Ultraviolet Photodissociation of Protonated, Fixed Charge, and Charge-Reduced Peptides

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1. Abstract

The fragmentation behavior of three peptides (RGAFSTFGAS, GAFSTFGASR, and GAFSTFGASS) was evaluated by collisional activated dissociation (CAD), higher energy collisional activated dissociation (HCD), and ultraviolet photodissociation (UVPD). In particular, hybrid methods combining electron transfer with CAD, HCD, or UVPD were used to assess the impact of the location of a basic site at the C- or N-terminus, the presence of a fixed charge at the C- or N-terminus, and the presence of a radical site. The release of a mobilized proton occurred for those peptides modified with a quaternary amine at the C- or N-terminus, thus resulting in formation of C- and/or N-terminus fragment ions that would not otherwise be expected if the fixed charge remained static. Activation of charge-reduced peptides (i.e. peptide radical ions) resulted in simplified spectra compared to the corresponding even electron peptides. Interestingly, UVPD of the charge-reduced species generated similar spectra to CAD and HCD of the same precursor ions, suggesting a shift to dominant radical-induced dissociation rather than the more diverse pathways common upon photodissociation.

Keywords:

Hybrid activation

Ultraviolet photodissociation

Electron transfer

Fixed charge

Collisional activation

Quaternary amine

2. Introduction

Bottom-up proteomic strategies that rely on proteolytic digestion of proteins and tandem mass spectrometry to characterize the resulting peptides are the most popular approaches for implementation of high throughput, broad-scale identification of proteins in complex mixtures [1-3]. The typical bottom-up workflow has proven successful for identifying hundreds to thousands of proteins in complex mixtures based on matching peptides to proteins via *in silico* database search algorithms. The foundation of the database search algorithms is largely based on the ability to generate accurate theoretical tandem mass spectra based on predictive rules of peptide fragmentation [4-7]. Understanding the factors that modulate peptide dissociation remains an important challenge in developing the best correlations between experimental MS/MS spectra and *in silico* predictions. Both the location and type of peptide charge sites, as well as the ion activation method, influence the resulting fragmentation patterns. Bottom-up protocol commonly involves proteolytic digestion using trypsin, cleaving peptide bonds at basic lysine and arginine residues [8]. The presence of basic amino acids can alter MS/MS fragmentation patterns by sequestering a proton [5], thus reducing proton mobility and simplifying fragmentation spectra. Others have noted the enhancement of specific peptide backbone cleavage sites, such as cleavages adjacent to acidic residues and at the N-terminal side of proline, among others, which lead to prominent fragment ions in the MS/MS spectra of protonated peptides [6-9].

Attaching fixed charges to peptides through derivatization, originally as a means to enhance ionization efficiencies [10,11], has been shown by a number of groups to modulate the formation of N- and C-terminal fragment ions via charge-remote mechanisms [10-33]. It was noted that the resulting fixed-charge peptides exhibited differences in their fragmentation behavior compared to the analogous non-fixed charge counterparts. For example, the Reilly group has investigated several fixed charge reagents and found that the presumed fixed charge site of a quaternary amine modification can cleave generating a mobile proton, which may lead

to fragment ions that do not originate from the terminus of the original fixed charge site [12]. Another fixed charged reagent, tris(2,4,6-trimethoxy phenyl)phosphonium acetyl (TMPP), did not result in a similar mobile proton effect upon collision activated dissociation (CAD). High energy ultraviolet photodissociation (UVPD) using 157 nm photons of TMPP-tagged peptides promoted consistent charge-remote fragmentation [13]. Affixing a fixed charge tag to the N-terminus of a peptide has also been reported to improve sequence coverage obtained by electron transfer dissociation (ETD) for tryptic-like peptides (ones containing arginine at the C-terminus) [14]. Thus, evidence for variations in peptide fragmentation patterns has been correlated with the type of charge site (e.g. fixed charge versus mobile proton), the locations of charge sites (e.g. C-terminus, N-terminus, side-chain, backbone site), and the nature of the energization (e.g. low energy step-wise CAD versus high energy UVPD).

