RESEARCH ARTICLE

Electron Induced Dissociation of Singly Deprotonated Peptides

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Abstract

Dissociation of singly charged species is more challenging compared with that of multiply charged precursor ions because singly charged ions are generally more stable. In collision activated dissociation (CAD), singly charged ions also gain less kinetic energy in a fixed electric field compared with multiply charged species. Furthermore, ion–electron and ion–ion reactions that frequently provide complementary and more extensive fragmentation compared with CAD typically require multiply charged precursor ions. Here, we investigate electron induced dissociation (EID) of singly deprotonated peptides and compare the EID fragmentation patterns with those observed in negative ion mode CAD. Fragmentation induced upon electron irradiation and collisional activation is not specific and results in the formation of a wide range of product ions, including *b*-, *y*-, *a*-, *x*-, *c*-, and *z*-type ions. Characteristic amino acid side chain losses are detected in both techniques. However, differences are also observed between EID and CAD spectra of the same species, including formation of odd-electron species not seen in CAD, in EID. Furthermore, EID frequently results in more extensive fragmentation compared with CAD. For modified peptides, EID resulted in retention of sulfonation and phosphorylation, allowing localization of the modification site. The observed differences are likely due to both vibrational and electronic excitation in EID, whereas only the former process occurs in CAD.

Key words: Electron induced dissociation, Collision activated dissociation, Singly charged peptide anions

Introduction

The dissociation of peptides in tandem mass spectrometry (MS/MS) is a widely used technique for obtaining sequence information, with the vast majority of tandem mass spectrometric techniques utilizing positive ion mode and frequently relying on fragmentation of doubly protonated

species generated by electrospray ionization (ESI) [1]. Biomolecules can also be introduced into the gas phase by matrix-assisted laser desorption/ionization (MALDI) [2, 3], which is more tolerant to salts and impurities, and shows less ion suppression compared with ESI. However, because MALDI produces predominantly singly charged ions, generation of informative tandem mass spectra is more challenging compared with the dissociation of multiply charged precursor ions [4–7]. Specifically, low-energy collision activated dissociation (CAD) MS/MS of singly protonated peptides containing lysine and arginine often results in highly selective bond cleavages and poor sequence coverage, [8–11] presumably due to the absence of a mobile proton that can promote backbone bond cleavages [8–10, 12].

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To overcome this limitation, Lebrilla and co-workers employed a combination of infrared and collisional activation (CIRCA) to access higher energy fragmentation pathways [9, 10]. These authors demonstrated that for singly protonated model and tryptic peptides, CIRCA provided greater sequence coverage and higher quality spectra compared with CAD or infrared multiphoton dissociation (IRMPD) alone. In a different approach, Reilly and co-workers have shown that 157 nm photodissociation of singly protonated peptides produces informative spectra from which structural information can be obtained [13, 14]. The fragmentation pathways observed in 157 nm photodissociation are different compared with those observed in vibrational excitation of precursor ions: *x*-, *v*-, and *w*-type product ions were observed when the charge was localized at the C-terminus, whereas *a*- and *d*-type product ions were detected when the charge was localized at the N-terminus [13]. Photodissociation at 193 nm has also been exploited for fragmentation of singly protonated peptides [15–18]. Similar to 157 nm dissociation, formation of *a*- and *d*-type product ions was observed when arginine residues were in close proximity to the N-terminus and *y*-, *x*-, *v*- and *w*-type fragments were detected when Arg residues were located near the C-terminus [17, 19]. Detailed investigations of 193 nm photodissociation revealed that the fragmentation behavior is significantly affected by the presence and location of arginine residues [16], and that product ion types and abundances vary depending on precursor ion charge location [20].

All the aforementioned work focuses on fragmentation of singly protonated peptides, i.e., positive ion mode MS/MS. However, utilization of negative ion mode is often desired, given that ~50% of naturally occurring proteins are acidic. Peptides containing numerous acidic residues (e.g., glutamic and aspartic acid) may be challenging to detect in positive ion mode [21–23]. On the other hand, it has been shown that highly basic peptides can easily form singly deprotonated ions with sufficient intensities for fragmentation experiments [24]. Furthermore, negative ion mode provides enhanced sensitivity for peptides containing acidic post-translational modifications such as sulfonation [25] and phosphorylation [26].

