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RESEARCH ARTICLE

A Comparison of the Effects of Amide and Acid Groups at the C-Terminus on the Collision-Induced Dissociation of Deprotonated Peptides

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Abstract

The dissociative behavior of peptide amides and free acids was explored using low-energy collision-induced dissociation and high level computational theory. Both positive and negative ion modes were utilized, but the most profound differences were observed for the deprotonated species. Deprotonated peptide amides produce a characteristic c_{m-2}^- product ion (where m is the number of residues in the peptide) that is either absent or in low abundance in the analogous peptide acid spectrum. Peptide acids show an enhanced formation of c_{m-3}^-; however, this is not generally as pronounced as c_{m-2}^- production from amides. The most notable occurrence of an amide-specific product ion is for laminin amide (YIGSR-NH_2) and this case was investigated using several modified peptides. Mechanisms involving 6- and 9-membered ring formation were proposed, and their energetic properties were investigated using G3(MP2) molecular orbital theory calculations. For example, with C-terminal deprotonation of pentaglycine amide, formation of c_{m-2}^- and a 6-membered ring diketopiperazine neutral requires >31.6 kcal/mol, which is 26.1 kcal/mol less than the analogous process involving the peptide acid. The end group specific fragmentation of peptide amides in the negative ion mode may be useful for identifying such groups in proteomic applications.

Key words: C-terminal peptide amide, Deprotonated peptides, CID, Peptide sequencing

Introduction

Hydrophobic amide groups at the C-termini of peptides are prolific in nature and generally thought to be one of the key binding sites on biologically active peptides [1–4]. For example, a C-terminal amide group is a post-translational modification found in peptides that have the C-terminus extended by a glycine residue [5–7]. In addition, there are several groups of well-known biologically active peptides that are C-terminal amides; for example, the tachykinins, a family of neurological peptides. Substance P, discovered by Von Euler and Gaddum in 1931, belongs to the broad tachykinin family and is one of the peptides used in our study [8]. There are several groups of peptides with antimicrobial and anticancer properties that possess a C-terminal amide [9]. Additional biologically active C-terminal amide peptides include the adrenomedullins, amylins, calcitonin, cholecystokinins, endokinins, growth hormone releasing factor, and oxytocins [10–17]. Furthermore, C-terminal amide dipeptides are easily converted to 2,5-diketopiperazines, a 6-membered ring species, in the presence of an acid [18], and the formation of diketopiperazines from the N-terminal amino group has been reported [19, 20]. Wang et al. [21] studied endomorphin-1 and endomorphin-2, both C-terminal amides, and their activity when the C-terminus was modified. Their results showed that the functionality of the C-terminus was

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important and that derivatizing the amide by converting the terminus to an ester has a detrimental effect on the activity of the endomorphin peptide.

There has been a great deal of research comparing the properties of peptide acids and amides by chemical methods other than mass spectrometry. Dennison et al. [9] studied the effects of changing the C-terminus on peptides that exhibit antimicrobial and anticancer activity via physical and computational methods. Their work demonstrated an unpredictable effect on the efficacy and no effect on the selectivity of the peptides studied. Krstenansky et al. [22] synthesized several C-terminal analogs of hirudin (54-65) to probe the structure–activity relationship. The C-terminal amide was among the modifications studied and its presence lessened the activity of the antithrombin peptide. Chemical means have been used to determine the C-terminal end group of peptides and proteins by converting the C-terminus from a carboxylic acid to an amide via ammonolysis or hydrazinolysis [23, 24].

There have been few reports comparing acid and amide analogous peptides by mass spectrometry. Kim and Kim [25] employed a carboxypepsidase Y digestion to identify amino acid amides using fast atom bombardment (FAB) mass spectrometry. Brinkworth and Bowie [26] investigated the maculatin family of C-terminal amide peptides by negative ion mode electrospray ionization (ESI) mass spectrometry. Although their work did not consider the acid versions of the peptides, it did demonstrate the dissociation patterns of deprotonated peptide amides. Enjalbal and coworkers [27] performed an extensive study comparing the differences in positive ion mode collision-induced dissociation (CID) fragmentation patterns of both peptide acids and amides. Their work included 76 pairs of peptide acids and amides with a variety of amino acid compositions. For their study of protonated peptides, the presence of the amide group at the C-terminus had little effect on the resulting CID spectra, apart from an increased loss of ammonia from the protonated peptide amide. This minor difference is likely influenced by the peptide composition and is not solely due to the C-terminal end group. To date, there have been no published reports of a comparison of the acid/amide analogous peptides via negative ion mode CID.

Conversion of a C-terminal carboxylic acid group to an amide group may remove a possible charge site in negative ion mode. Consequently, absence of this carboxylic acid group should alter the fragmentation pattern of the deprotonated peptide. Complimentary to the positive ion mode work of Enjalbal and coworkers [27], our effort focuses on the differences observed in negative ion mode fragmentation. Differing fragmentation patterns between nearly identical deprotonated species will allow for greater identification of unknown peptides.

Experimental

Peptides

Table 1 shows the amino acid sequences of each peptide studied and the monoisotopic masses for the protonated and deprotonated ions. Substance P acid and amide, cholecysto-

Table 1. Peptide Sequences, Precursor Ion Monoisotopic Masses, and Predominant Backbone Cleavage Ions from CID of $[M - H]^-$

Peptide Sequence (name)	Monoisotopic mass[a] of $[M - H]^-$		Base peak of spectrum[a]		Observed CID backbone product ions[b,c]	
	Amide	Acid	Amide	Acid	Amide	Acid
AAAAA (penta-alanine)	371.1	372.2	c_3^-/y_3^-	c_2^-	a_3^-, a_4^-, a_5^-, c_2^-, **c_3^-**, y_2^-, **y_3^-**, y_4^-	a_3^-, a_4^-, a_5^-, **c_2^-**, c_3^-, y_2^-, y_3^-, y_4^-
YIGSR (laminin)	592.3	593.3	$[M - H - CH_2O]^-$	$[M - H - CH_2O]^-$	**c_3^-**	c_2^-, c_3^-
YIGSA	507.2	508.2	$[M - H]^-$	$M - H - NH_3]^-$	c_2^-, **c_3^-**	c_2^-, c_3^-
FPARVGI	756.4	757.4	$[M - H]^-$	$[M - H - NH_3]^-$	c_2^-, c_3^-, c_4^-, **c_5^-**, y_3^-, y_4^-, y_5^-, y_6^-	c_2^-, c_3^-, c_4^-, c_5^-, y_3^-, y_4^-, y_5^-, y_6^-
DYMGWMDF (cholecystokinin)	1061.4	1062.4	$[M - H - H_2O]^-$	$[M - H - H_2O]^-$	c_6^-, y_7^-	c_6^-, y_7^-
LMYVHWVK	1072.6	1073.6	y_6^- and c_6^-	c_5^-	c_3^-, **c_4^-**, c_5^-, **c_6^-**, y_3^-, **y_4^-**, **y_5^-**, **y_6^-**, y_7^-	c_3^-, c_4^-, **c_5^-**, c_6^-, y_3^-, y_4^-, **y_5^-**, **y_6^-**, y_7^-
LMYVHWVR	1100.6	1101.6	c_6^- and $[M - H - HN{=}C{=}NH]^-$	$[M - H - HN{=}C{=}NH]^-$	c_3^-, **c_4^-**, c_5^-, **c_6^-**, **c_7^-**, y_4^-, **y_5^-**, **y_6^-**, y_7^-	c_3^-, c_4^-, c_5^-, c_6^-, c_7^-, y_4^-, y_5^-, y_6^-, y_7^-
MLGFRSVGYA	1097.5	1098.5	$[M - H]^-$	$[M - H - CH_2O]^-$	c_3^-, c_4^-, c_5^-, c_6^-, c_7^-, c_8^-, c_9^-, y_3^-, y_4^-, y_5^-, y_6^-, y_7^-, y_8^-, y_9^-, y_{10}^-	c_3^-, c_4^-, c_5^-, c_6^-, c_7^-, c_8^-, c_9^-, c_{10}^-, y_3^-, y_4^-, y_5^-, y_6^-, y_7^-, y_8^-, y_9^-, y_{10}^-
WFAPPRVGYL	1202.6	1203.6	$[M - H]^-$	$[M - H - NH_3]^-$	c_2^-, c_5^-, c_6^-, c_7^-, **c_8^-**, y_3^-, y_4^-, y_5^-, y_6^-, y_7^-, y_8^-, y_9^-, y_{10}^-	c_5^-, c_6^-, c_7^-, c_8^-, c_9^-, y_3^-, y_4^-, y_5^-, y_6^-, y_7^-, y_8^-, y_9^-, y_{10}^-
RPKPQQFFGLM (Substance P)	1345.7	1346.7	c_9^-	$[M - H - HN{=}C{=}NH]^-$	c_4^-, c_5^-, c_6^-, c_7^-, c_8^-, **c_9^-**, y_4^-, y_5^-, **y_6^-**, y_7^-, **y_8^-**, y_9^-, y_{10}^-	c_8^-, c_9^-, y_4^-, y_5^-, y_6^-, y_7^-, **y_8^-**, y_9^-, y_{10}^-

