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Secondary fragmentation of linear peptides in electron capture dissociation

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Abstract

Inspection of the electron capture dissociation (ECD) spectra of doubly-protonated peptides, Leu_4-Sar-Leu_3-Lys-OH, Leu_4-Ala-Leu_3-Lys-OH, Gly_4-Sar-Gly_3-Lys-NH_2 and Gly_3-Pro-Sar-Gly_3-Lys-NH_2, reveals extensive secondary fragmentation. In addition to w ions, entire, and in some cases multiple, cleavages of amino acid side chains from backbone fragments are observed. Extensive water loss from backbone fragments is observed for the glycine-rich peptides. For Leu_4-Ala-Leu_3-Lys, the preferred fragmentation channel is cleavage of the amide bond to produce b_7 and b_8 ions. ECD of Gly_3-Pro-Sar-Gly_3-Lys-NH_2 results in amine bond (c/z) cleavage in the proline residue accompanied by C–C (or secondary N–C) cleavage in the proline side chain. That fragmentation channel has not been observed previously. The peptides were also subjected to "hot" electron capture dissociation (HECD) and the resulting spectra differed markedly from those obtained under standard ECD conditions. In contrast to HECD, secondary fragmentation observed under standard ECD conditions cannot be attributed to excess energy arising from the kinetic energy of the electrons prior to capture. The results suggest that the fragmentation channels available following electron capture depend somewhat on the individual peptide structure and have mechanistic implications.

Keywords: FT-ICR; FT-MS; Electron capture dissociation; ECD; Secondary fragmentation; MS/MS; Tandem MS

1. Introduction

The introduction of electron capture dissociation (ECD) [1,2] to electrospray ionization [3] Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry [4–6] has had a great impact on the analysis of peptides and proteins. Typically, ECD results in cleavage of the backbone amine bond to produce c and $z^{\bullet}$ fragments [1,7,8]. Amino acid side chain cleavages are also observed [9]. The extent of cleavage along the backbone is in general far greater than that for other fragmentation techniques. ECD backbone fragments have a tendency to retain post-translational modifications so that sites of glycosylation [10–12] and phosphorylation [13,14], for example, can be identified. A further feature of ECD is its ability to cleave disulfide bonds [7,15]. Recently, Zubarev and coworkers introduced a technique coined "hot" electron capture dissociation (HECD)

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[16,17]. Standard ECD employs slow (<0.2 eV) electrons whereas in HECD, dissociative capture follows irradiation with hot (3–13 eV) electrons. In addition to amine bond cleavage, the excess energy associated with HECD resulted in backbone cleavage to form b- and y-type ions, and most interestingly d and w ions. The d and w ions are products of secondary fragmentation. The analytical import of that finding is that leucine and isoleucine can be distinguished.

In this paper, we show that, for some peptides, significant secondary fragmentation is observed under standard ECD conditions. Kjeldsen and Zubarev [18] have observed formation of w ions under standard ECD conditions. In this study, entire, and in some cases multiple, cleavages of amino acid side chains from backbone fragments are observed, in addition to extensive w cleavage. Extensive water loss from backbone fragments is observed for the glycine-rich peptides. Perhaps the most important observation described here is amine bond (c/z$^{\bullet}$) cleavage in the proline residue accompanied by C–C (or secondary N–C) cleavage in the proline side chain in the peptide, Gly_3-Pro-Sar-Gly_3-Lys-NH_2. Such a cleavage has not been observed previously and suggests that it may be possible to sequence proline-containing peptides entirely. Unlike HECD, secondary fragmentation observed under standard ECD conditions cannot be attributed to excess energy arising from the electron kinetic energy prior to capture. The results suggest that the fragmentation channels available following electron capture depend somewhat on the individual peptide structure.

