86 kJ mol⁻¹ relative to 14) and is accompanied by a concomitant elimination of CO₂ forming fragment ion 21. Note that although 21 is 18 kJ mol⁻¹ less stable than the isomeric fragment ion 13, the formation of the former requires a substantially lower TS energy (64 kJ mol⁻¹ from 10) and is likely to be kinetically preferred.

A notable feature of cation-radical 21 is that it is not an aminoketyl radical in spite of the presence of a C(OH)NH moiety from the decarboxylated Asp residue. Spin analysis [35] of 21 indicates 60% of odd-electron density at the C_β-methylene of the former Asp residue whereas the enolimine carbon atom has only 36%. In addition, the Asp enolimine moiety in 21 is planar, which contrasts the typically pyramidal geometries of aminoketyl radicals [36]. These structure and electronic properties of 21 undoubtedly affect its reac-

tivity. For example, CID-MS⁴ of the respective *m/z* 395 ion resulted in major losses of water, C₃H₇, and C₄H₈, combined with elimination of CO₂ (Figure S2). In contrast, an *m/z* 209 fragment from an N- C_α bond dissociation between the decarboxylated Asp and Ala residues was completely absent.

Although the occurrence of CO₂ loss from Asp-containing *z* ions seems to indicate that the Asp carboxyl group is involved, other pathways are possible starting from the *z*₄ conformer 22, but involving the C-terminal COOH group. The first such pathway we explored (Scheme S5) starts with *trans-cis* isomerization, 22 → TS16 → 23 to form a *cis*-amide, which is 11 kJ mol⁻¹ above 22 (Table 2). Migration of the Asp-H_α to the His- C_α position, 23 → TS17 → 24, forms the Asp- C_α radical, which is practically isoenergetic with 22. Migration of the C-terminal carboxyl hydrogen atom to Asp- C_α requires 183 kJ mol⁻¹ in TS18, which represents another high-energy transition-state, and results in a spontaneous loss of CO₂ forming ion 25 (Scheme S5).

Alternatively, the C-terminal carboxyl can directly reach the His- C_α radical center in 22 in a conformation that retains all *trans* amide bonds and facilitates carboxyl hydrogen atom transfer (Scheme 6). The pertinent TS19 requires 85 kJ mol⁻¹ from 22, which is substantially lower than the energies for TS10, TS12, and TS13, and comparable to that in TS14. The Leu carboxyl H-atom transfer through TS19 leads to a concomitant

Table 2. Relative energies of $^*\text{HDAL } z_4$ ions

Ion/reaction	Relative energy ^{a,b}			
	B3LYP	B3LYP	PMP2	B3-PMP2
	6-31+ G(d,p)	6-311++ G(2d,p)		6-311++ G(2d,p)
10 → 11	41	41	26	33
10 → TS9	69	69	66	68
10 → TS10	157	158	180	169
10 → 12	81	81	118	99
10 → 13 + CO ₂	18	5	−6	−0.5
11 → TS11	55	55	60	58
11 → 14	−62	−64	−48	−56
14 → TS12	152	154	200	177
14 → 20	42	42	33	38
10 → 15	1	0.1	19	10
15 → 16	23	23	22	22
15 → 17	7	9	1.5	5
17 → TS13	133	134	162	148
17 → 18	68	69	104	87
15 → TS14	88	87	77	82
15 → 19 + CO ₂	16	5	−25	−10
14 → TS15	98	91	81	86
10 → TS15	77	68	59	64
14 → 21 + CO ₂	51	39	43	41
10 → 21 + CO ₂	30	16	21	18
10 → 22	21	23	16	19
22 → TS16	60	59	63	61
22 → 23	11	10	11	11
22 → TS17	100	100	94	97
22 → 24	−2	−3	4	0.5
22 → TS18	167	166	199	183
22 → 25 + CO ₂	9	−6	13	3
22 → TS19	77	78	93	85
22 → 26	−53	−66	−52	−59
16 → TS20	93	93	82	88
15 → 28	32	31	33	32
16 → TS21	89	84	111	98
15 → 29 + 30	72	66	117	92

^aIn units of kJ mol^{−1}.^bIncluding B3LYP/6-31+G(d,p) zero-point energies and referring to 0 K.

Leu C $_{\alpha}$ –COO bond dissociation forming an ion–CO₂ complex (26) at 59 kJ mol^{−1} below 22. The CO₂ binding energy in 26 is only 20 kJ mol^{−1}, so that the complex is

presumed to spontaneously dissociate by loss of CO₂ to fragment ion 27 when formed through TS19. The absence of a C-terminal COO radical as an intermediate from TS19 is consistent with the low stability of analogous carboxylate radicals from amino acids [37, 38].