[a] Units are Daltons
[b] Neutral loss products are excluded from the listing
[c] Ions in bold red have an intensity of >40 % of base peak

kinin acid, and laminin acid and amide were purchased from Anaspec (San Jose, CA, USA). Cholecystokinin amide was purchased from American Peptide (Sunnyvale, CA, USA). Penta-alanine and the laminin analogs were synthesized in our laboratory using standard Fmoc protocol [28] on an Advanced ChemTech Model 90 peptide synthesizer (Louisville, KY, USA). Wang and Rink amide resins (Advanced ChemTech) were used to produce the acid (–COOH) and amide functionalities ($–CONH_2$), respectively. The Sieber resin (Anaspec, San Jose, CA, USA) was used to produce the secondary amide functionality (–NHEt). YIGSR-NMe_2 was custom synthesized by NEO BioScience (Cambridge, MA, USA). YIGSR-OMe was produced in our laboratory via methyl esterification [29] of the commercially available laminin acid. The remaining peptides were provided by Christine Enjalbal's group at the University of Montpellier (Montpellier, France).

Sample Preparation

Stock peptide solutions were prepared in ultrapure water from solid peptide at a concentration of 1–2 mg/mL. Peptides that had low solubility in water were prepared in a 50:50 mixture (by volume) of ultrapure water and methanol. Solutions for mass spectral analysis were obtained by diluting the stock solutions to 1–5 µM in a 50:50 mixture of acetonitrile and ultrapure water. A 0.1 %–1 % volume of either acetic acid or ammonium hydroxide was added to the sample solution to assist in protonation or deprotonation, respectively.

Mass Spectrometry

Low-energy CID experiments were performed using a Bruker HCTultra PTM Discovery System (Billerica, MA, USA) high capacity quadrupole ion trap (QIT) equipped with ESI. Peptide samples were introduced into the ESI source using a KD Scientific syringe pump (Holliston, MA, USA) at a flow rate of 100–250 µL/h. Ions were produced by ESI using a spray voltage of ±3000–4000 V. Nitrogen was used as the ESI drying gas and was heated to a temperature of 250 °C and set to flow at a rate of 5 L/min. The nebulizer gas, also nitrogen, was set to a pressure of 10 psi.

For low-energy CID experiments, precursor ions were allowed to accumulate in the trap for up to 200 ms and then mass-selected using ejection pulses. The isolation window was 4 *m/z* wide in most cases, but was narrowed to 1 *m/z* for a couple of situations that had interfering peaks nearby. Narrowing the isolation window had a negligible effect on the intensity of the ion of interest and effectively removed the interfering peak. Helium was the collision gas. A collision energy sweep of 30 %–200 % with amplitude between 0.8 and 1.0 V was used to maximize CID fragmentation. Care was taken to use the same CID conditions for each member of an acid amide peptide pair.

To describe the observed fragmentation of the peptides in the study, nomenclature introduced by Roepstorff and Fohlman [30] is used. The symbols a, b, c, x, y, and z refer to cleavage sites on the peptide backbone, the subscript denotes the residue at which backbone cleavage occurs, and the prime symbol is used to indicate loss or gain of hydrogen (s) relative to the cleavage site. A prime symbol to the left of the alphabetical symbol indicates loss of a hydrogen. A prime symbol to the right indicates the gain of a hydrogen. This nomenclature can be used to describe both positive and negative ion mode cleavages, but the number of associated hydrogens differs for b-, c-, y-, and z-type cleavages. For example, in positive ion mode, a y-type cleavage produces $y_n''^+$, whereas y_n^- is produced in negative ion mode. A diagram illustrating (in more detail) the common types of peptide cleavages has been included in the supplementary materials (Supplementary Figure S1).

Computations

The calculations were performed at the density functional theory (DFT) and correlated molecular orbital (MO) theory levels with the program Gaussian-03 [31]. The geometries were initially optimized at the DFT level with the B3LYP exchange-correlation functional [32, 33] and the DZVP2 basis set [34]. Vibrational frequencies were calculated to show that the structures were minima and to provide zero point and thermal corrections to the enthalpy and entropies so that free energies could be calculated for direct comparison to experiment. A range of structures were optimized to determine the most stable conformers. In our previous work on amino acid acidities [35–37] [and Bokatzian-Johnson, S. S.; Stover, M. L.; Dixon, D. A.; Cassady, C. J., unpublished results] we showed that the high level G3(MP2) correlated molecular orbital method [38] gave agreement for the acidities with the experimental values to within about ±1 kcal/mol. G3(MP2) has an additional advantage over DFT methods in terms of reliable predictions for these types of compounds because the correlated molecular orbital methods in G3(MP2) perform better in the prediction of hydrogen bond energies as well as steric non-bonded interactions than do most widely-used DFT exchange-correlation functionals.

Results and Discussion

CID of Peptide Acid/Amide Pairs

Protonated, $[M+H]^+$, and deprotonated, $[M-H]^-$, peptides were fragmented by low-energy CID. For protonated peptides, loss of NH_3 was more prominent in the amide spectra than in the peptide acid spectra (data not shown), but few other differences were observed. This is consistent with the C-terminal acid and amide groups not being charge sites in the positive ion mode. These results are in agreement with

those of Enjalbal and coworkers for CID on protonated ions from 76 acid/amide pairs [27].

