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Breakthroughs and Views

Electron capture dissociation mass spectrometry in characterization of post-translational modifications

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Abstract

Electron capture dissociation (ECD) represents a significant advance in tandem mass spectrometry for the identification and characterization of post-translational modifications (PTMs) of polypeptides. In comparison with the conventional fragmentation techniques, such as collisionally induced dissociation and infrared multi-photon dissociation, ECD provides more extensive sequence fragments, while allowing the labile modifications to remain intact during backbone fragmentation. This unique attribute offers ECD as an attractive alternative for detection and localization of PTMs. The success and rapid adoption of ECD recently led to the culmination of The 1st International Uppsala Symposium on Electron Capture Dissociation of Biomolecules and Related Phenomena (October 19–22, 2003, Stockholm, Sweden). Herein, we present a general overview of the ECD technique as well as selected applications in characterization of post-translationally modified polypeptides.

Keywords: Electron capture dissociation; Mass spectrometry; Peptide sequencing; Fourier transform; Electrospray ionization; Post-translational modifications

Post-translational modifications (PTMs) are of significance in the regulation of biological activities and signal transduction. PTMs may alter physical and chemical properties, structural conformation, distribution, stability, activity; and hence, function of the cellular proteins. Well-known examples of the biological consequences of protein modification include phosphorylation for signal transduction, ubiquitination for proteolysis, and attachment of fatty acids for membrane anchoring, glycosylation for protein half-life, targeting, and cell–cell interactions. Despite the importance of PTMs, their global identifications (e.g., in proteomic studies) and characterization have been hampered by the lack of a rugged universal method. Among the various techniques employed for identification of PTMs, mass spectrometry (MS) has played an increasingly important role. The popularity of mass spectrometry has been mainly due to the introduction of soft ionization techniques, namely electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) [1–6]. Typically, for the identification of PTMs, ESI or MALDI mass spectrometry is used to determine the molecular weight (MW) of the intact protein. The discrepancy between the measured mass and the sequence-predicted value indicates the presence of a covalently bound modification. Subsequently, identification of modification site(s) is performed by tandem mass spectrometry (MS/MS).

In a typical MS/MS experiment, the molecular ions of polypeptides are dissociated in the gas-phase and its modifications can be revealed from the resulting fragments. The most common method for ion dissociation involves the collision of the peptide ion of interest with neutral, non-reactive, gas-phase target atoms or

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molecules such as argon, helium, or molecular nitrogen. These collisions result in excitation of the analyte ions and subsequent fragmentation. The nomenclature used for assignment of fragment ions in MS/MS spectra of peptides and proteins is depicted in Fig. 1. When a peptide is cleaved (in vacuo) and the charge is retained by the N-terminal fragment, the descriptors a_n, b_n, and c_n are used, where n represents the position of the fragmentation in the amino acid chain. Likewise, if the charge is retained by the C-terminal portion of the molecule, x_n, y_n, and z_n designations are assigned to the fragment ions. Additionally, a superscript denotes the charge state of each fragment ion. Therefore, since the fragment or product ions are a direct consequence of dissociation of the parent or precursor ion upon collision with a target gas, this process is referred to as the collision-induced dissociation (CID) or collisionally activated dissociation (CAD) [7]. The CID spectrum of a peptide yields valuable information on its amino acid sequence, often composed of complementary pairs of C- and N-terminal product ions (Fig. 1). Despite its utility, there are two major limitations associated with CID in the characterization of PTMs. First, CID often selectively cleaves certain inter-residue bonds, leaving sequence gaps, which can be highly predominant for larger polypeptides [8–11]. Second, the labile modifications often have the propensity to undergo elimination prior to the peptide backbone fragmentation. Electron capture dissociation (ECD) [12,13], introduced by McLafferty and co-workers in 1998, largely overcomes these limitations by providing more extensive peptide sequence fragments, while allowing the labile modifications to remain intact during the peptide backbone fragmentation. In the remaining segment of this communication, we will present a brief overview of ECD as well as selected examples of its utility in characterization of post-translational modifications.

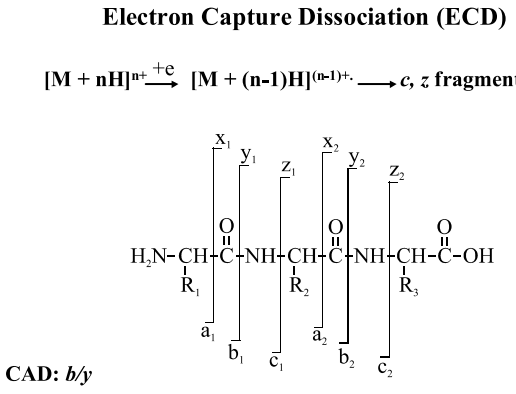

Fig. 1. A simplified representation of the ECD process and the nomenclature used for assignment of product ions in the CID and ECD spectra of peptides and proteins.