Whether the peptides are odd electron radical ions or even electron species is an additional mitigating factor that has generated considerable interest in recent years with the emergence of electron-activated dissociation methods: electron capture dissociation (ECD) [34,35] and electron transfer dissociation (ETD) [36,37]. Electron-activated methods lead to peptides with an extra electron via an exothermic electron transfer process that facilitates radical-induced dissociation and production of *c/z*-type fragment ions that contrast to the conventional *b/y*-type fragment ions generated by collision-based methods. Although the electron-activated methods often favor the formation of non-dissociated charge-reduced precursor ions (i.e. ETnoD), fragmentation efficiency has been significantly enhanced by applying supplemental energy or by following electron-activation with an auxiliary activation method [38,39]. Because of the greater propensity of ions in low charge states to undergo charge reduction rather than fragmentation into *c/z* ions, a number of groups have developed means to increase the charge states of ions via using super-charging ESI conditions or by covalent attachment of fixed charge sites [40-43].

“Hybrid” activation methods combine electron transfer reactions with CAD, higher-energy collisional dissociation (HCD), infrared multiphoton dissociation (IRMPD) or UVPD and have been reported to circumvent some of the potential charge-dependent shortcomings of ECD or ETD alone to generate richer fragmentation patterns [38,39,44-51]. UVPD has been previously shown to produce rich fragmentation patterns alone compared to other methods [52,55-72]. We have previously used 193 nm UVPD to explore the fragmentation of radical peptide cations generated by ETnoD [52]. A key feature that influenced the types of products was the position of the charge site as evidenced by the location of a sequestered proton (via a basic Arg residue) at either the N- or C-terminus. A recent study utilized ETD and subsequent activation by CAD or UVPD to determine the impact of the location of a specific amino acid (serine) within a peptide sequence on the resulting peptide fragmentation patterns [53]. For peptides containing histidine, electron transfer reactions caused a chromophore effect that specifically enhanced absorption of 355 nm photons [54]. We have recently reported an increase in sequence information obtained for intact proteins by combining ETD and 193 nm UVPD to produce more evenly distributed fragment ion types [44], in contrast to the dominant *a*-type fragment ions observed upon UVPD alone [55,56].

In the present study we evaluate the variations in fragmentation of fixed-charge peptides in comparison to analogous peptides containing a highly basic, proton-sequestering arginine at either the N- or C-terminus. The three reactions used to attach fixed charges are shown in **Scheme 1**. Three methods, CAD, HCD, and UVPD, are used to characterize the conventional protonated and fixed-charge peptides, and three hybrid activation methods, ETcaD, EThcD, and ETuvPD, are used to produce and analyze the corresponding peptide radical ions after charge reduction. To explore the impact of the location and type of charge on the fragmentation pathways of peptides, three peptides (RGAFSTFGAS, GAFSTFGASR, and GAFSTFGASS) were evaluated. Two of these peptide sequences were specifically designed to have a highly basic site (Arg) at the N- or C- terminus, and the third was an analogous peptide with no basic

site. For all three peptides, the remainder of the peptide sequence was kept constant to mitigate the impact of other residue-specific effects. In the present study, it is shown that simplified spectra are produced by photodissociation of the charge-reduced fixed-charge peptides generated by ETnoD.

3. Experimental

2.1 Materials

N,N-Dimethylformamide (DMF), HPLC grade water, methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxy-7-azabenzotriazole (HOAt) were purchased from AAPPTec, LLC (Louisville, KY, USA). N-Succinimidyl [tris(2,4,6-trimethoxyphenyl)phosphonio]acetate bromide (TMPP-AC-OSu) was obtained from Toronto Research Chemicals Inc. (Toronto, ON, CAN). Girard's Reagent T and trifluoroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Peptides RGAFSTFGAS, GAFSTFGASR, and GAFSTFGASS, were synthesized in-house using solid phase reaction and Fmoc amino acids from Novabiochem (Billerica, MA, USA).