2. Experimental methods

2.1. Samples

Luteinizing hormone releasing hormone (LHRH) and melittin were purchased from Sigma (St. Louis, MO, USA) and used without further purification. The peptides L_4SarL_3K-OH, L_4AL_3K-OH, G_3PSarG_3K-NH_2 and G_4SarG_3K-NH_2 were synthesized by the BASS facility at Florida State University. The samples were microelectrosprayed at 10 μM from solutions of 1:1 water:methanol (J.T. Baker, Philipsburg, NJ, USA), 2% acetic acid (Aldrich, Milwaukee, WI, USA).

2.2. Electron capture dissociation FT-ICR mass spectrometry

The peptide samples were analyzed with a homebuilt, passively shielded, 9.4 T FT-ICR mass spectrometer [19] equipped with an external microelectrospray ionization source [20]. Samples were infused at a flow rate of 500 nL/min through an electrospray emitter consisting of a 50 μm i.d. fused silica capillary, which had been mechanically ground to a uniform thin-walled tip [21]. 2.0 kV was applied between the microspray emitter and the capillary entrance. The electrosprayed ions were delivered into the mass spectrometer through a Chait-style atmosphere-to-vacuum interface [22,23] and externally accumulated for 2 s in an rf-only octopole [20]. The ions were transferred through multipole ion guides and trapped in an open [24] cylindrical cell. The peptide ion under investigation was isolated by use of a resolving quadrupole [25], prior to transfer and trapping in the ICR cell.

An indirectly heated dispenser cathode [26,27], situated ∼24 in. behind the ICR cell, provided the electrons for ECD [1,2]. A voltage of −1.6 or −4 V (ECD) and −10 or −16 V (HECD [17]) was applied to the cathode during the irradiation event. A grid situated in front of the filament was kept at −200 V for most of the experiment and pulsed to 6 V during the ECD event. The isolated parent ions were irradiated with electrons for 17 ms (ECD) and 250 ms (HECD). The (H)ECD fragment ions were frequency-sweep (“chirp”) [28,29] excited (72–640 kHz at 150 Hz/μs) and detected in direct mode (512 Kword time-domain data). Between 100 and 250 time-domain data sets were co-added, Hanning apodized, zero-filled once and subjected to fast Fourier-transform (FFT) followed by magnitude calculation. The experimental event sequence was controlled by a modular ICR data acquisition system (MIDAS) [30]. The FT-ICR mass spectra were internally frequency-to-*m*/*z* calibrated [31,32] with respect to

parent $[M+2H]^{2+}$ and $[M+H]^{\bullet+}$ ions. The FT-ICR mass spectra were analyzed by use of the MIDAS analysis software package [33].

3. Results and discussion

We first determined instrumental conditions under which ECD and HECD of standard peptides (luteinizing hormone releasing hormone (LHRH) and melittin) occurred. Fig. 1 shows ECD (top) and HECD (bottom) ESI FT-ICR mass spectra of the doubly-protonated peptide, Leu_4-Sar-Leu_3-Lys-OH. (Note that for this peptide, and all other examples herein, the ECD spectra obtained did not change on further reduction of electron energy.) As expected, the two spectra differ markedly. The HECD spectrum is characterized by more fragment ions, particularly at low *m*/*z*. The major peaks in the HECD spectrum correspond to y_5 and y_7. In contrast, the ECD spectrum shows the expected c and $z^{\bullet}$ ions. The remarkable feature of the ECD spectrum is the presence of secondary fragments. Peaks corresponding to w_4 and w_6–w_9 ions

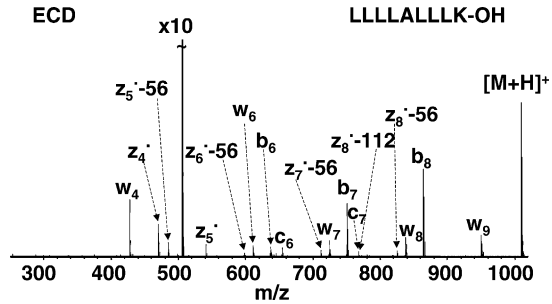

Fig. 2. ECD ESI FT-ICR mass spectrum of doubly-protonated Leu_4-Ala-Leu_3-Lys-OH. Losses of entire Leu side chains from $z^{\bullet}$ ions are designated ($z_n^{\bullet}$-56). $z_n^{\bullet}$-112 indicates loss of two Leu side chains from $z^{\bullet}$ ions.