Electron capture dissociation mass spectrometry

In an ECD experiment, the multiply charged ions produced by ESI are trapped within the confine of a combination of magnetic and electrostatic fields of a Fourier transform mass spectrometer (FTMS) instrument, and are irradiated with a beam of relatively low energy ($\leqslant$0.2 eV) electrons generated by an electron gun [14,15]. The partial neutralization of multiply protonated ions with low energy electrons typically renders extensive inter-residue backbone cleavage to yield c and $z^{\bullet}$ fragment ions (Fig. 1). This unique c/z fragmentation is in sharp contrast to the b/y fragmentation by low energy MS/MS. While y and b ions originate from the dissociation of the amide bond, the c and $z^{\bullet}$ fragments result from a cleavage of the N–C_{α} amine backbone bond; this unique c/z dissociation pathway was first observed in the experiment of 193 nm photo-dissociation of polypeptides [12] and was the result of recombination of multiply charged polypeptide ions with the low-energy photoelectrons ejected from the FTMS cell walls. More intriguingly, even labile modifications remain intact during backbone fragmentation, which makes ECD particularly amenable to detection and localization of post-translational modifications (e.g., glycosylation, sulfation, oxidation, and phosphorylation) [16–19].

ECD has been proposed as a "non-ergodic" fragmentation process, in which the intramolecular energy randomization is slower than the ECD cleavages [15,20,21]. This unique non-ergodic feature has been exemplified by the ability of ECD in preserving the labile side chain modifications groups on the peptide backbone fragments. This is in contrast to CID and other conventional fragmentation techniques, which typically eject the labile modifications prior to the peptide backbone dissociation [21].

Selected applications

O-linked glycosylation

Protein glycosylation is one of the major post-translational modifications with significant effects on protein folding, conformation distribution, stability, and activity. Carbohydrates in the form of asparagine-linked (N-linked) or serine/threonine (O-linked) oligosaccharides are major structural components of many cell surfaces and secreted proteins. In contrast to the N-linked glycosylation (occurring at asparagine residues in an Asn-Xxx-Ser/Thr, in which Xxx could be any amino acid except proline), there is no sequence consensus for the O-linked glycosylation. Moreover, the characterization of O-linked glycosylation by tandem mass

spectrometry has been particularly difficult, because the conventional MS/MS methods (e.g., CID) yield weak or no signal for glycan-carrying ions. This is mainly due to a higher labile nature of glycosidic bonds relative to peptide bonds. Zubarev and colleagues [22] first demonstrated that ECD could be applicable to the analysis of O-glycosylated peptides. The backbone fragmentation, which resulted form the ECD experiment, led to unequivocal determination of five GalNAc sites in a 3 kDa glycopeptide isolated from a mixture of glycoforms. Haselmann et al. [23] demonstrated that four of six GalNAc monosaccharides could be mapped in a 6.8 kDa peptide. Additionally, characterization of a 25-residue peptide containing up to five sialic acid and six GalNAc modifications was achieved.

Phosphorylation

Phosphorylation pathways constitute an integral part of the signal transduction mechanism in the cell. The addition and loss of phosphate groups mediated by kinases and phosphatases, respectively, have been subjected to an enormous body of research. In recent years, mass spectrometry has been one of the widely utilized methods to detect and characterize protein phosphorylation [24].

A common MS/MS process observed for phosphorylated peptides which contain phosphoserine and/or phosphothreonine is the loss of neutral phosphoric acid (H_3PO_4, 98 Da) corresponding to the formation of dehydroalanine or dehydroaminobutyric acid via gas-