For the deprotonated peptides, common negative ion mode CID backbone cleavages occur; that is, c_n^- and y_n^- [39–42]. Penta-alanine also produces a_n^- and $''b_n^-$, which is typical of oligoalanine peptides in the negative ion mode [41]. Neutral loss peaks common to specific amino acid residues are abundant in the spectra; for example, elimination of H_2O and CH_2O from serine [42–49], the guanidino group (HN=C=NH) from arginine [40, 49, 50], H_2O from aspartic acid [51–54], $O=C_6H_4=CH_2$ from tyrosine [54], and CH_3SH, CH_3SCH_3, and $CH_2CH_2SCH_3$ from methionine [55].

In the negative ion mode, a dominant backbone cleavage product ion forms for most of the peptides studied and is typically several times more abundant than other backbone product ions. The deprotonated peptide amides produce abundant c_{m-2}^-, where m is the number of residues in the peptide sequence. Many of the peptide acids produce c_{m-3}^- in abundance, which was also observed in prior work by Cassady and coworkers [40].

Data from the deprotonated peptide CID experiments have been compiled in Table 1. Three sets of example spectra are given in Figures 1, 2, and 3. The negative ion mode CID spectra for the remaining peptides are in the Supplemental Material. These example spectra illustrate the differences observed between the acid and amide peptides. Laminin amide (Figure 1a), which is a pentapeptide (m=5), produces c_3^- as the only backbone cleavage ion in abundance. This c_{m-2}^- type ion is far more pronounced in the amide spectrum than in the acid spectrum. Laminin acid (Figure 1b), whose sequence is YIGSR, produces a very simple spectrum consisting mainly of neutral loss peaks and c_2^- and c_3^- in low abundance. Another example can be seen in the spectra of FPARVGI (m=7, Figure 2). Dissociation of the FPARVGI amide (Figure 2a) produces several c_n^- and y_n^-, as well as many neutral loss peaks. A very intense c_5^- (a c_{m-2}^- type ion) and products from internal valine (V) residue elimination (e.g., $[y_4 - V]^-$ and neutral loss products from this ion) are unique to the amide spectrum. The acid form of FPARVGI (Figure 2b) produces a spectrum similar to the amide, without the intense c_5^- and valine loss. Another significant aspect of the FPARVGI acid spectrum is a more abundant c_4^-, a c_{m-3}^- type ion. Penta-alanine (AAAAA, m=5) is also shown to demonstrate the differences between acids and amides without interaction from amino acid side chains. Penta-alanine amide (Figure 3a) has c_3^-/y_3^- as the base peak of the spectrum (masses are identical due to symmetry) and a very prominent water loss peak associated with this ion. The remaining product ions have an intensity of less than 20 % relative to the base peak. Penta-alanine acid (Figure 3b) has c_2^- as the base peak of its CID spectrum, which is a c_{m-3}^- ion. While other peptide acids show enhanced c_{m-3}^-, this effect is most pronounced for penta-alanine. Other backbone fragment ions are observed for penta-alanine, but they are all less than 20 % relative to the base peak.

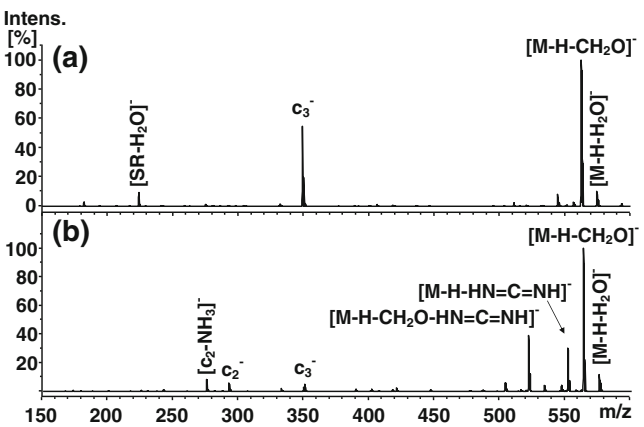


Figure 1. CID spectra of $[M - H]^-$ from laminin (YIGSR) **(a)** amide and **(b)** acid

These selected spectra demonstrate the differences in dissociative behavior of deprotonated peptide acids and their amide analogs. Formation of c_{m-3}^- in CID of the peptide acids has been observed previously [40, 56]. However, the production of c_{m-2}^- in the CID spectra of the peptide amides has not been addressed. There are several factors that must be considered in this case. Since conversion of the C-terminus to an amide removes a highly acidic site on a peptide, the site of deprotonation may be a factor in c_{m-2}^- formation. Another factor is the reaction enthalpy, which accounts for the difference in energies between the deprotonated peptide precursor ion and the products (neutral and c-ion).

Peptide Composition Effects on c_{m-2}^- Formation

To elucidate the involvement of a C-terminal amide group in formation of c_{m-2}^-, we chose to modify laminin and study these peptides by CID. Several laminin analogs were synthesized with altered C-termini to explore the involvement of the C-terminus. Other analogs were studied to test amino acid residue and backbone site interactions. Table 2 shows the C-terminal modifications as well as the absolute and relative (to the base peak, $[M - H - CH_2O]^-$) intensities of c_3^-.

The first modification is replacement of one of the hydrogens on the C-terminal amide moiety with an ethyl group, producing YIGSR-NHEt. CID of deprotonated YIGSR-NHEt produces a spectrum (Supplementary Figure S2a) nearly identical to that of the original laminin amide (Figure 1b), with the exception of product ions related to the C-terminal ethyl group. The intensity of c_3^- is reduced in half when one amide hydrogen is substituted by an ethyl group, from ~60 % of the base peak for laminin amide to ~30 % of base peak for the –NHEt analog.

The second modification was to completely remove both C-terminal amide hydrogens and replace them with methyl groups, producing YIGSR-NMe_2. Although its intensity has

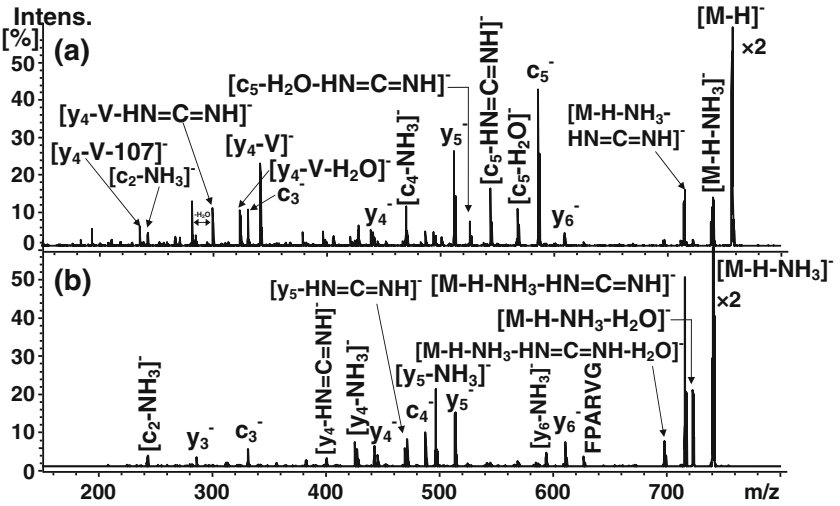


Figure 2. CID spectra of $[M - H]^-$ from FPARVGI **(a)** amide and **(b)** acid

dropped by about a third relative to unmodified laminin amide, c_3^- is still present at ~40 % of the base peak (Supplementary Figure S2b). This suggests that a precursor ion structure involving deprotonation at the C-terminal amide group is responsible for some, but not all, c_{m-2}^- formation.