Cassady and co-workers have examined the dissociation of singly charged basic and acidic peptides in positive and negative ion mode post source decay (PSD) and showed that negative ion PSD results in structurally informative spectra, complementary to those obtained in positive ion mode [22, 24]. The main product ion types in negative ion mode PSD correspond to *y*-, *b*-, and *c*-type ions. Bowie and co-workers have examined extensively the dissociation of singly deprotonated peptides in negative ion mode CAD and demonstrated that sequence information can be derived [27, 28]. For example, comparison of positive and negative ion mode CAD of singly charged bioactive peptides containing four to five amino acids showed that negative ion spectra were as informative or at least complementary to the positive ion spectra [28]. Harrison has shown that low-

energy CAD of singly deprotonated peptides resulted in sufficient fragmentation to establish the amino acid sequence of di- to pentapeptides containing H or alkyl side chains and he concluded that negative ion mode CAD provided as much information as positive ion mode CAD of protonated peptides [29]. However, one drawback of negative ion mode peptide PSD and CAD is extensive neutral and side chain loss, and the formation of internal product ions. Neutral losses do not provide sequence information and the diverse fragmentation processes yield complex spectra, thereby reducing MS/MS sensitivity and rendering spectral interpretation more difficult.

Electron capture dissociation (ECD) [30] and electron transfer dissociation (ETD) [31] have been shown to be complementary and result in more extensive fragmentation compared with slow heating techniques such as CAD [32–34]. However, ECD and ETD are only applicable to positively charged precursor ions carrying at least two charges. Electron detachment dissociation (EDD) [35, 36] involves dissociation of multiply charged anions and results in the formation of *a*- and *x*-type product ions. A major advantage of EDD compared with slow heating techniques is the retention of phosphorylation [37] and sulfonation [35]. Similar fragmentation pathways, i.e., formation of *a*- and *x*-type product ions, was observed in negative electron transfer dissociation (NETD) [38, 39], although phosphate loss was prevalent when xenon was used as reagent [38, 39]. EDD and NETD spectra are less complex compared with those obtained from PSD and CAD because neutral and side chain losses are significantly less prevalent. However, both EDD and NETD require at least doubly negatively charged precursor ions, limiting their applicability for singly charged species.

Ion–electron reactions of singly charged cations were previously examined for small organic molecules [40–42]. For such cationic species, interaction with electrons in the energy range 2–70 eV, produced by a continuous electron beam, resulted in excitation followed by fragmentation similar to that obtained in CAD. This technique was originally termed electron impact excitation of ions from organics (EIEIO) [41, 42] and later termed electron induced dissociation (EID) [40], which is the term we use here. Wang and McLafferty performed 70 eV EID of sodium cationized, $[M + Na]^+$, precursor ions from the cyclic peptide gramicidin S [43]. Similar to the case of small organic molecules, the fragmentation observed in EID was similar to that in CAD.

More recently, O'Hair and co-workers explored EID of singly protonated aromatic amino acids, cystine, and small peptides. These authors showed that EID yielded different and complementary fragmentation compared with CAD and suggested that ion activation in EID proceeds via both electronic and vibrational excitation [44]. Julian and co-workers extended this work to larger protonated peptides and reported that several fragmentation pathways observed in EID of singly charged peptide cations are radical-induced

[45]. Furthermore, the fragmentation of singly protonated and sodiated betaine dimers in EID was examined by O'Hair and co-workers who reported that betaine loss was the dominant fragmentation pathway for both the protonated and sodiated species [46]. Zubarev and co-workers performed EID of protonated and sodiated oligosaccharides and showed that full sequence coverage could be obtained, similar to CAD of the same species, and to ECD of the corresponding doubly protonated species [47]. EID has also recently been applied for structural elucidation of singly charged ions of oxo-centred trinuclear carboxylate-bridged iron complexes [48], octaethylporphyrin and its iron(II) complex [49], and small organic cationic molecules [50]. In a different approach, Zubarev and co-workers recently demonstrated that irradiation of molecular cations with higher energy electrons (>20 eV) can cause double ionization followed by subsequent fragmentation to produce extensive backbone dissociation. This fragmentation technique has been termed electron ionization dissociation [51] (also abbreviated EID) and is different from electron induced dissociation, which is utilized here.

In contrast to EID of singly cationized species, very few reports focused on negative ion mode EID. Yoo and Hakansson applied negative ion mode EID to phosphate-containing metabolites and showed that EID results in complementary fragmentation compared with CAD and IRMPD [52]. In addition, Amster and co-workers applied EID to singly charged anions from sulfonated glycosaminoglycans [53]. These authors showed that EID resulted in fragmentation similar to that observed in EDD, but different from that observed in CAD and IRMPD of the same molecule. Both odd- and even-electron product ions were observed in EID [53]. More recently, EID of doubly deprotonated tryptophan clusters was investigated [54] and it was shown that EID proceeds via different pathways depending on the electron energy. In agreement with previous reports [52, 53], the complementarity of EID and CAD was also demonstrated [54].

Here, we examine the applicability of EID for the dissociation and characterization of singly deprotonated peptides and compare the fragmentation patterns with those observed in negative ion mode CAD. These experiments aimed to examine the fragmentation pathways in EID of peptide anions and also to address whether EID provides complementary information compared with CAD for peptide analysis.