2.2 Fixed Charge Derivatization of Peptides

Aliquots of each peptide were derivatized to contain a phosphonium N-terminal fixed charge via reaction with TMPP-AC-OSu using a previously described method (see **Scheme 1A**) [13]. The molar stoichiometric ratio of the reaction was reduced to 5:1 (reagent:peptide) to decrease the residual TMPP that carried over after C₁₈ cleanup. An additional peptide N-terminus fixed charge reagent, trimethyl-ammonium butyric acid with an amine-reactive N-hydroxysuccinimide group (TMAB-NHS), was synthesized in-house according to a previously reported method [30]. TMAB-NHS was reacted in a 5:1 stoichiometric ratio (reagent:peptide) in 0.2 M NaHCO₃ in 20% acetonitrile, pH = 9, using the reaction procedure also used to derivatize

peptides with TMPP-AC-OSu (see **Scheme 1B**) [13]. Quaternary amine fixed charges were attached to the C-termini of peptides by incubating a 100 μ L aliquot of peptide (1 mM) in water with 100 μ L of 10 mg/mL Girard's Reagent T (in 50% DMF). The reaction entailed mixing 100 μ L of 15 mg/mL EDC in DMF, 100 μ L of 15 mg/mL HOAt in DMF, and 30 μ L of 10% TFA for a net pH of 6 (see **Scheme 1C**). The mixture was allowed to react for 12 hours at 35°C and dried under vacuum overnight. The resulting derivatized peptides were desalted using standard SPE C₁₈ clean-up procedures.

2.3 Instrumentation and data collection

Derivatized and underivatized peptides were diluted to 1 μ M with methanol/water/formic acid (70/30/0.1, v/v/v) before direct infusion into a Thermo Fisher Scientific Orbitrap Elite mass spectrometer (Bremen, Germany) modified to allow UVPD in the HCD cell as described previously [55]. A 500 Hz Coherent ExciStar ArF (193 nm, 5 nsec per pulse) excimer laser (Santa Clara, CA, USA) was used for UVPD. The ion trap control language (ITCL) was customized to allow MS³ combining ET reactions and UVPD. Hybrid MS³ activation was undertaken by performing ETD of the +2 charge state precursor in the linear ion trap, followed by CAD (also in the linear ion trap), HCD (in the HCD cell), or UVPD (in the HCD cell) of the resulting isolated charge-reduced species. For comparisons of MS/MS behavior, CAD, HCD, and 193 nm UVPD spectra of each singly protonated or fixed-charge peptide were collected. The CAD parameters were as follows: 23 - 35 NCE, activation time of 10 ms, and an activation q of 0.25. The HCD parameters were as follows: 23 – 38 NCE, activation time of 0.10 ms. 193 nm UVPD was undertaken by exposing the ions to 4 – 8 laser pulses at 2 - 3 mJ per pulse. The laser pulses were applied at 500 Hz during an 8 - 16 ms activation period.

4. Results

The fragmentation patterns of three peptides RGAFSTFGAS, GAFSTFGASR, and GAFSTFGASS were compared. A fixed charge was attached at either termini, and each one

analyzed as either a singly protonated species (those not modified with a fixed charge tag), a fixed charge ion (not protonated; the charge is appended via the phosphonium TMPP⁺ or a quaternary amine QA⁺), or a charge-reduced protonated radical ion (formed via an electron attachment reaction of a protonated species). The peptide radical ions were generated in the dual-pressure linear ion trap of the Orbitrap Elite mass spectrometer through electron transfer (ET) reactions of the doubly charged peptides initially generated by ESI, resulting in charge-reduced peptide radical ions (i.e. initially 2⁺ but with an extra electron attached) [38]. Subsequent activation of the resulting peptide ions was undertaken either in the high pressure trap (CAD) or in the HCD cell (for HCD or UVPD). Whether protonated, fixed charge, or charge-reduced, in each case the net charge state of the precursor ion was 1⁺, thus maintaining charge parity throughout the study. Representative examples of the MS/MS spectra are shown in **Figures 1 - 5** and **Supplemental Figures 1 - 4**.

3.1 Unmodified Peptides

As initial benchmark data, examples of the UVPD spectra obtained for singly protonated peptides containing a basic amino acid (arginine) at the N- or C-terminus are shown in **Figure 1**, and the corresponding ETuvPD spectra (i.e. formation and analysis of the charge-reduced peptides) are shown in **Figure 2**. The UVPD mass spectra of protonated peptides are rich, containing *a,b,c*, *x*, *y*, and *z* ions, as well as side-chain loss ions (*d*, *v*). Neutral losses of water or ammonia from these primary fragment ions are also observed. These types of complicated fragmentation patterns are one of the hallmarks of UVPD and are attributed to the multiple mechanisms of UVPD, occurring both directly from excited states and after intramolecular vibrational redistribution after internal conversion [57,73]. As expected, the presence of the terminal arginine shifts the distribution of N- and C-terminal fragments due to proton sequestration [5]. The presence of an N-terminal arginine results in production of solely N-terminal fragments (*a,b,c,d*) (**Figure 1A**), whereas a C-terminal arginine results in predominantly

C-terminal fragments (v,x,y,z) and a few b ions (**Figure 1B**). For the peptide lacking an arginine, a more even distribution of N-terminal and C-terminal ions was observed (**Figure 1C**).