A third C-terminal modification was to form the methyl ester of laminin acid. The CID spectrum of YIGSR-OMe (Supplementary Figure S3) is dominated by products formed from elimination of CH_3OH from the methyl ester functional group. There have been previous reports of CH_3OH loss in CID of peptide methyl esters [57, 58]. Laminin acid (Figure 1a) produces $[c_2 - NH_3]^-$ and c_2^-; these ions do not form for the laminin methyl ester. However, the laminin methyl ester produces small amounts of $y_3'^-$, c_3^-, and $c_3'^-$. The $c_3'^-$ ion (i.e., $[c_3+H]^-$) appears adjacent to c_3^- and is not observed in other spectra, which suggests that this product ion stems from the presence of the C-terminal methyl ester group.

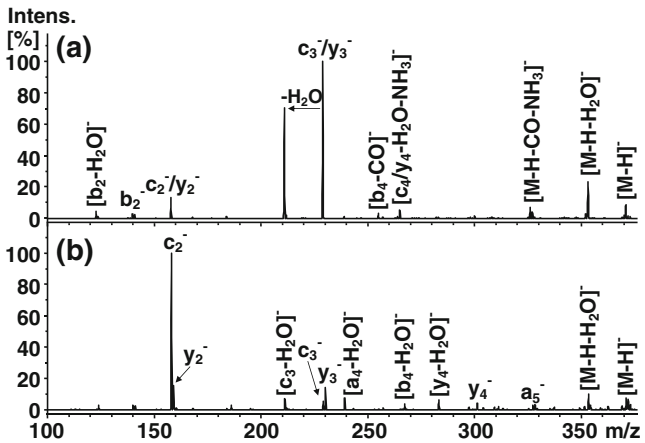


Figure 3. CID spectra of $[M - H]^-$ from penta-alanine (AAAAA) **(a)** amide and **(b)** acid

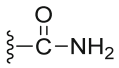
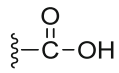
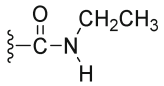
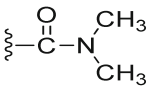
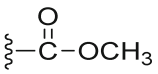
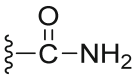
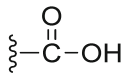
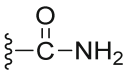
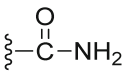
Laminin was also modified internally by replacing specific residues in the sequence to test their involvement in the formation of c_{m-2}^-. The first of these modifications was replacement of arginine with a neutral alanine residue, to form YIGSA. This was done to rule out the possibility of a salt bridge interaction involving the highly basic arginine residue [50, 59]. Both acid and amide analogs were synthesized and fragmented by CID (Supplementary Figures S4a and S4b, respectively). The acid and amide analogs of YIGSA yield spectra nearly identical to the spectra for laminin (YIGSR) analogs except without the neutral loss peaks related to the side chain of arginine. These spectra indicate that arginine is not participating in the mechanism that leads to production of c_{m-2}^- or c_{m-3}^-.

Additional internally modified peptides, YIGA′R-NH_2 and YIGG′R-NH_2, were studied to test the involvement of the backbone alpha carbon and the amide nitrogen on formation of c_{m-2}^-. The penultimate residue of these laminin amide analogs is either 2-methylalanine (A′), which has two methyl groups on the alpha carbon, or sarcosine (G′), which is glycine substituted with a methyl group on the amide nitrogen. Both of these laminin analogs (Supplementary Figures S5 and S6, respectively) produce abundant c_3^- as the base peak in the CID spectra. These results indicate that at least one mechanism of c_{m-2}^- formation does not require hydrogen substitution on the methylene carbon or amide nitrogen of the penultimate residue. Thus, c_{m-2}^- can form even when it is not possible to deprotonate the backbone amide site at the penultimate residue.

Peptide Deprotonation Site

In general, gas-phase deprotonation of a peptide occurs at an acidic site such as the carboxylic acid groups of the glutamic acid (E) and aspartic acid (D) side chains and the C-terminus [46]. In the peptide amides, the carboxylic acid group at the C-terminus is replaced with a less acidic terminal amide

Table 2. Comparison of CID Fragmentation Observed for Laminin (YIGSR) and Related Derivatives

Peptide	C-terminus	Relative intensity[a] of c_3^-	Absolute intensity of c_3^-
YIGSR-NH_2		60%	73108
YIGSR-OH		<5%	7218
YIGSR-NHEt		30%	2770
YIGSR-NMe_2		40%	38710
YIGSR-OMe		<6%[b]	252
YIGSA-NH_2		80%	371294
YIGSA-OH		8%	50060
YIGA′R-NH_2		100%[c]	103225
YIGG′R-NH_2		100%[c]	75374

[a]Relative to the base peak, $[M - H - CH_2O]^-$
[b]Base peak is $[M - H - MeOH - HN{=}C{=}NH]^-$
[c]Because this peptide does not contain a serine residue, the c_3^- ion is the base peak

group. A comparison of the gas-phase acidities (GAs) of simple compounds like acetic acid and acetamide reveals a distinct difference in the energy required for the deprotonation of carboxylic acid versus amide functional groups. Acetic acid has a GA of 341.1±2.0 kcal/mol, whereas acetamide has a GA of 355.0±2.0 kcal/mol [60], an energy difference of 13.9 kcal/mol with acetic acid being more acidic (i.e. lower GA). Yet, the peptide amides studied here produce abundant $[M - H]^-$ by ESI. These peptide amides deprotonate even though (with the exception of cholecystokinin) they are composed of amino acid residues without acidic side chains. This is consistent with past work by Gao and Cassady [61], where peptides with no carboxylic acid groups deprotonate readily by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. In addition, Bowie and coworkers [42, 46, 62] have reported instances of deprotonation occurring at the amide nitrogen for peptide and organic amides. Their experimental results are supported by lower level theoretical calculations (HF/6-31 G*//AM1) using model systems [62]. Thus, amide nitrogens and alpha carbons of the peptide backbone must be considered as alternative deprotonation sites.

We are currently undertaking a comprehensive experimental and high level G3(MP2) computational study of the GAs for all possible deprotonation sites of numerous small peptides. This will be the subject of a future publication. We have found that the amide nitrogens are the most energetically favorable peptide backbone deprotonation sites due to their ability to form an –(O⁻)–C=N– resonance structure. Therefore, these sites need to be considered in the present study.

Table 3 includes the calculated G3(MP2) heats of formation (ΔH_f) for several deprotonated peptide ions produced from acid and amide analogs of tri- to pentaglycine and the acid analog of penta-alanine. The lowest energy structure for each deprotonated peptide has been calculated by sampling a variety of conformers. For the peptide amides, the ΔH_f values indicate that there is only a few kcal/mol difference between deprotonation at the C-terminal amide nitrogen and deprotonation at a backbone amide nitrogen. For example, consider pentaglycine amide: ΔH_f of the anion is –261.8 kcal/mol when deprotonated at the carboxylic acid group of the C-terminus, –265.7 kca/mol when deprotonated at the amide nitrogen of the central residue, and –263.7 kcal/mol when deprotonated at the amide nitrogen of the penultimate residue. Given this relatively small difference in energies, the ESI process in the mass spectrometer should be capable of deprotonation at all three sites. The fact that multiple precursor ion structures are potentially involved explains why removal of both C-terminal amide hydrogens in the YIGSR-NMe_2 experiments lessens, but does not eliminate, c_{m-2}^- formation by CID and why removing both backbone amide hydrogens in the YIGA′R-NH_2 experiments also does not eliminate c_{m-2}^- formation.