are observed. Peaks are also observed at *m*/*z* 824.574, 711.489, 598.405, and 471.305 corresponding to loss of C_4H_8, i.e., the entire Leu side-chain, from $z_8^{\bullet}$, $z_7^{\bullet}$, $z_6^{\bullet}$ and $z_5^{\bullet}$. Furthermore, fragments corresponding to the loss of *two* Leu side chains from $z_5^{\bullet}$ and $z_6^{\bullet}$, i.e., fragments requiring cleavage of *three* covalent bonds, are seen at *m*/*z* 415.243 and 542.342.

Fig. 2 shows the ECD ESI FT-ICR mass spectrum obtained from the peptide, Leu_4-Ala-Leu_3-Lys-OH.

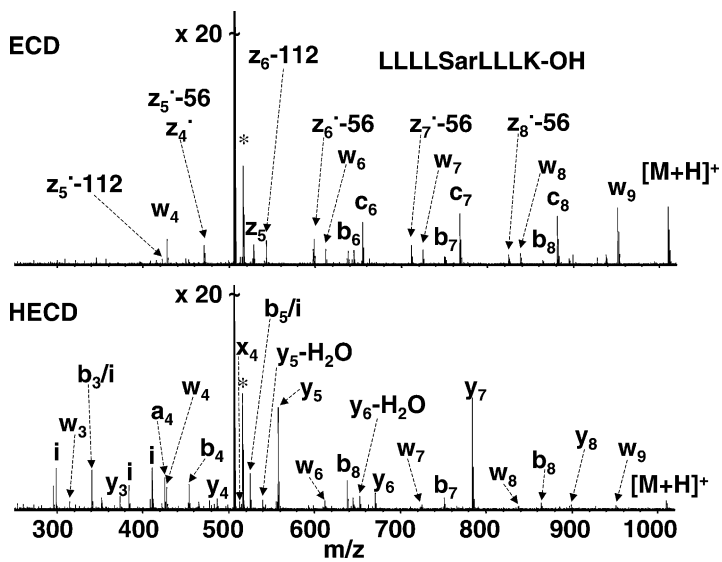

Fig. 1. ECD (top) and HECD (bottom) ESI FT-ICR mass spectra of quadrupole-isolated doubly-protonated Leu_4-Sar-Leu_3-Lys-OH. Losses of entire Leu side chains from $z^{\bullet}$ ions are designated ($z_n^{\bullet}$-56). $z_n^{\bullet}$-112 indicates loss of two Leu side chains from $z^{\bullet}$ ions. (i) Denotes internal (b/y type) fragment ions; (*) denotes $[M+H+Na]^{2+}$ not removed during isolation.

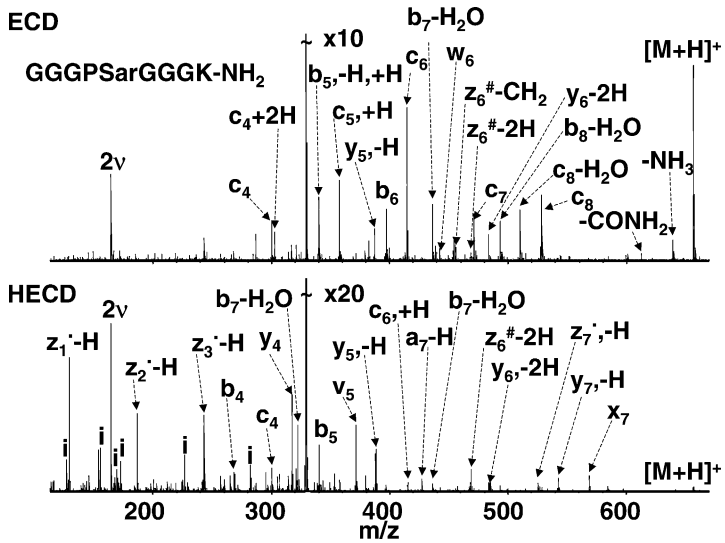

Fig. 3. ECD (top) and HECD (bottom) ESI FT-ICR mass spectra of doubly-protonated Gly_3-Pro-Sar-Gly_3-Lys-NH_2. (#) Denotes a diradical ion. (i) Denotes internal (b/y type) fragment ions.