Experimental

Sample Preparation

The peptides H-RPKPQQFFGLM-NH₂, H-RPKPQQFFGLM-OH, CH₃CO-RRRA(pS)VA-OH (phosphorylated at serine), H-WHWLQL-OH, pEHWSYGLRPG-NH₂, pEHWSYGLRPG-OH, H-PPGFSPFR-OH, pEQWFWWM-NH₂, pEVNFSPGWGT-NH₂, and H-GNLWATGHFM-NH₂ were purchased from Sigma (St. Louis, MO, USA) and the peptides H-DY*MGWMDF-NH₂

(sulfonated at tyrosine) and H-DYMGWMDF-NH₂ were obtained from Advanced Chemtech (Louisville, KY, USA) and used without further purification. H-WHWLQL-OH was diluted to a final concentration of 1 μ M, CH₃CO-RRRA(pS)VA-OH to 1.7 μ M, and H-PPGFSPFR-OH to 3 μ M. All other peptides were diluted to 5 μ M. All peptides, except H-WHWLQL-OH, were electrosprayed from a spraying solvent containing 1:1 isopropanol:water (Fisher Scientific, Fair Lawn, NJ, USA) and 10 mM ammonium acetate (Fisher Scientific). For H-WHWLQL-OH, 0.4% piperidine (Acros Organics, Morris Plains, NJ) was used instead of 10 mM ammonium acetate.

Mass Spectrometry

All experiments were performed with an actively shielded 7 T quadrupole Fourier transform ion cyclotron resonance (Q-FT-ICR) mass spectrometer (Apex-Q; Bruker Daltonics, Billerica, MA, USA), which has been previously described [55]. The peptides were electrosprayed in negative ion mode at a flow rate of 70 μ L/h. Ions were accumulated in the first hexapole for 0.1 s, transferred through the mass selective quadrupole (10 m/z isolation window), mass selectively accumulated in the second hexapole for 0.1 to 3 s to optimize ion abundances for MS/MS experiments, transferred through high-voltage ion optics, and captured in the ICR cell by dynamic trapping. This accumulation sequence was looped twice. EID was performed with an indirectly heated hollow dispenser cathode at a bias voltage of 10–12 V and an irradiation time of 5, 8, or 10 s. A lens electrode located in front of the hollow cathode was kept 0.3–1.0 V lower than the cathode bias voltage. CAD was performed in the hexapole collision cell with argon as collision gas.

All spectra were acquired with XMASS (ver. 6.1; Bruker Daltonics) using 256 or 512 K data points and summed over 32 (for CAD experiments) or 64 (for EID experiments) scans. Data processing was performed with the MIDAS [56] analysis software. Internal frequency-to-mass calibration was performed by Microsoft Excel with a two-term calibration equation. The calculated masses of the precursor ions and abundant product ions were used for calibration. The product ions used for calibration are indicated in Tables S1A to S12B in Supplemental Data. Product ion spectra were interpreted using the MS Product function (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct>) in Protein Prospector. Only peak assignments with a mass accuracy better than 15 ppm were accepted.

Results and Discussion

EID and CAD of Singly Deprotonated Amidated and Free Acid Forms of Substance P

EID of singly deprotonated Substance P, H-RPKPQQFFGLM-NH₂, is shown in Figure 1a. CAD of the same species was performed for comparison (Figure 1b). All

Figure 1. EID (a) and CAD (b) spectra of the singly deprotonated amidated form of Substance P (H-RPKPQQFFGLM-NH₂). Following both electron irradiation and collisional activation, *b*- and *y*-type product ions are observed. However, in EID, several radical species are detected. By contrast, no odd-electron species are formed in CAD. Characteristic side chain losses from phenylalanine and methionine are detected in EID. Several product ions in the CAD spectrum could not be assigned based on known fragmentation pathways. ? = unidentified product ions, $\nu_3=3^{\text{rd}}$ harmonic

assigned product ions are summarized in [Supplemental Data](#), Tables [S1A](#) and [S1B](#). EID of the Substance P anions resulted in formation of several *b*- and *y*-type product ions, which dominate in slow heating techniques such as CAD and IRMPD. However, for this peptide, several odd-electron species, $z_7^\bullet$, $z_9^\bullet$, $a_9^\bullet$, and $a_{10}^\bullet$ were also detected. Formation of *a*-type radical ions has also been observed in CAD of radical anions formed by UV photodetachment of electrons from doubly deprotonated peptides [57]. The a_8 product ion was a mixture of odd- and even-electron species (see inset, Figure 1a). These radical ions were unique to EID (absent following CAD). Formation of odd-electron product ions from even-electron precursor ions, although not common, has been previously observed in EID of glycosaminoglycan anions and it was suggested that these odd-electron products arise from electronic excitation of the even-electron precursor ions [53]. Alternatively, irradiation of molecular ions with high energy electrons may result in hydrogen atom ejection followed by subsequent fragmentation to produce