For the peptide acids, the ΔH_f values of Table 3 indicate that C-terminal deprotonation is more favorable than

Table 3. G3(MP2) Calculated Heats of Formation

	ΔH_f (kcal/mol)		
	Deprotonation site		
	C-terminus	Backbone for 6-membered ring formation[a]	Backbone for 9-membered ring formation[b]
Precursor Ions			
[GGG-NH_2 – H]⁻	–156.5	–164.6	NA[c]
[GGG-OH – H]⁻	–220.4	–210.4	NA
[GGG-NHEt – H]⁻	–166.9	–172.7	NA
[GGGG-NH_2 – H]⁻	–209.5	–213.9	–216.4
[GGGG-OH – H]⁻	–271.5	–258.7	–254.2
[GGGG-NHEt – H]⁻	–214.2	–221.6	–224.4
[GGGGG-NH_2 – H]⁻	–261.8	–263.7	–265.7
[GGGGG-OH – H]⁻	–325.2	–298.7	–312.8
[GGGGG-NHEt – H]⁻	–264.5	–271.5	–271.0
[AAAAA-OH – H]⁻	–369.4	–341.4	–353.2
Product Ions[d]			
c_{m-2}^- from GGG peptides	–55.4		
c_{m-2}^- from GGGG peptides	–108.6		
c_{m-3}^- from GGGG peptides	–55.4		
c_{m-2}^- from GGGGG peptides	–156.5		
c_{m-3}^- from GGGGG peptides	–108.6		
c_{m-2}^- from AAAAA peptide	–182.4		
c_{m-3}^- from AAAAA peptide	–125.7		
Neutral products[e]			
Diketopiperazine (DKP)	–73.7		
1-Ethylpiperazine-2,5-dione (DKP-Et)[f]	–83.6		
1,4,7-Triazonane-2,5,8-trione (TAT)	–105.1		
1-Ethyl-1,4,7-triazonane-2,5,8-trione (TAT-Et)[g]	–114.6		
Diketomorpholine (DKM)	–111.0		
1,4,7-Oxadiazonane-2,5,8-trione (ODAT)	–147.1		
1,4-Dimethyl-2,5-diketomorpholine (DKM(Me_2))[h]	–130.5		
3,5,9-Trimethyl-1,4,7-oxadiazone-2,5,8-trione (ODAT(Me_3))[i]	–175.1		

[a]Deprotonation is at the backbone amide nitrogen adjacent to the N–C bond cleavage site for formation of c_{m-2}^- and the 6-membered ring DKP and DKM species

[b]Deprotonation is at the backbone amide nitrogen adjacent to the N–C bond cleavage site for formation of c_{m-3}^- and the 9-membered ring TAT and ODAT species

[c]NA=not applicable. For triglycine (GGG), loss of a neutral incorporating a 9-membered ring containing all three residues would not leave sufficient atoms to form a c-ion

[d]Changing the C-terminal end group (–OH, –NH_2, –NHEt) does not change ΔH_f of the product ions. All of the calculations involve c-ions with linear structures where the negative charge is on the amide nitrogen at the C-terminal side

[e]Neutral structures are shown in Schemes 1 and 2. The neutral products do not possess a deprotonation site

[f]Structure is equivalent to DKP with an ethyl group on a ring nitrogen

[g]Structure is equivalent to TAT with an ethyl group on a ring nitrogen

[h]Structure is equivalent to DKM with a methyl group on each ring carbon

[i]Structure is equivalent to ODAT with a methyl group on each ring carbon

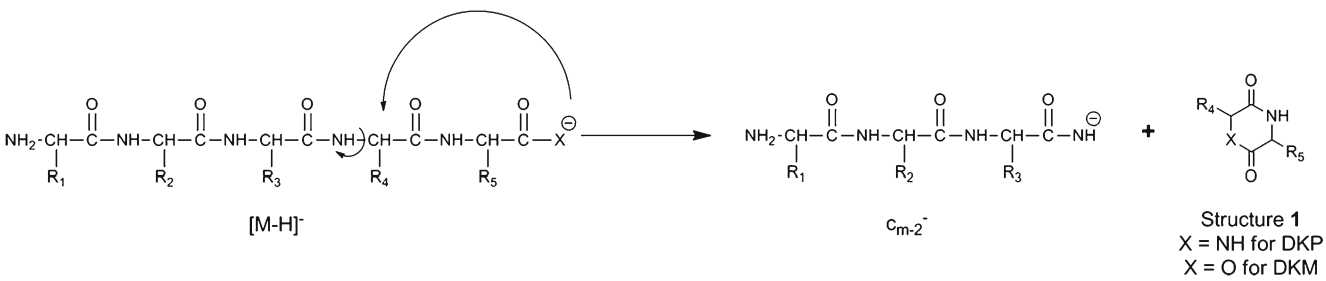
backbone deprotonation by 10–30 kcal/mol. For example, with pentaglycine acid, ΔH_f for the peptide ion deprotonated at the C-terminal carboxylic acid group is –325.2 kcal/mol, which is more favorable than ΔH_f's for the ions deprotonated at the central amide nitrogen (–312.8 kcal/mol) and at the penultimate amide nitrogen (–298.7 kcal/mol). ESI is a very soft ionization technique that produces ions will little excess energy and, therefore, preferentially generates ions with the lowest energy structure [63–67]. As a result, the majority of the precursor ions produced by ESI on the peptide acids should be deprotonated at the C-terminal carboxylic acid group. However, the presence of some peptide acid anions with backbone amide deprotonation cannot be ruled out. In particular, after ion activation by CID, the location of negative charge on the ion may move in an analogous manner to the "mobile proton model" [68] that occurs during CID on protonated peptides in the positive ion mode.

Proposed Dissociation Mechanisms

We propose a substituted 2,5-piperazinedione (or diketopiperazine, DKP) as the stable heterocyclic leaving group for the formation of c_{m-2}^- by deprotonated peptide amides. The general DKP structure is shown in Structure 1 of Scheme 1 for X=NH. DKP analogs frequently form as a by-product in solid-phase peptide synthesis [69–71]. These 6-membered ring dipeptides are often biologically active and commonly present in nature as secondary metabolites [72, 73]. DKP analogs have been the subject of much debate in the formation of b_2^+ from protonated peptides [74–77], but there have been few reports of the role of this structure in deprotonated peptide spectra. Harrison and co-workers [78] proposed involvement of a DKP neutral in the formation of $''b_2^-$ from a_3^- in low-energy CID of tripeptides, but their theoretical calculations suggested this was not a key pathway [79]. Harrison and Young [80] mentioned the possible formation of DKPs in their study involving formation of oxazolones in CID of deprotonated *N*-benzoylpeptides. Bowie and coworkers [57] proposed the formation of a deprotonated DKP from diglycine methyl ester in high-energy CID. There have also been reports of CID fragmentation of deprotonated DKPs, as well as theoretical calculations of fragmentation pathways [81, 82]. A diketomorpholine (DKM) is the analogous leaving group for a deprotonated peptide acid. This compound is identical to DKP, except that DKM has an oxygen in place of one ring nitrogen (Structure 1 with X=O). Diketomorpholines are frequently used in synthetic applications and are occasionally biologically active [83, 84].