The analogous CAD and HCD spectra for each of the three singly protonated peptides are shown in **Supplemental Figures 1, 2, and 3**. CAD spectra of the peptides that contained an N- or C-terminal arginine (**Supp. Figures 1A and 2A**) followed previous trends of dominant loss of NH_3 from the protonated arginine side-chain [7]. HCD spectra of those same peptides (**Supp. Figures 1B and 2B**) showed higher conversion efficiencies of precursor to backbone fragment ions. As expected, CAD and HCD of the N-terminal arginine peptide generated N-terminal fragment ions; CAD and HCD of the C-terminal arginine peptide generated a mixture of N- and C-terminal fragments due to the possibility of two protonation sites (N-terminal amine and Arg). CAD and HCD of GAFSTFGASS produced a mixture of N- and C-terminal fragments (**Supp. Figure 3**).

The UVPD mass spectra of the corresponding charge-reduced peptides are shown in **Figure 2**. In this case, doubly protonated peptides (2+) were subjected to electron transfer reactions to generate charged-reduced species, then the charged-reduced peptides (1+) were subjected to UVPD (in a net hybrid process termed ETuvPD). The fragmentation patterns were generally much simpler than the patterns of the conventional protonated peptides in terms of the number of fragment ions produced, displaying mainly c - and z -type ions, ones traditionally observed upon electron activation. For all three peptides and their fixed charge analogs, this spectral simplification was especially true for ETuvPD (in comparison to ETcaD and EThcD) for which the number of different fragment ions typically decreased by ~50% for the charge-reduced peptides.

We were particularly interested in evaluating the impact on the UVPD behavior of fixed charge attachment to these peptides in comparison to conventional protonation, in addition to assessing the impact of charge-reduction of the peptides by electron attachment, yielding odd

electron peptides. Comparative examples of the UVPD spectra for the resulting peptide ions are shown in **Figures 3, 4, 5, Supplemental Figure 4**, and summarized in **Figure 6** and **Table 1**, with key results described in the following sections.

3.2 N-Terminal Arginine (RGAFSTFGAS)

Examples of the UVPD mass spectra for the C-terminal quaternary amine fixed-charge peptide RGAFSTFGAS-QA⁺ are shown in **Figure 3**. This is an interesting peptide since the basic Arg residue at the N-terminus is known to sequester a proton (resulting in the predominance of N-terminal fragment ions observed in **Figure 1A**), whereas the attachment of the fixed charge quaternary amine at the C-terminus localizes a single charge at the opposite end of the peptide. UVPD of the fixed-charge peptide results in a mixture of N- and C-terminal fragments although a prominent neutral loss of trimethylamine (-59 Da) and addition neutral loss of CO (-87 Da) occurred (**Figure 3A**). These latter pathways were also dominant in the CAD and HCD spectra (spectra not shown, see **Table 1**). UVPD of the corresponding charged-reduced peptide resulted in a substantially simplified spectrum (**Figure 3B**), comprised of only c- and z-type fragment ions which are radical-mediated products commonly observed upon ETD. The z-type ions are more abundant than the c-type, suggesting greater retention of the charge at the C-terminus, and there is a notable reduction in the loss of trimethylamine that was a dominant process upon UVPD of the even electron fixed-charge peptide (**Figure 3A**).

Attachment of a quaternary amine tag to the N-terminus of peptide RGAFSFGAS resulted in a fixed-charge peptide (QA⁺-RGAFSFGAS) in which the loss of trimethylamine was dominant upon CAD, HCD or UVPD, as summarized in **Table 1A**. In addition, UVPD generated over 20 N-terminal fragment ions (*a,b,c*), which are summarized in the top section of **Figure 6C** and **Table 1A**. In contrast to the prominent loss of trimethylamine for QA⁺-RGAFSFGAS upon CAD or HCD, ETcD and EThcD (i.e. interrogation of the odd electron peptide created after electron attachment to the protonated fixed-charge peptide) resulted in a series of diagnostic c

and z ions (**Table 1A**). ETuvPD produced a similar series of c/z ions (**Table 1A**), but the total number of different fragment ions (8 fragment ions) was significantly lower in comparison to UVPD of the even electron fixed-charge peptide (20 fragment ions) (**Figure 6C**).