Again, extensive secondary fragmentation produces w ions, w_4, w_6–w_9, and losses of entire Leu side chains from $z_5^\bullet$ through $z_8^\bullet$. A peak at *m*/*z* 768.516 corresponds to the loss of two Leu side chains from $z_8^\bullet$. We conclude, therefore, that the presence of an N-methylated amino acid residue (sarcosine) is not responsible for the secondary fragmentation behavior. The major products of ECD of Leu_4-Ala-Leu_3-Lys-OH, b_7 and b_8, derive from fragmentation of backbone amide bonds, a non-standard fragmentation channel in ECD. We have previously observed that fragment ions due to amide bond cleavages, particularly those known to be facile, e.g., cleavage N-terminal to proline, are sometimes detected in ECD spectra. Such ions were observed in experiments in which the parent ion was isolated by stored waveform inverse Fourier-transform (SWIFT) ejection [34,35] and subjected to 30 s irradiation with electrons provided by a filament electron gun. b ions arose when parent ions, excited during the SWIFT event, underwent collision-induced dissociation (CID) following collision with background gas during that long ECD event. In the present experiments, parent ions were isolated by use of a resolving quadrupole [25] prior to trapping in the ICR cell, and were irradiated for 17 ms with electrons provided by a dispenser cathode [27]. Those instrumental conditions eliminate the possibility that CID processes are contributing to the observed spectrum.

Fig. 3 shows the ECD (top) and HECD (bottom) ESI FT-ICR mass spectra of the peptide, Gly_3-Pro-Sar-Gly_3-Lys-NH_2. Again, the two spectra are noticeably different: the HECD spectrum is characterized by more peaks at low *m*/*z*, including a number of peaks corresponding to internal (b/y type) cleavages. Opportunity for secondary fragmentation within amino acid side chains is limited in this peptide. However, the incidence of backbone fragments $\pm n$ H atoms, in which $n = 1, 2$, in the ECD spectrum, suggests that rearrangement mechanisms, similar to side chain expulsion, occur. Extensive secondary fragmentation in the form of water loss is also observed. Similar results (not shown) were obtained for the peptide, Gly_4-Sar-Gly_3-Lys-NH_2. For the proline-containing peptide, peaks observed at *m*/*z* 468.257, 456.257, and 442.240 are of particular interest. Those peaks correspond to ($z_6^{\#}$-2H), ($z_6^{\#}$-CH_2) and ($z_6^{\#}$-C_2H_4) (or w_6) ions (# denotes diradical) (calculated *m*/*z* 468.257,

456.257, 442.241, respectively), i.e., amine bond ($c/z^{\bullet}$) cleavage at proline accompanied by C–C (or secondary N–C) cleavage in the proline side chain. That cleavage has not previously been observed under standard ECD conditions. Thus, complete c, $z^{\bullet}$ sequence coverage for proline-containing peptides with ECD may be possible. In the present case, all cleavages except $c_2/z_7^{\bullet}$ were observed. Peaks corresponding to ($z_6^{\#}$-2H), ($z_6^{\#}$-CH_2) and ($z_6^{\#}$-C_2H_4) (or w_6) ions were also observed under HECD conditions. Under those conditions, complete c, $z^{\bullet}$ sequence coverage was observed.

4. Conclusion

In summary, the present results show that secondary fragmentation can occur within linear peptides under standard ECD conditions and suggest that the fragmentation channels available to a peptide following electron capture are related to its structure. The observation of amine bond cleavage accompanied by C–C (or secondary N–C) cleavage within the proline side chain is perhaps the most significant observation. Full characterization of the effect of peptide sequence on ECD will be valuable both mechanistically and analytically. Such studies are in progress.

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