Scheme 1 illustrates our proposed mechanism to form c_{m-2}^- from either the acid or amide form of a pentapeptide with involvement of the C-terminus. The process proceeds by a nucleophilic attack of the deprotonated C-terminus on the alpha carbon of the penultimate residue. When the C-terminus is a carboxylic acid, the neutral leaving group is a DKM. In instances where the C-terminus is an amide, the leaving group is a DKP. A factor of importance to the proposed mechanism is the nucleophilicity of the deprotonated functional group on the C-terminus of the peptide. As neutrals, both groups are weakly nucleophilic, with the carboxylic acid being a somewhat better nucleophile [85]. However, deprotonation renders the amide, NH^-, to be a much stronger nucleophile than the deprotonated acid, COO^-.

Reaction enthalpies (ΔH_{rxn}) give a more complete energetic explanation for the processes occurring here, especially as they are endothermic processes. G3(MP2) calculations have been performed for the thermodynamics of a variety of dissociation processes of deprotonated peptides. The calculated structures and the respective processes for GGGG are shown in Figure 4, the G3(MP2) heats of formation are given in Table 3, and the reaction enthalpies are given in Table 4. For each peptide, deprotonation has been considered at the C-terminus and also at the backbone amide nitrogen adjacent to the cleavage site. Heats of formation have been calculated for the backbone fragment ions, c_{m-2}^- and c_{m-3}^-, as these are two prominent features of the peptide amide and acid spectra, respectively. Heats of formation have also been calculated for the neutral leaving groups resulting from the CID processes. When c_{m-2}^- forms, the proposed neutral leaving groups are the 6-membered ring species DKP (Figure 4a and Scheme 1) for the peptide amide and DKM for the peptide acid (Figure 4b and Scheme 1). As shown in Scheme 2, formation of c_{m-3}^- is proposed to involve elimination of a 9-membered ring neutral, which is 1,4,7-triazonane-2,5,8-trione (TAT, Structure 2 with X=NH) for peptide amides, and 1,4,7-oxadiazonane-2,5,8-trione (ODAT, Structure 2 with X=O) for



Scheme 1.

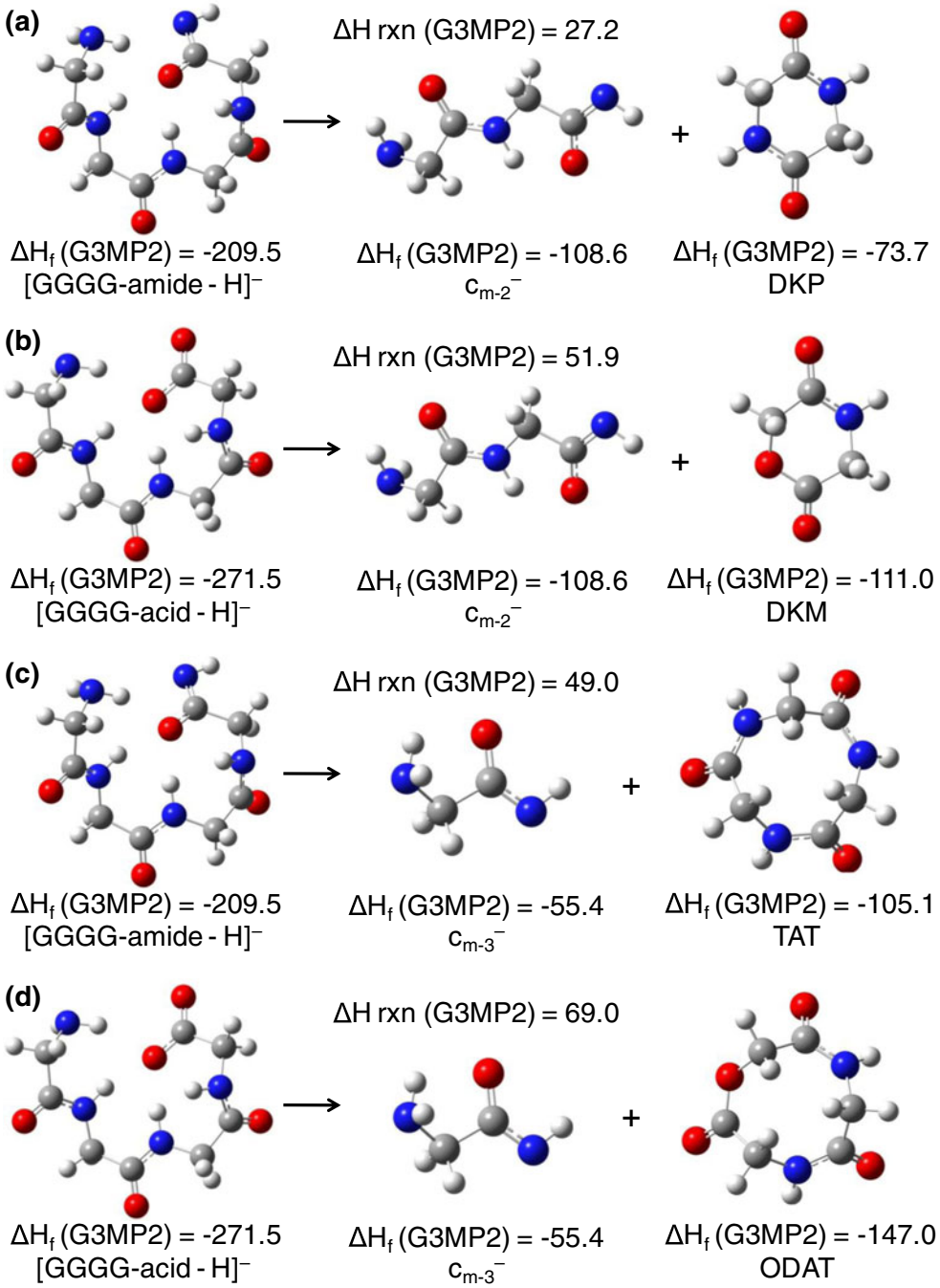


Figure 4. G3(MP2) calculations of enthalpies of formation (ΔH_f) and of reaction enthalpies (ΔH_{rxn}) in kcal/mol for **(a)** GGGG-CONH$^-$ forming c_{m-2}^- and DKP, **(b)** GGGG-COO$^-$ forming c_{m-2}^- and DKM, **(c)** GGGG-CONH$^-$ forming c_{m-3}^-, and TAT, **(d)** GGGG-COO$^-$ forming c_{m-3}^- and ODAT

peptide acids. For comparison, G3(MP2) calculations for the C-terminal ethyl amide (–NHEt) analogs of the oligoglycines are also included in Tables 3 and 4.

The reaction enthalpies shown in Table 4 are in most cases substantially endothermic, which explains the need for collisional activation and dissociation to form the fragmentation products. Several noteworthy trends can be obtained from these values. Many of the examples discussed below will involve the pentaglycine analogs because all of the peptides studied experimentally by CID had five or more residues.