A second N-terminal fixed-charge peptide was generated by attachment of the TMPP tag to RGAFSTFGAS. Examples of UVPD and ETuvPD mass spectra for the resulting TMPP⁺-RGAFSTFGAS peptide are shown in **Supplemental Figure 4**, and the CAD, HCD, ETcaD, and EThcD results are summarized in **Figure 6A, 6B** and **Table 1A**. The even electron fixed-charge peptide dissociated predominantly by the loss of the charged TMPP tag (resulting in the TMPP⁺ ion of m/z 573) in addition to the formation of some low abundance TMPP-containing N-terminal fragment ions (a,c,d ions) (**Supplemental Figure 4A**). UVPD of the corresponding odd electron charge-reduced peptide produced a series of N-terminal c ions, giving nearly full sequence coverage, with a significant reduction in the abundance of the TMPP tag ion (**Supplemental Figure 4B**). The number and types of N-terminal c ions produced by ETcaD, EThcD, and ETuvPD for charge-reduced TMPP⁺-RGAFSTFGAS are summarized in the bottom sections of **Figures 6A, 6B**, and **6C**, respectively, and in **Table 1A**, and were almost identical for all three hybrid methods. In general, attachment of the TMPP fixed charge tag to the N-terminus resulted in the exclusive formation of N-terminal fragment ions for all peptides and for all activation methods as well as for both odd and even electron precursors.

3.3 C-Terminal Arginine (GAFSTFGASR)

The number and types (C-terminal versus N-terminal) of fragment ions produced upon CAD, ETcaD, HCD, EThcD, UVPD, and ETuvPD for protonated GAFSTFGASR and its fixed-charged variants are summarized in the center sections of **Figure 6** and **Table 1B**. Protonated or charge-reduced GAFSTFGASR produced a mixture of N-terminal and C-terminal fragment ions upon CAD, HCD, or UVPD, as summarized in the top section of **Table 1B**. Attachment of a quaternary ammonium fixed charge to the N-terminus (QA⁺-GAFSTFGASR) led to significant

loss of trimethylamine upon UVPD (as noted previously for other quaternary amine-tagged peptides), as well as formation of an extensive array of N- and C-terminal fragment ions (a, b, c, x, y, z) (**Figure 4A**). In contrast, UVPD of charge-reduced QA⁺-GAFSTFGASR resulted solely in c and several N-terminal a -type ions (**Figure 4B**).

UVPD of the peptide created by replacement of the quaternary amine tag by the TMPP fixed charge tag at the N-terminus (TMPP⁺-GAFSTFGASR) yielded only N-terminal fragment ions. The UVPD spectrum of the charge-reduced TMPP⁺-GAFSTFGASR is very similar to that of QA⁺-GAFSTFGASR: showing a series of c and less abundant a -type ions. Moving the fixed charge to the C-terminus (GAFSTFGASR-QA⁺) led to the prominent loss of trimethylamine upon UVPD, in addition to low abundances of N- and C-terminal fragment ions (b, x, y, z). In contrast, UVPD of the charge-reduced peptide resulted solely in production of C-terminal z ions, similar to those observed upon CAD or HCD of the same charge-reduced peptide.

3.4 No Arginine (GAFSTFGASS)

A final peptide containing no amino acids with basic side-chains, GAFSTFGASS, was evaluated. Upon UVPD of the protonated or charge-reduced peptide, both N- and C-terminal ions were produced; a, b , and x, y for the protonated peptide and c and z for the charge-reduced peptide (top section of **Table 1C**). Attachment of a quaternary ammonium fixed charge to the N-terminus (QA⁺-GAFSTFGASS) again yielded a prominent loss of trimethylamine upon UVPD, in addition to a substantial series of N-terminal fragment ions (a, b, c). UVPD of charge-reduced QA⁺-GAFSTFGASS likewise resulted solely in N-terminal product ions; however in this case only a and c ions, no b ions, were formed (see **Table 1C**), and the number of different fragment ions was reduced by roughly a factor of three compared to UVPD of even electron QA⁺-GAFSTFGASS (**Figure 6C**).