odd-electron $z^\bullet$ - and $a^\bullet$ -type product ions. In EID, the *y*-type product ions corresponding to cleavages at the N-terminal side of proline were observed as y_8 and $[y_8 - 2H]$ and y_{10} and $[y_{10} - 2H]$. These hydrogen deficient species were not observed in CAD. Side chain losses from phenylalanine (PhCH₂, 91.055 Da) and methionine (CH₂CH₂SMe, 75.027 Da) were only detected following EID. Such side chain losses have been previously reported in CAD of singly deprotonated dipeptides formed by fast atom bombardment (FAB) [58, 59]; however they were absent in our low energy CAD of Substance P-NH₂ (Figure 1b). CAD of C-terminally amidated Substance P anions resulted in the formation of mainly *b*- and *y*-type product ions although some *c*-type products, c_7 , c_8 , and c_9 , were also detected. In EID, only the c_9 product ion was observed. Formation of *c*-type product ions is generally observed in negative ion mode CAD [21, 29, 60]. Some *y*- and *b*-type ions, y_4 , b_4 , and b_7 , observed in CAD were absent in EID. Interestingly, several CAD product ions could not be identified based on known

fragmentation pathways (*a*-, *b*-, *c*-, *x*-, *y*-, and *z*-type product ions, NH_3 and H_2O losses from precursor and product ions, and amino acid side chain losses from precursor and product ions). In contrast, EID resulted in less complex spectra in which all product ions were identified.

EID and CAD of the singly deprotonated free acid form of Substance P, H-RPKPQQFFGLM-OH, are displayed in Figure 2a and b (complete list of all assigned product ions are shown in Supplemental Data, Tables S2A and S2B). Similar to EID and CAD of the singly deprotonated amidated form, extensive *b*- and *y*-type product ions are formed. For this peptide, only one radical species, $z_9^\bullet$, was detected in EID. Some *a*-type product ions, a_8 , a_9 , a_{10} , are also observed; however they are even-electron species whereas, in EID of amidated Substance P, the *a*-type products were detected as odd-electron species. These product ions, $z_9^\bullet$, a_8 , a_9 , and a_{10} , were absent in the CAD spectrum. Also, the product ions x_4 , x_5 , and y_3 were present

only in the EID spectrum. As observed for the amidated form of Substance P, *y*-type product ions, y_8 and y_{10} , corresponding to cleavage at the N-terminal side of proline were detected as both y_n and $[y_n - 2\text{H}]$ -type species. These hydrogen deficient species were not observed following CAD, whereas the c_5 , c_6 , c_8 , and b_8 product ions were only detected following CAD. For both the amidated and free acid form of Substance P, several product ions were unique in the EID or CAD spectra, suggesting that EID and CAD can provide complementary structural information.

The differences observed between the EID spectra of the amidated and free acid singly deprotonated forms of Substance P, and also between the CAD spectra of these two singly deprotonated forms, suggest that charge location can affect fragmentation patterns in both EID and CAD. For example, side chains losses from arginine (100.087 Da), leucine (57.070 Da), glutamine (72.045 Da), and methionine (62.019 Da), and *x*-type product ions were only detected in

Figure 2. EID (a) and CAD (b) spectra of the singly deprotonated free acid form of Substance P (H-RPKPQQFFGLM-OH). EID resulted in more extensive fragmentation compared with CAD. *a*- and *x*-type product ions were solely formed in EID. Characteristic side chain losses from the precursor and product ions are dominant in EID. Some product ions formed in EID could not be assigned based on known fragmentation pathways. Only the major peaks are labeled in the figure, complete lists of all observed product ions are given in Supplemental Data, Tables S2A and S2B. ? = unidentified product ions, * = noise peak, v_3 = 3rd harmonic

EID of the free acid form. Also, the y_3 and y_4 product ions were only present in EID of the free acid form whereas the b_6 , a_7 , and c_9 product ions were only detected for the amidated form. Several product ions in the EID spectrum of the free acid form could not be assigned based on known fragmentation pathways (as defined above) whereas, in EID of the amidated form, all product ions were assigned. Differences in fragmentation patterns between the amidated and free acid form of Substance P were also reported by Tsybin et al. in ECD of doubly protonated species: ECD of the free acid form resulted in formation of several a - and y -type product ions, not detected following ECD of the amidated form [61]. These authors suggested that charge location affects the ECD outcome [61]. Differences observed in negative ion mode CAD (Figures 1b and 2b) of the two forms of Substance P include the product ions b_4 , b_7 , c_7 , z_7 , x_5 , and c_9 , only detected for the amidated form whereas the product ions c_5 , c_6 , c_8 , and b_8 were present only in CAD of the free acid form. Furthermore, the free acid form produced a less complex spectrum compared with that of the amidated form. For the free acid form, the C-terminal carboxylic acid is likely the deprotonation site whereas, for the amidated form, the charge location is less predictable because no acidic groups are present. It has been suggested that backbone amide hydrogens are the next most acidic hydrogens after the hydrogen atoms of carboxylate groups [36]. Therefore, for the amidated form of Substance P, possible deprotonation sites include any of the backbone amide hydrogens. Regardless of the exact position of the deprotonation site, it appears that charge location influences the fragmentation outcome in both EID and CAD of singly deprotonated species.