For the mechanism shown in Scheme 1 with deprotonation at the C-terminus, formation of DKP from a peptide amide is considerably less endothermic than production of DKM from a peptide acid, regardless of peptide length. For example, with C-terminal deprotonation and dissociation to yield c_{m-2}^- and a 6-membered ring neutral, pentaglycine amide requires greater than 31.6 kcal/mol, whereas penta-

Table 4. G3(MP2) Calculated Reaction Enthalpies

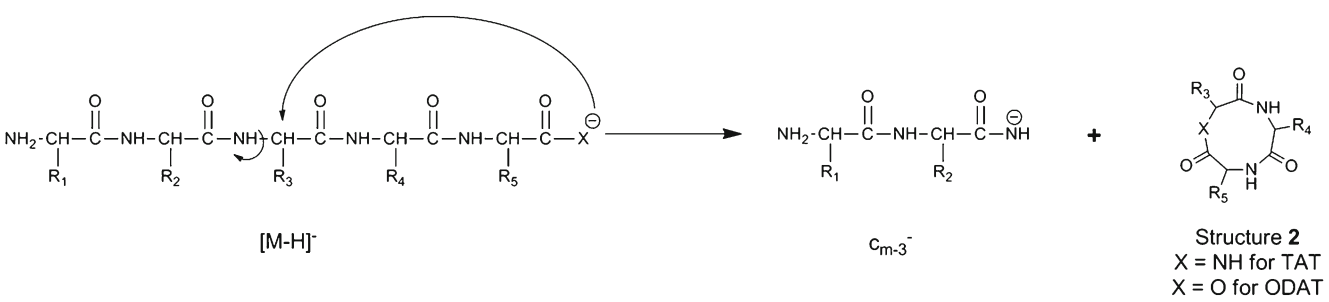
Reaction	ΔH_{rxn} (kcal/mol)			
	GGG m=3	GGGG m=4	GGGGG m=5	AAAAA m=5
C-terminus deprotonation of precursor ion				
[Peptide amide – H]$^-$→c_{m-2}^-+DKP	27.4	27.2	31.6	–[d]
[Peptide ethyl amide – H]$^-$→c_{m-2}^-+DKP-Et	27.9	22.0	24.4	–
[Peptide acid – H]$^-$→c_{m-2}^-+DKM	54.0	51.9	57.7	56.5[e]
[Peptide amide – H]$^-$→c_{m-3}^-+TAT	NA[a]	49.0	48.1	–
[Peptide ethyl amide – H]$^-$→c_{m-3}^-+TAT-Et	NA	44.2	41.3	–
[Peptide acid – H]$^-$→c_{m-3}^-+ODAT	NA	69.0	69.5	68.6[f]
Backbone deprotonation of precursor ion				
[Peptide amide – H]$^-$→c_{m-2}^-+DKP[b]	35.5	31.6	33.5	–
[Peptide ethyl amide – H]$^-$→c_{m-2}^-+DKP-Et[b]	33.7	29.4	31.4	–
[Peptide acid – H]$^-$→c_{m-2}^-+DKM[b]	44.0	39.1	31.2	28.5[e]
[Peptide amide – H]$^-$→c_{m-3}^-+TAT[c]	NA	55.9	52.0	–
[Peptide ethyl amide – H]$^-$→c_{m-3}^-+TAT-Et[c]	NA	54.4	47.8	–
[Peptide acid – H]$^-$→c_{m-3}^-+ODAT[c]	NA	51.7	57.1	52.4[f]

[a]NA=not applicable. Due to the small size of triglycine (GGG), c-ions cannot form with a 9-membered ring neutral
[b]Backbone deprotonation for the precursor ion is at the amide nitrogen adjacent to the N–C bond cleavage site for formation of c_{m-2}^- and the 6-membered ring neutrals
[c]Backbone deprotonation for the precursor ion is at the amide nitrogen adjacent to the N–C bond cleavage site for formation of c_{m-3}^- and the 6-membered ring neutrals
[d]Not calculated for this species
[e]The DKM species formed in this case is $DKM(Me)_2$
[f]The ODAT species formed in this case is $ODAT(Me)_3$

glycine acid requires greater than 57.7 kcal/mol. This is consistent with the CID results where c_{m-2}^- forms in much greater abundance for peptide amides than for peptide acids. When the peptide amides are deprotonated at the backbone nitrogen adjacent to the site of N–C_α bond cleavage, the process is 2–8 kcal/mol more endothermic than for C-terminal deprotonation. Also, as the size of the peptide increases, the energy difference between processes involving C-terminus deprotonation and backbone deprotonation decreases. The process to form c_{m-2}^- and DKP with C-terminus deprotonation of the dissociating ion is less endothermic than the analogous process with backbone deprotonation by 8.1 kcal/mol for triglycine amide, 4.4 kcal/mol for tetraglycine amide, and 1.9 kcal/mol for pentaglycine amide. This suggests that for larger peptide amides both C-terminal and backbone deprotonation sites may contribute to c_{m-2}^- formation.

For the mechanism shown in Scheme 2, formation of a 9-membered ring neutral from a peptide amide is ~20 kcal/mol less endothermic than from a peptide acid. This is true for both tetraglycine and pentaglycine. (Triglycine analogs, m=3, cannot form c_{m-3}^- because of their short sequences.) For example, pentaglycine amide requires greater than 48.1 kcal/mol to form c_{m-3}^- and TAT when the precursor ion has C-terminal deprotonation and greater than 52.0 kcal/mol with backbone deprotonation. The analogous process to produce ODAT from pentaglycine acid requires greater than 69.5 kcal/mol with C-terminal deprotonation and greater than 57.1 kcal/mol with backbone amide deprotonation.

The reaction enthalpies indicate that processes leading to the formation of 6-membered ring neutrals are always lower in energy (when considering ΔH_{rxn}) than those that produce 9-membered rings, regardless of peptide length or deprotonation site of the precursor ion. With pentaglycine amide, formation of c_{m-2}^- and DKP is less endothermic than production of c_{m-3}^- and TAT by 16.5 kcal/mol for C-terminal deprotonation and 18.5 kcal/mol for backbone amide deprotonation. For pentaglycine acid deprotonated at the C-terminus, formation of c_{m-2}^- and DKM is less endothermic than production of c_{m-3}^- and ODAT by 11.8 kcal/mol. The predicted reaction enthalpy trend is interesting because it is the opposite of the trend in our CID experiments where peptide acids generally produce more c_{m-3}^- than c_{m-2}^- (although this effect is not as pronounced as



Scheme 2.

the enhancement of c_{m-2}^- for peptide amides). The difference between the predicted thermodynamic trend and experiment may relate to the ability of ESI to generate the deprotonated peptide precursor ion and to the contribution of backbone deprotonation in these fragmentation pathways. As the ΔH_f values in Table 3 indicate for pentaglycine acid anions, [GGGGG-OH – H]$^-$, deprotonation at the amide nitrogen of the central residue (which is a precursor for c_{m-3}^-) requires 12.4 kcal/mol more energy than deprotonation at the carboxylic acid group of the C-terminus; however, deprotonation at the penultimate residue (which is a precursor for c_{m-2}^-) requires a significantly larger amount of energy, 26.5 kcal/mol more than C-terminal deprotonation. Therefore, for peptide acids with five residues or more, the ESI process may preferentially generate the deprotonated backbone precursor for the c_{m-3}^- pathway, resulting in a more intense c_{m-3}^- in the CID spectra. We note that the calculated energy differences for dissociation processes starting from the same anion are much larger than the estimated ±1 to ±2 kcal/mol errors for such calculated energy differences.