Substitution of the quaternary amine tag by the TMPP tag at the N-terminus (TMPP⁺-GAFSTFGASS) yielded nearly an identical series of UVPD fragment ions as observed upon

UVPD of QA⁺-GAFSTFGASS (**Table 1C**). ETuvPD of the charge-reduced peptide also resulted in nearly identical fragmentation patterns for QA⁺-GAFSTFGASS and TMPP⁺-GAFSTFGASS. Moving the quaternary amine fixed charge to the C-terminus of the peptide resulted in an UVPD spectrum (**Figure 5A**) similar to that of the unmodified peptide (**Figure 1C**) for which both C-terminal and N-terminal fragments were produced. In contrast, ETuvPD of the charge-reduced GAFSTFGASS-QA⁺ peptide resulted solely in a clean series of C-terminal z ions in a manner consistent with radical-mediated pathways (**Figure 5B**).

5. Discussion

Table 1 provides a global summary of the types of fragmentation pathways observed for the peptides, in terms of formation of conventional sequence ions (*a,b,c,x,y,z*), prominent neutral losses (-59 Da for those containing a quaternary trimethyl ammonium terminus), or formation of a significant TMPP ion (for the TMPP-modified peptides). Shown in **Figure 6** is a bar-graph summary of the number of N-terminal (*a,b,c*) and C-terminal (*x,y,z*) fragment ions for the three peptides in their protonated, fixed charge, or odd electron charge-reduced forms. The presence of an N-terminal arginine or an N-terminal fixed charge led to the predominant formation of N-terminal fragments for all activation methods, whereas the presence of a C-terminal arginine or C-terminal fixed charge produced more C-terminal fragments. In each case, 193 nm UVPD produced a greater number of diagnostic fragments compared to CAD or HCD for the protonated or fixed-charge peptides. For the charge-reduced peptides (ones produced by electron transfer), the number and types of fragment ions were very similar for all three activation methods: ETcaD, EThcD, and ETuvPD.

For RGAFSTFGAS, N-terminal fragments were always favored for the unmodified peptide and for both types of N-terminal modified versions (TMPP⁺-RGAFSTFGAS and QA⁺-RGAFSTFGAS), whether activated as even-electron or charge-reduced odd electron species (see **Table 1A**). However, for RGAFSTFGAS-QA⁺ with a fixed charge at the C-terminus, both

C- and N-terminal ions were observed for the charge-reduced peptides upon ETcaD, EThcD, UVPD, and ETuvPD. Elimination of trimethylamine (-59 Da, neutral tag loss) has also been previously shown as a prominent loss allowing a proton to be liberated in the process [12]. UVPD of singly charged RGAFSTFGAS and RGAFSTFGAS-QA⁺, **Figures 1A** and **3A** respectively, resulted in formation of similar N-terminal fragment ion series, along with a dominant tag loss in **Figure 3A**. The unexpected formation of N-terminal product ions in **Figure 3A** despite the fixed charge at the C-terminus is rationalized through the elimination of trimethylamine which re-generates a mobile proton that may migrate to the N-terminal Arg, via a process identical to that reported by Reilly *et al.* [12]. Although not shown, this neutral loss of trimethylamine was also observed for CID and HCD of the RGAFSTFGAS-QA⁺ peptide, which supports the conclusion that this is not a radically driven neutral loss of an arginine side-chain [74] but rather due to a rearrangement, possibly involving cyclization with the N-terminal Arg, occurring when the fixed charge tag is lost.

For GAFSTFGASR and GAFSTFGASS, there was greater variation in whether C- or N-terminal fragments or both types were formed, and the outcome depended on the activation method (CAD, HCD versus UVPD). Activation of unmodified peptides resulted in a mixture of C- and N-terminal fragments by CAD, HCD, and UVPD. Modification of these two peptides by attachment of TMPP at the N-terminus resulted solely in formation of N-terminal fragment ions, confirming the dominant role of the fixed charge on the fragmentation behavior. In contrast, addition of a quaternary amine at the N-terminus of GAFSTFGASR and GAFSTFGASS led to formation of both C- and N-terminal ions, but yielded only N-terminal ions for the charge-reduced peptides. The quaternary amine tag underwent cleavage to eliminate trimethylamine and generate a mobile proton that facilitated formation of the unexpected C- or N-terminal ions upon CAD and HCD despite the location of a charge-sequestering Arg residue. However, the N-terminal quaternary amine-tagged GAFSTFGASR and GAFSTFGASS peptides yielded only N-

terminal ions for the charge-reduced species, cleavage of trimethylamine was no longer dominant, and the resulting fragment ions were dictated by radical-mediated pathways.