EID and CAD of Singly Deprotonated Amidated and Free Acid Forms of LHRH

EID and CAD of the singly deprotonated amidated form of luteinizing hormone releasing hormone (LHRH, pEHW-SYGLRPG-NH₂) showed relatively similar fragmentation, including formation of mainly b - and y -type product ions (Supplemental Data, Tables S3A and S3B). Products corresponding to loss of HCHO (30.011 Da) from the serine residue were present in both spectra, although this loss was more dominant in the CAD spectrum. Characteristic side chain loss from serine- (formaldehyde, HCHO) and threonine- (acetaldehyde, CH₃CHO) containing peptides have previously been reported in negative ion mode CAD [27, 60, 62, 63].

Neutral loss from tryptophan side chains was observed in both CAD and EID of singly deprotonated LHRH-NH₂. In CAD, loss of 129 Da (C₉H₇N, 129.058 Da) was seen whereas, in EID, loss of 130 Da (C₉H₈N, 130.066 Da) was observed. In agreement with our results, loss of 129 Da from Trp residues has been previously observed in CAD of singly deprotonated peptides formed by FAB and was proposed to result from charge migration (producing a deprotonated N-terminus) followed by formation of an indole anion and

hydride transfer [59]. This loss was also reported in EID of singly protonated amino acids [44]. In ECD, loss of 131 Da (C₉H₉N, 131.074 Da) from Trp residues has been reported [64] and we have documented loss of 129 Da from the Trp side chain in EDD [65].

A major difference between CAD and EID of singly deprotonated LHRH-NH₂ is the detection of two radical species, $a_8^\bullet$ and $a_9^\bullet$, in EID, similar to EID of Substance P-NH₂. The [y_9 - NH₃] product ion was only detected in EID whereas the c_3 and b_8 product ions were only observed in CAD.

The free acid form of LHRH was also investigated (Supplemental Data, Tables S4A and S4B). For this peptide, no odd-electron products were detected in EID of the singly deprotonated species. Some a -type ions, a_7 and a_8 , were formed but they were detected as even-electron species, similar to EID of the free acid form of Substance P. These product ions were absent in CAD. Also, the c_5 product ion was present only in the EID spectrum. Some b - and y -type product ions, y_8 , y_5 , and b_7 , were only formed in CAD. Side chain losses and H₂O and NH₃ losses from both the precursor and product ions were significantly more pronounced in CAD compared with EID (Supplemental Data, Tables S4A and S4B). The observed differences between CAD and EID spectra of LHRH suggest that these two fragmentation techniques proceed via different activation pathways.

Similar to EID of the amidated and free acid forms of Substance P, differences are observed between the EID spectra of the amidated and free acid forms of LHRH. For example, formation of odd-electron species was not observed for the free acid form. Furthermore, the y_6 , c_5 , and a_7 product ions were only detected for the free acid form whereas the y_8 product ion was only observed for the amidated form. Similar trends were apparent in CAD spectra, in which some product ions were only observed for the amidated or free acid form, respectively. Neutral losses from the precursor and product ions were more abundant in CAD of the free acid form. Therefore, as discussed above, the charge location appears to influence the fragmentation outcome in both EID and CAD. Differences in fragmentation behavior were also reported in ECD of the amidated and free acid forms of LHRH [61]. For example, for the free acid form, several y -type product ions were detected, which were not observed in ECD of the amidated form.