The above discussion assumes that the reactions are under thermodynamic control. It is of course possible that the reactions that are under kinetic control. For the highly endoergic dissociations, this seems to be unlikely as the location of the barrier will be displaced towards the products and the barrier will be small [86]. We did search for transition states for [GGGG-OH – H]$^-$ to form the 6-member and 9-member rings. Initially, we used a semi-empirical molecular orbital method with the PM6 parameterization scheme [87] to search for the transition states with the AMPAC version 9 program [88]. This gave PM6 barrier heights of 76.5 and 78.9 kcal/mol for the dissociation to the 6-member and 9-member rings respectively. Using these geometries, single point calculations at the DFT/B3LP/DZVP2 level gave respective barriers of 85 and 100 kcal/mol for the 6- and for the 9-member rings. These energy differences are in the order of the dissociation energies and the differences in the barriers to the dissociated products is almost the same for both rings (31–33 kcal/mol). Numerous subsequent optimizations of these transition states at the DFT level always led back to the low energy chain structure so it is likely that there is no or only a small barrier to dissociation at the higher computational level. Thus, we have no evidence for why there could be a kinetic preference for the 9-member ring unless there are dynamic effects which would not be found by the calculation of specific points on the potential energy surface.

The G3(MP2) calculations show that production of c_{m-2}^- and DKP from C-terminally deprotonated peptide amide ions requires only 27.4 kcal/mol, 27.2 kcal/mol, and 31.6 kcal/mol, respectively, for the amides of tri-, tetra-, and pentaglycine. Calculations were also performed for oligoglycine amides where one hydrogen on the amide C-terminus was replaced with an ethyl group. When comparing the processes that led to DKP and DKP-Et, the reactions involving triglycine are very close in energy (27.4 kcal/mol versus 27.9 kcal/mol). However, for tetra- and pentaglycine analogs, the processes that led to DKP-Et are less endothermic by 5–7 kcal/mol than that of DKP. These same trends can be seen in the reactions that produce TAT-Et and TAT; TAT-Et production is less endothermic by 5–7 kcal/mol. Steric hindrance from the relatively bulky ethyl group apparently lowers the reaction energy for the tetraglycine and pentaglycine ethyl amides; however, in triglycine ethyl amide the ethyl group causes the reaction to be more endothermic and comparable to that for the triglycine amide. Steric hindrance may also be a factor in the CID experiments where replacement of the $-NH_2$ group on the laminin with a –NHEt group decreased the intensity of c_3^- by approximately half.

We used the same G3(MP2) computational approach to study the thermodynamics of the penta-alanine anion decomposition. For C-terminus deprotonation, the effect of the five methyl groups (from the alanine side chains) is small, on the order of 1 kcal/mol decreasing both reaction energies (ΔH_{rxn}=68.6 kcal/mol for [AAAAA-Peptide Acid – H]$^-$→c_{m-3}^-+ODAT(Me$_3$) and ΔH_{rxn}=56.5 kcal/mol for [AAAAA-Peptide Acid – H]$^-$→c_{m-2}^-+DKM(Me$_2$)) relative to the same processes for pentaglycine. Backbone deprotonation at the third nitrogen from the C-terminus of penta-alanine is 11.8 kcal/mol more probable than deprotonation of the second nitrogen from the C-terminus. Deprotonation from the third nitrogen leads to the formation of the nine-member ring ODAT(Me$_3$) (see Supplemental Figure S14 for a molecular drawing) and from the second nitrogen to the 6-member ring DKM(Me$_2$). The effect of the methyl groups are to lower the endothermicity of the 9-member ring formation by 4.7 kcal/mol (ΔH_{rxn}=52.4 kcal/mol for AAAAA-Peptide Acid – H]$^-$→c_{m-3}^-+ODAT(Me$_3$)) and that of the 6-member ring by 2.7 kcal/mol (ΔH_{rxn}=28.5 kcal/mol for AAAAA-Peptide Acid – H]$^-$→c_{m-2}^-+DKM(Me$_2$)) as compared to the unsubstituted reactions. These effects are larger than for the C-terminus deprotonation as the methyl groups have more of an impact on the anion site in the backbone deprotonated penta-alanine.

Internal Valine Residue Loss

An interesting aspect of the CID spectrum for FPARVGI amide (Figure 2a) is the incidence of [y_4 – V]$^-$ and related products such as [y_4 – V – H_2O]$^-$ and [y_4 – V – HN=C=NH]$^-$. This is not observed in the CID of FPARVGI acid (Figure 2b). Loss of an internal valine residue was originally reported by Bowie and coworkers when studying CID of deprotonated citropin 1.1 and its synthetic analogs; these peptides all have C-terminal amide groups [89, 90]. They proposed that the helical structure of the peptide allows a conformational rearrangement to take place and found that the ESI solvent can affect the process. Elimination of valine has also been observed by Jai-nhuknan [91] in a study of protonated transform growth factor (TGF) α [34–43] using sustained off-resonance irradiation (SORI) CID in a Fourier

transform ion cyclotron resonance (FT-ICR) mass spectrometer. The valine loss appears as $[M+2\ H-V]^{2+}$ in the SORI-CID spectrum of $[M+3\ H]^{3+}$ from TGF α (34–43), which has a C-terminal carboxylic acid group and a disulfide bond [91]. Jai-nhuknan [91] also studied the reduced form of TGF α [34–43], where the disulfide bond is cleaved; this ion did not eliminate the valine residue. This suggests that the intact disulfide bond in TGF α [34–43] facilitates a peptide ion conformation that contributes to a unique loss of an internal valine residue [91].

Peptides with No Difference in CID Spectra Between Acid and Amide Analogs

Although most of the peptide amides in this study produced abundant c_{m-2}^-, there were a few that did not follow this trend. The peptide amides of SWAMVR, MLGFRSVGYA, and cholecystokinin produce only a small amount of c_{m-2}^-. (These spectra can be found in the Supplemental Materials.) In these cases, backbone fragmentation is overshadowed by neutral loss products from specific amino acid side chains. Each of the peptides contains at least one methionine residue, which is known for its abundant side chain loss in negative ion mode [55]. Cholecystokinin also has two aspartic acid residues in its sequence; acidic amino acid residues yield significant H_2O loss [51, 54]. In addition, serine is responsible for the production of abundant CH_2O loss in the spectra of deprotonated SWAMVR and MLGFRSVGYA [43, 53, 58, 90].

Conclusions

The effects of differing C-termini were studied using positive and negative ion mode CID on a group of peptides. Key differences were observed in the fragmentation of deprotonated Substance P, laminin, penta-alanine, WFAPPRVGYL, LMYVHWVR, LMYVHWVK, and FPARVGI peptide acids and amides. G3(MP2) calculations show that formation of c_{m-2}^- and a diketopiperazine neutral leaving group from a deprotonated peptide amide requires significantly less energy than the analogous process of a deprotonated peptide acid leading to the formation of a diketomorpholine. The presence of a prominent c_{m-2}^- in a negative ion mode CID spectrum is a strong indicator that a peptide has an amide group at the C-terminus. However, in some cases, side chain eliminations can suppress this distinctive fragmentation.

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