One of the most notable findings from the collection of results for the three peptides in the fixed-charge forms was the similarities of the fragmentation patterns, both in terms of number and types of fragment ions, obtained upon CAD, HCD and UVPD of the charge-reduced species (i.e. odd electron peptides). This result contrasted with the substantially greater diversity of fragment ions produced by UVPD relative to CAD and HCD for the protonated and fixed-charge peptides. Reference ETD spectra collected for the doubly-charged peptides (those with one proton and one fixed charge site) displayed c or z fragment ions depending on the location of the fixed charge site. Activation of charge-reduced peptide ions, whether by collisions or photons, led to the same c and or z ions observed upon ETD, thus recapitulating the dominance of the radical-mediated pathways regardless of activation method.

Since formation of a salt bridge between amino acids may occur for low charge states [75], the adoption of zwitterionic states was considered, especially in the context of the potential impact on fragmentation pathways. According to a previous study that evaluated the existence of zwitterionic peptides by UVPD, the competitive neutral loss of 45 Da (COOH) versus 44 Da (CO₂) allowed a means to quantify the portion of zwitterions [76]. Based on the CO₂-loss factor equation where $K_{CO_2} = ([-44] - [-45]) / ([-44] + [-45])$ [76], zwitterions yielded positive K_{CO_2} values up to 1.0, whereas non-zwitterions produced negative values down to -1.0. For peptides that were modified at the C-terminus (quaternary amine, QA⁺) in the present study, the carboxyl-terminus was no longer free and thus zwitterion formation was not possible.

K_{CO_2} values were calculated to assess the existence of zwitterionic states for unmodified peptides during 193 nm UVPD (**Figure 1**). UVPD of RGAFTSTFGAS, GAFSTFGASR, and GAFSTFGASS yielded K_{CO_2} values of 0.04, -0.27, and 0.05 respectively. These values are quite small and do not indicate a large population of zwitterionic states. UVPD of charge-reduced states of the unmodified peptides did not demonstrate losses of 45 Da or 44 Da loss

from the precursor ion. In fact, UVPD of charge-reduced precursors with and without fixed-charge tags did not undergo substantial losses of COOH or CO₂, presumably due to neutralization of a charged basic site (by electron transfer reaction) that would otherwise allow zwitterionic states.

Those peptides containing a fixed charge tag located on the N-terminus afforded a greater opportunity for formation of zwitterionic states, as in **Figures 4A** and **Supplemental Figure 4A**. K_{CO_2} values for each of these peptides were 0.49 and 0.53, respectively. These positive values suggest that zwitterionic states may be adopted for those peptides with an N-terminal quaternary amine fixed charge (QA⁺-GAFSTFGASR). Although not shown, CAD and HCD spectra of QA⁺-GAFSTFGASR contained dominant neutral losses of the trimethylamine (-59 Da) as well as a neutral loss of CO₂ (-44 Da) characteristic of zwitterions.

Furthermore, UVPD of the peptide containing a phosphonium fixed charge tag attached to the N-terminus (TMPP⁺-RGAFSTFGAS) generated a K_{CO_2} value of 0.53, one of the higher values found in the present study. The Reilly group observed the formation of TMPP⁺ upon the loss of the tag from TMPP-tagged FSWGAEQQR and RFSWGAEQ peptides, with the TMPP⁺ ion much more prominent for the peptide with the Arg at the N-terminus [28]. For the latter, they suggested the possible formation of a salt bridge between the arginine side-chain and the C-terminal carboxylic acid group, forming a cyclized structure. Blocking the C-terminus by esterification resulted in little change in the prominent loss of the fixed charge tag, leading to the conclusion that the potential salt bridge was not responsible for enhancing the dominant loss of TMPP⁺.