EID and CAD of Other Singly Deprotonated C-terminally Amidated Peptides without Acidic Sites

Because C-terminally amidated Substance P and LHRH (neither of which contains acidic sites) yielded more radical (unique) product ions in EID compared with their free acid forms, we proceeded to characterize EID of other C-terminally amidated peptides without acidic sites. Product ions observed in EID and CAD of singly deprotonated neuromedin B, H-GNLWATGHFM-NH₂, are listed in Supplemental Data, Tables S5A and S5B. The precursor ions and the majority of product ions exhibited abundant

neutral loss of CH_3CHO (44.026 Da) from the threonine residue. y -type product ions were detected in both CAD and EID. The y_7 product ion was observed only in EID whereas the c_8 product ion was only present in CAD. Furthermore, for this peptide, side chain loss from tryptophan was only detected in EID. By contrast, in the case of LHRH, side chain loss from tryptophan was present in both CAD and EID. Two radical species, $z_6^\bullet$ and $a_9^\bullet$, were only formed in EID, although the $a_9^\bullet$ product ion was detected with low abundance. As discussed above, formation of radical species in EID can be due to electronic excitation of molecular ions, or due to hydrogen atom ejection followed by radical-driven fragmentation. Similar to CAD of Substance P- NH_2 , several product ions detected in CAD of neuromedin B could not be assigned based on known fragmentation pathways.

EID of the singly deprotonated peptide pEVNFSPGWGT- NH_2 resulted in several product ions not detected in CAD, including $a_9^\bullet$, c_7 , $[b_7 - \text{H}]^-$, $[b_6 - 30]$ (serine side chain loss), a_5 and c_4 (Supplemental Data, Tables S6A and S6B). In CAD,

only two product ions corresponding to backbone bond cleavages were observed, y_7 and y_8 . Similar to the previously examined peptides containing Ser or Thr residues, characteristic HCHO (30.0106 Da) and CH_3CHO (44.026 Da) losses were highly abundant in CAD and EID spectra of pEVNFSPGWGT- NH_2 . The EID spectrum of pEVNFSPGWGT- NH_2 was more informative compared with CAD because it contained more backbone product ions.

The peptide pEQWFWWM- NH_2 was the only peptide examined here for which EID (Supplemental Data, Table S7A) resulted in less extensive fragmentation compared with CAD (Supplemental Data, Table S7B). Four product ions, y_3 , $[z_4 - 75]$, $[x_5 - 62]$, and $[z_6 - 130]$, detected in CAD, were absent in the EID spectrum. Several neutral losses (see Supplemental Data, Tables S7A and S7B) are observed in the CAD and EID spectra.

We have previously examined EDD of the doubly deprotonated peptide pEQWFWWM- NH_2 [65]. EID of the $[M - \text{H}]^-$ precursor ions from this peptide (Table S7A, Supplemental

Figure 3. EID (a) and CAD (b) spectra of singly deprotonated bradykinin (H-PPGFSPFR-OH). EID resulted in a highly informative spectrum: a series of b - and y -type product ions is observed, and a -, x -, and z -type product ions are also detected. Product ions corresponding to loss of serine and phenylalanine side chains are also present. In CAD, few sequence specific product ions were formed. Instead, side chain and small molecule neutral losses are mainly observed. Only the major peaks are labeled in the figure, complete lists of all observed product ions are given in Supplemental Data, Tables S8A and S8B. ? = unidentified product ions, $v_3=3^{\text{rd}}$ harmonic

Data) showed similar fragmentation as EDD of the $[M - 2H]^{2-}$ precursor ions; however, some differences between these spectra were also observed. For example, formation of c - and x -type product ions was observed in both techniques whereas formation of y -type product ions was detected only in EID. Neutral loss of the tryptophan side chain and neutral loss of the amidated C-terminus were present in both spectra; however, these losses were more predominant in EDD spectra. The higher yield of Trp side chain loss observed in EDD can be due to the low vertical ionization energy of Trp, which may allow direct electron detachment from Trp and, therefore, steer the fragmentation in EDD [65]. By contrast, EID of singly deprotonated species is unlikely to occur via electron detachment because this would result in neutralization of the precursor and product ions.

In our previous work [65], we also performed EDD of the peptides pEVNFSPGWGT-NH₂ and H-GNLWATGHFM-NH₂. For these two peptides, only one backbone product ion was observed and, instead, neutral loss corresponding to

cleavage of the tryptophan side chain was dominant. By contrast, EID and CAD of the corresponding singly deprotonated species (Table S5A for H-GNLWATGHFM-NH₂ and Table S6A for the peptide pEVNFSPGWGT-NH₂, both in Supplemental Data) produced more informative spectra, allowing structural information to be derived although Trp side chain loss was also present for the peptide H-GNLWATGHFM-NH₂.