Competitive Kinetics of Asp and C-Terminal CO₂ Eliminations

The loss of Asp CO₂ through pathway *d* (Scheme 5) and the loss of the C-terminal CO₂ through TS19 (Scheme 6) have comparable TS energies, which raises the question of whether these pathways are kinetically competitive. We carried out RRKM calculations for the pertinent steps and the results are plotted in Figure S1. The loss of the C-terminal CO₂ through TS19 shows a very shallow log *k*(*E*) curve indicating an entropically disfavored reaction. Consequently, the log *k*(*E*) curve reaches the kinetically relevant value (log *k* = 1.06 for 90% dissociation on the 0.2 s experimental time scale) at internal energies >370 kJ mol^{−1}. The loss of the Asp CO₂ through pathway *d* shows two kinetically relevant steps, TS11 and TS15 (Scheme 5), which show comparable rate constants (Figure S1). Treating the pathway *d* dissociation kinetics as a system of two consecutive reactions (Scheme 5) gave 90% dissociation at 0.2 s for reactant internal energies >196 kJ mol^{−1}. In addition, the *k*(*E*) curves for TS11 and TS15 showed substantially greater slopes than that for TS19 (Figure S1). The RRKM data imply that the loss of Asp CO₂ through pathway *d* is kinetically favored by ~4 orders of magnitude over the loss of C-terminal CO₂. This is consistent with the experimental data, which show that only the Asp-containing *z* ions lose CO₂ upon collisional activation.

Asp-C $_{\alpha}$ –CO Bond Cleavage

According to the mechanism suggested in Scheme 2, this site-specific backbone cleavage is presumably activated by a previous formation of a radical center at the

Scheme 6

C_β carbon of the Asp side chain. The formation of this radical requires a backbone amide *trans-cis* isomerization; starting from **15**, the isomerization to the *cis*-amide **16** is 22 kJ mol⁻¹ endothermic (Scheme 7, Table 2). The following transfer of an Asp methylene hydrogen atom in **16** requires 88 kJ mol⁻¹ in **TS20** forming the C_β radical **28** at 32 kJ mol⁻¹ above **15**. Cleavage of the Asp C_α -CO bond in **28** proceeds through **TS21** (98 kJ mol⁻¹) to form fragment ion **29** (m/z 210) and neutral radical fragment **30** at 92 kJ mol⁻¹ above **15**.

Discussion

The computational analysis of the competing dissociations underscores the importance of conformational effects in affecting the transition-state energies and thus directing the ion reactivity. In contrast, dissociation thermochemistry can be a poor predictor of the reaction competitiveness. For example, the loss of C_3H_7 from the AAHAL z_4 ion is 45 kJ mol⁻¹ more endothermic than the elimination of C_4H_8 , and yet the former dissociation is kinetically more favorable. Likewise, the endothermic backbone dissociation of the Asp- C_α -CO bond competes favorably with the exothermic elimination of CO_2 . These effects are comprehensible only on the basis of a detailed examination of the reaction coordinates, including multiple conformational changes,

to pinpoint the rate determining steps and assess their TS energies.

Amide Rotational Barriers

Owing to the stiffness of the peptide *trans*-amide backbone, *cis-trans* amide rotations are often necessary to allow remote groups to approach each other for bond-forming reactions such as H-atom transfers (e.g., Scheme 3, S2, S5). The amide rotational barriers in peptides depend on the amino acid residue and can be of both steric and electronic origin [39–41]. In z ions, we observe a marked decrease of energy barriers for amide rotation in the presence of an C_α radical site. For example, the His amide bond rotation in **1** has $E_{TS1} = 79$ kJ mol⁻¹, which is close to those in neutral peptides [40] and peptide ions in the gas phase [42]. In contrast, rotation of the His amide bond in C_α radicals **10** and **22** requires only 68 and 61 kJ mol⁻¹, respectively. This barrier lowering is understandable considering canonical amide bond structures (Scheme S6), which show a partial C=N double-bond in amides, but less so in amide C_α radicals. This is corroborated by the calculated changes in the C–N amide bond lengths. These increase from 1.325 Å in **1** to 1.434 Å in the TS for amide rotation (**TS1**), whereas a smaller increase is found for amide bond rotation in **10** ($d(C-N) = 1.376$ Å) and

Scheme 7

the pertinent transition-state, **TS9**, where $d(\text{C}-\text{N}) = 1.443 \text{ \AA}$.

Hydrogen Atom Migrations

The calculated TS energies indicate that H-atom migrations often are the rate determining steps in z ion dissociations, e.g., **Figure 4**. In all cases studied here, the H-atom receptor was a C_α radical site whereas the donors were Asp- C_α -H, Leu- C_α -H, Asp- C_β -H, Leu- C_γ -H, and Asp and C-terminal COOH groups. The TS energies can be expected to be affected by steric effects or ring strain and electronic effects due to the types of covalent bonds to be broken and formed and the associated changes in hydrogen bonding interactions when proceeding from the reactant to the TS. Steric effects are dominant in the Leu- C_α -H $\rightarrow$ Ala- C_α transfer through **TS1** and **TS4**. Here, the strain imposed on the all-*trans* amide backbone contributes a 66 kJ mol^{-1} energy increase from $E_{\text{TS4}} = 66 \text{ kJ mol}^{-1}$ in the *cis* amide to $E_{\text{TS1}} = 132 \text{ kJ mol}^{-1}$ in the *trans* amide. The steric strain is somewhat relieved in the Leu- C_γ -H $\rightarrow$ Ala- C_α transfer ($E_{\text{TS6}} = 103 \text{ kJ mol}^{-1}$) due to the flexibility of the Leu side chain.