6. Conclusions

The influence of four key factors on the fragmentation patterns of peptides has been examined: i) location of a basic arginine residue site, ii) impact of a fixed charge, iii) conversion of conventional even electron peptides to odd electron peptides by electron transfer, and iv)

activation method (collisional versus photoactivation). The two charge tags appended to the N-terminus (TMPP and QA) modulated the fragmentation pathways in different ways, with the TMPP acting as a true fixed charge site and leading to dominant and exclusive formation of N-terminal fragment ions, whereas the quaternary amine charge tag underwent cleavage from either peptide terminus to release a mobile proton in a manner noted previously by Reilly *et al.* [12,13]. For all peptides, whether containing a fixed charge site or not, the fragmentation patterns of the charge-reduced species (those created via electron transfer) resulted in clean production of *c* and *z* ions upon secondary activation of CAD, HCD, or UVPD. Hybrid activation spectra were remarkably similar for ETcaD, EThcD, and ETuvPD despite the well-established differences in the collisional activation and photoactivation processes. Although UVPD is well known to create rich fragmentation patterns for protonated peptides, this was not the case for UVPD of odd electron peptides, and instead the resulting spectra were much simpler than typical UVPD mass spectra and nearly identical to the spectra produced by ETD (*c/z* ions). The dominance of the radical-mediated fragmentation pathways underscored the overwhelming impact of the radical site, more-so than the presence of a fixed charge or the type of activation method used. From a practical standpoint, this outcome suggests a strategy for simplifying overly congested MS/MS spectra (particularly UVPD spectra) by incorporating an initial charge-reduction step to consolidate fragmentation into *c/z* ions.

7. Acknowledgements

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8. References

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A)

B)

C)

Scheme 1. A) Attachment of phosphonium fixed charge to the N-terminus of a peptide, B) attachment of a quaternary amine fixed charge to the N-terminus of a peptide, and C) attachment of a quaternary amine fixed charge to the C-terminus of a peptide.

Figure 1. UVPD of protonated peptides: A) RGAFSTFGAS, showing mainly N-terminal fragments, B) GAFSTFGASR, showing mainly C-terminal fragments, and C) GAFSTFGASS, showing a mixture of both N and C-terminal fragments. All spectra have the same magnification scale ($\times 8$). # = water loss, * = ammonia loss.

Figure 2. ETuvPD of protonated charge-reduced peptides: A) RGAFTSTFGAS, showing mainly N-terminal fragments, B) GAFSTFGASR, showing mainly C-terminal fragments, and C) GAFSTFGASS, showing a mixture of both N and C-terminal fragments. All spectra have the same magnification scale ($\times 3$).

Figure 3. A) UVPD of +1 ion of C-terminal fixed-charge peptide RGAFTSTFGAS-QA⁺ showing dominant loss of trimethylamine, and B) ETuvPD of charge-reduced species of C-terminal fixed-charge peptide RGAFTSTFGAS-QA⁺ simplified fragmentation. Δ = ions containing fixed-charge tag. All spectra have the same magnification scale (x5). # = water loss, * = ammonia loss.

Figure 4. A) UVPD of A) +1 ion of N-terminal fixed-charge peptide QA⁺-GAFSTFGASR, and B) ETuVPD of charge-reduced species of N-terminal fixed-charge peptide QA⁺-GAFSTFGASR showing simplified fragmentation. Δ = ions containing fixed-charge tag.

Figure 5. A) UVPD of A) +1 ion of C-terminal fixed-charge peptide GAFSTFGASS-QA⁺, and B) ETuvPD of charge-reduced species of C-terminal fixed-charge peptide GAFSTFGASS-QA⁺ showing simplified fragmentation. # = water loss, * = ammonia loss, and Δ = ions containing the fixed-charge tag.

Figure 6. Number of fragment ions identified for peptides RGAFSTFGAS, GAFSTFGASR, and GAFSTFGASS with no fixed charge, TMPP N-terminal fixed charge (TMPP-N), quaternary amine N-terminal fixed charge (QA-N), and quaternary amine C-terminal fixed charge (QA-C). A) CAD versus ETcaD, B) HCD versus EThcD, and C) UVPD versus ETuvPD. Light blue bars represent C-terminal (x,y,z) fragment ion types and dark blue bars represent N-terminal (a,b,c) fragment ion types. The gold bars indicate that no fragments were identified. Note that the scale of the y-axis in C is double the scale of A and B.

TOC Graphic