EID and CAD of Singly Deprotonated Peptides with a C-terminal Carboxylic Site but No Acidic Residues

Because most natural peptides are not C-terminally amidated, we proceeded to characterize the EID behavior of peptides with a carboxylate C-terminus but no other acidic sites. EID and CAD spectra of singly deprotonated bradykinin, H-PPGFSPFR-OH, are shown in Figure 3a and b, respectively. Complete lists of observed product ions are

Figure 4. EID (a) and CAD (b) spectra of the singly deprotonated peptide H-WHWLQL-OH. EID and CAD show similar fragmentation patterns; however, EID resulted in the formation of more backbone product ions. Several product ions are unique to EID or CAD, respectively. Product ions corresponding to tryptophan side chain loss are detected in both spectra. ? = unidentified product ions, v_3 = 3rd harmonic, * = noise peak

included in [Supplemental Data](#), Tables S8A and S8B. The observed fragmentation behavior for singly deprotonated bradykinin was similar to that of the peptide pEVNFSPGWGT-NH₂ (see previous section), for which EID resulted in more extensive fragmentation compared with CAD. Several product ions from backbone bond cleavage, including y_2 , x_2 , y_3 , y_4 , b_4 , $[z_4 - 30]$, z_6^+ , $[c_6 - 30]$, $[y_6 - 30]$, $[y_7 - H]^+$, $[c_7 - 30]$, and a_7 were detected in EID but absent in CAD. Therefore, for bradykinin, EID was more informative than CAD. CAD resulted in the formation of only two backbone product ions, b_7 and $[y_6 - 30]$, which were also formed in EID. The y_3 product ion, corresponding to cleavage on the N-terminal side of proline was detected as both y_3 and $[y_3 - 2H]$, as observed in EID of Substance P. The increased backbone fragmentation observed in EID compared with CAD may be attributed to differences in ion internal energy following activation. The higher energy in EID allows population of higher vibrational levels and possibly electronic excitation.

EID (Figure 4a and [Supplemental Data](#), Table S9A) of the peptide H-WHWLQL-OH showed similar fragmentation

to that observed in CAD (Figure 4b and [Supplemental Data](#), Table S9B), including formation of c -, y -, and b -type product ions. However, some product ions detected in EID were not observed in CAD. These include mainly even-electron species; y_2 , b_2 , $[b_3 - 129]$, $[z_5 - 129]$, and a_5 . Two odd-electron species, z_3^+ and z_5^+ , are also unique to EID. On the other hand, the product ions a_3 , a_4 , and y_5 , are unique to CAD. Similar to other tryptophan-containing peptides, loss of the Trp side chain was observed in both EID and CAD. Product ions formed in EID exhibited less neutral losses compared with CAD, resulting in a less complex spectrum. For example, in CAD, the product ions c_3 and c_4 exhibited multiple neutral losses whereas these product ions did not show any neutral losses in EID. We have also previously examined EDD of the peptide H-WHWLQL-OH [65]. We observed that similar to the peptides pEVNFSPGWGT-NH₂ and H-GNLWATGHFM-NH₂, loss of the tryptophan side chain dominated the EDD spectrum. In contrast, EID (Figure 4a, and Table S9A) and CAD (Figure 4b, and Table S9B) of this peptide provided more extensive fragmentation, as was observed for the peptides H-

Figure 5. EID (a) and CAD (b) spectra of the singly deprotonated serine-phosphorylated peptide CH₃CO-RRR(pS)VA-OH. The majority of product ions from EID retained the labile phosphate group, allowing its localization. By contrast, in CAD, all product ions exhibited loss of the phosphate group. ?=unidentified product ions, v_3 =3rd harmonic

GNLWATGHFM-NH₂ (Tables S5A and S5B) and pEVNFSPGWGT-NH₂ (Tables S6A and S6B)

EID and CAD of Singly Deprotonated Cholecystokinin

Cholecystokinin, H-DYMGWMDF-NH₂, which is C-terminally amidated but has two acidic residues, was also examined in our previous EDD experiments [65]. EDD of this doubly deprotonated peptide resulted solely in the formation of the charge-reduced species and CO₂ loss from this species. No backbone product ions were detected in EDD. By contrast, EID (Supplemental Data, Table S10A) and CAD (Supplemental Data, Table S10B) of the singly deprotonated species produced more informative spectra, allowing structural information to be derived. For this peptide, Trp side chain loss was absent in EID and CAD spectra, similar to the results obtained with EDD [65].

EID of singly deprotonated cholecystokinin resulted in the formation of several backbone product ions, *a*₅, *y*₅, *a*₆, and *z*₇, which were not observed following CAD of the same

species. All backbone product ions formed in CAD were also present in the EID spectrum; therefore CAD did not produce any additional structural information for this peptide.