H-atom transfer from the COOH groups seems to be mainly affected by electronic effects. Thus, the Asp-COOH $\rightarrow$ Asp- C_α transfer proceeds through a five-membered cyclic TS, which does not allow alignment of the $\text{O} \dots \text{H} \dots \text{C}_\alpha$ atoms in **TS12** and requires the highest energy ($E_{\text{TS12}} = 177 \text{ kJ mol}^{-1}$). The analogous Asp-COOH $\rightarrow$ His- C_α transfer proceeds through an eight-membered cyclic TS, which allows for a more favorable $\text{O} \dots \text{H} \dots \text{C}_\alpha$ angle (173° in **TS10**) and has a lower TS energy ($E_{\text{TS10}} = 136 \text{ kJ mol}^{-1}$). The Leu-COOH $\rightarrow$ His- C_α and Leu-COOH $\rightarrow$ Asp- C_α transfers proceed through large-ring cyclic TS (**TS18** and **TS19**, respectively) and both have low TS energies, $E_{\text{TS}} = 85 \text{ kJ mol}^{-1}$. It should be noted that both **TS18** and **TS19** gain some stabilization from hydrogen bonding of the donor COOH groups (**Scheme 6** and **Scheme S5**).

The C_α -H $\rightarrow$ C_α hydrogen migrations show a $58\text{--}86 \text{ kJ mol}^{-1}$ range of TS energies. The five-membered cyclic TS for the Asp- C_α -H $\rightarrow$ His- C_α (**TS11** and **TS17**) migrations appear to be electronically similar yet have different TS energies. The difference can be ascribed to the different stabilization of the TS by hydrogen bonding. Thus, the His- $\text{H}^+ \dots \text{O}=\text{C}$ hydrogen bond in **23** ($1.790 \text{ \AA}$, **Scheme S5**) is stretched to $1.805 \text{ \AA}$ in **TS17**. By contrast, the analogous H-bond in **11** ($1.631 \text{ \AA}$, **Scheme 5**) becomes shorter in **TS11** ($1.595 \text{ \AA}$, **Scheme S3**), presumably contributing to TS stabilization. The Asp- C_β -H $\rightarrow$ His- C_α migration proceeds through a six-membered cyclic transition-state (**TS20**, **Scheme 7**) in which there is loss of H-bonding of the Asp carboxyl group and weakening of the H-bonding of the protonated His ring. These effects presumably contribute to the TS energy, $E_{\text{TS20}} = 88 \text{ kJ mol}^{-1}$. We also note that the energy barriers for H-atom migrations in z ions are

substantially lower than those reported by Siu and coworkers for analogous H-atom migrations in triglycine C_α cation-radicals [43]. Whether this difference is of a general nature and applies to other peptide sequences remains to be explored.

Absence of Cascade Fragmentations

The fact that we observe no cascade fragmentations of the z_n ions from AAHAL, AHDAL, and AHADL is of interest because analogous $z_n \rightarrow z_m$ ($n > m$) transitions have been observed in ETD-CID spectra of other peptides [6]. Although neither the mechanism(s) of cascade fragmentations [18] nor the reason for their absence are understood, it is safe to say that they are not kinetically competitive in the peptide z ions studied here vis-a-vis the facile dissociations of the Leu and Asp side chains and backbone cleavage at the Asp residue. In contrast, spin-remote backbone dissociations [33] resulting in the eliminations of Leu, Ala-Leu, and Asp-Leu neutral molecules from the z ion C-termini occur competitively with the side chain and Asp- C_α -CO cleavages.

Conclusions

Collisional activation of z ions from electron-transfer dissociation of doubly protonated pentapeptides AAHAL, AHDAL, and AHADL produces two kinds of dissociations. Dissociations of the first kind are accompanied by proton migrations, occur at amide bonds, which are remote from the C_α radical site (spin-remote dissociations), and result in losses of amino acids or small peptides from the C-terminal residues. Dissociations of the second kind involve the C_α radical sites and are accompanied by hydrogen atom migrations and *trans-cis* amide group isomerizations. These radical dissociations result in side-chain losses of C_3H_7 , C_4H_8 , CO_2 , and a specific Asp- C_α -CO backbone cleavage. Quantum chemistry calculations explain detailed mechanisms of the radical-induced dissociations and, when combined with RRKM calculations, provide branching ratios, which are in close quantitative agreement with experimental data.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2010.02.018](https://doi.org/10.1016/j.jasms.2010.02.018).

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