EID and CAD of Singly Deprotonated Modified Peptides

Lastly, two modified peptides were examined with EID: the first peptide, CH₃CO-RRA(pS)VA-OH, contains a phosphorylated serine residue and the second peptide, H-DY*MGWMDF-NH₂, contains a sulfonated tyrosine residue. Both these modifications are acidic and, therefore, may benefit from negative ion mode analysis. In EID of the singly deprotonated Ser4-phosphorylated peptide, a major product ion corresponding to neutral phosphoric acid (H₃PO₄) loss from the precursor ions is observed (Figure 5a), in agreement with the results obtained in CAD (Figure 5b). All assigned product ions are displayed in Supplemental Data, Tables S11A and S11B. However, in EID, the majority of backbone product ions, *y*₄, *a*₄, *z*₅, and *a*₅,

Figure 6. EID (a) and CAD (b) spectra of the singly deprotonated tyrosine-sulfonated peptide cholecystokinin, H-DY*MGWMDF-NH₂. EID produced *b*, *y*, *c*, and mainly *a*-type product ions that retained the sulfonate group, allowing its localization. By contrast, CAD resulted in sulfonate loss from all, but one, product ions. For complete lists of all generated product ions see Tables S12A and S12A (Supplemental Data) [?]=unidentified product ions, ^{v3}=3rd harmonic

retained the labile phosphate group, allowing localization of the phosphorylation site. Retention of phosphorylation has been previously reported for multiply charged protonated precursor ions in ECD [37, 66–68] and ETD [31], and it was demonstrated that these electron-based reactions can be successfully applied for localization of phosphorylation sites. In sharp contrast, following negative ion CAD, only one product ion, b_5 , retained the phosphorylation.

Similar results were observed for the Tyr2-sulfonated peptide (complete lists of all assigned product ions are provided in [Supplemental Data](#), Tables S12A and S12B). Facile SO_3 loss is observed from the precursor ions following EID (Figure 6a) and CAD (Figure 6b). However, in EID, almost all backbone product ions containing the sulfonated tyrosine residue retained the labile sulfonate group, analogous to the behavior of the phosphorylated peptide discussed above. Only one backbone product ion, y_7 , displayed loss of the sulfonate moiety, and the c_6 product ion was detected as both a sulfonated and non-sulfonated species. These results are similar to those observed in EDD of a Tyr-sulfonated peptide, in which a mixture of sulfonated and non-sulfonated products was observed [35]. Following CAD, SO_3 loss from both the precursor and product ions dominated the spectrum. All, but one, product ions containing the sulfonated tyrosine residue lost the modification. Furthermore, for this peptide EID resulted in more extensive fragmentation compared with CAD. A series of a -type product ions, a_2 , a_3 , a_5 , a_6 , and a_7 , was observed for this peptide. These a -type product ions were absent in CAD. The c_3 and x_7 product ions were also only present following EID.

Conclusions

EID of singly-charged peptide anions was examined and compared with CAD of the same species. A wide range of product ions, including b -, y -, a -, c -, and some x - and z -type product ions, was detected in both EID and CAD, and some product ions were common to both fragmentation techniques. However, unique product ions, observed solely in EID or CAD spectra, respectively, were also detected and, in some cases, significant differences were observed between EID and CAD spectra of the same species. The different fragmentation pathways observed between EID and CAD suggest differences in the manner of ion activation in the two techniques. Based on our results, we postulate that EID of singly deprotonated peptides proceeds via both electronic and vibrational excitation. As a result of vibrational excitation, common product ions are observed between EID and CAD spectra, whereas product ions formed following electronic excitation are only detected in EID. Furthermore, the higher ion internal energy in EID can explain the richer fragmentation observed for several of the examined peptides. Freiser and co-workers suggested that ion activation during ion-electron collisions proceed through excitation of electronic modes, whereas CAD proceeds via excitation of vibrational modes [40]. Fragmentation via

electronic excitation in EID has also been proposed by Amster and co-workers [53] and O'Hair and co-workers suggested that EID proceeds via both electronic and vibrational excitation. However, as these authors mention, the exact mechanism of EID is likely to be complex [44, 46].

Regardless of the fragmentation mechanism, we show that electron irradiation of singly charged deprotonated peptides results in formation of several unique backbone product ions, not detected following CAD of the same species and, thus, providing more extensive structural information. Interestingly, for some peptides CAD provided poor fragmentation, whereas EID resulted in very informative spectra. Furthermore, characteristic losses from the precursor ions, corresponding to side chain loss from Phe, Met, Arg, and Trp residues, were present in the majority of EID spectra of peptides containing these amino acid residues. These characteristic amino acid side chain losses were less consistent in CAD spectra. Some uninformative small neutral losses from the precursor and product ions were also observed, but these losses were less pronounced in EID spectra, rendering them less complex compared with CAD. Formation of several radical species was also unique to EID. In EID of a phosphorylated and a sulfonated peptide, abundant neutral loss of H_3PO_4 and SO_3 was observed from the precursor ions, similar to CAD. However, in EID, the majority of product ions retained the phosphorylation and sulfonation, respectively. Therefore, EID can reveal the presence of phosphorylation, or sulfonation, and also identify their location.

The results presented here demonstrate that EID is a useful fragmentation technique for the analysis of singly deprotonated peptides, as it provides extensive sequence information, and also shows that EID can provide complementary structural information to that observed in negative ion mode CAD.

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