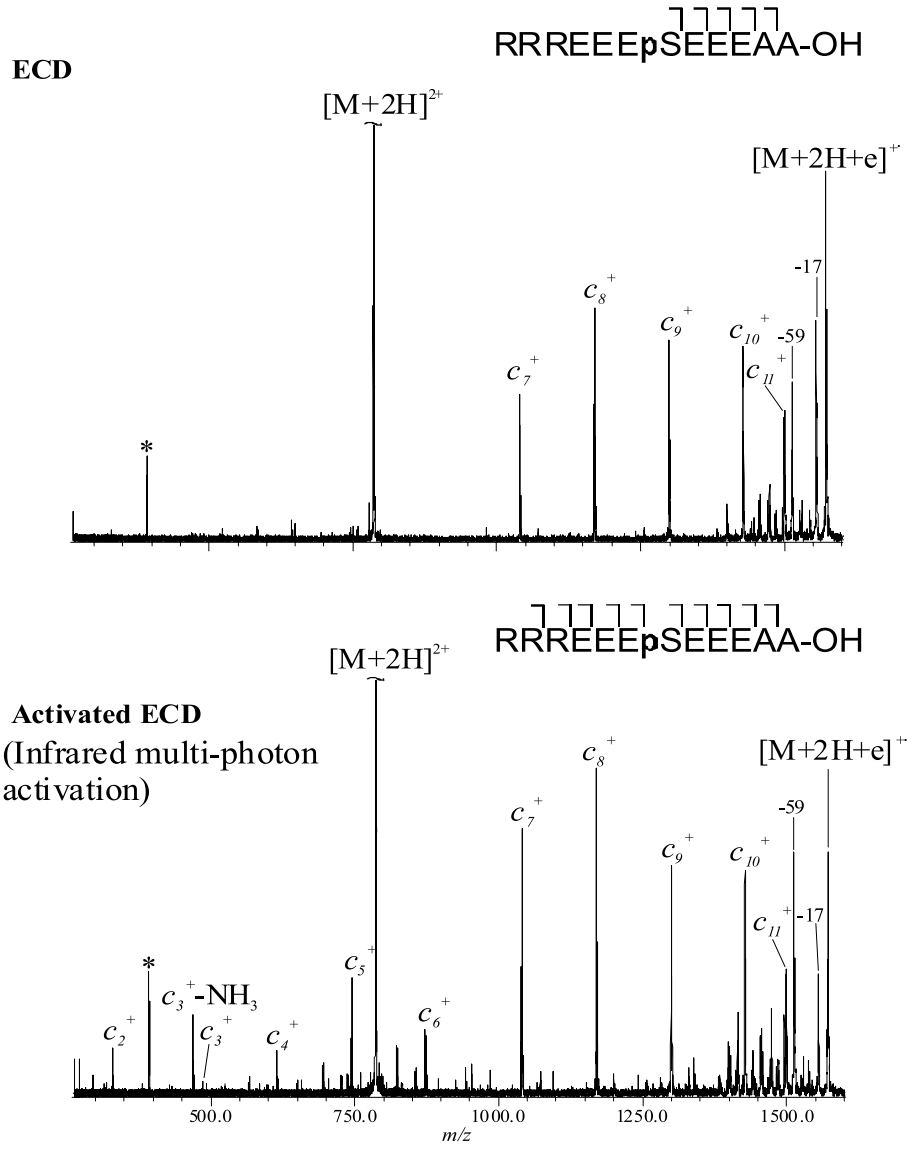

Fig. 2. ECD and activated ECD (see text for more details) spectra of $[M+2H]^{2+}$, a model phosphopeptide (containing a phosphoserine) with the corresponding product ion map. The asterisk at lower *m/z* corresponds to a noise peak.

phase β-elimination [24,25]. On the other hand, the elimination of HPO_3 (80 Da) is observed in the case of phosphotyrosine. The ECD mass spectra obtained from the analysis of intact homogeneous phosphoproteins can be a valuable complementary approach to that of conventional CID. This approach could consist of direct MW determination of the intact phosphoprotein followed by the analysis of its resulting peptides from enzymatic cleavage using liquid chromatography–tandem mass spectrometry. The latter step entails a combination of CID and ECD to yield a complete map of sites of phosphorylation, since in contrast to the CID process, the labile phosphate moiety is retained subsequent to the ECD experiment [26]. McLafferty and co-workers [17] reported the successful mapping of phosphorylated Ser and Thr residues in the 24 kDa heterogeneous β-casein protein with 87 of 208 (42% sequence coverage) ECD cleaved backbone bonds detected. Recently, Marshall and co-workers reported the characterization of phosphorylated bovine protein kinase A (average mass: 40,489 Da). In a single FTMS analysis of about 30 protein kinase A tryptic peptides, three sites of phosphorylation at Ser and Thr residues were rapidly identified [27].

The top panel in Fig. 2 depicts the product ion spectra obtained for a multiply charged ($[M+2H]^{2+}$) phosphopeptide using ECD on an FTMS instrument in these authors' laboratory. In contrast to the CID process, no evidence of β-elimination was observed and the labile phosphate moiety remained intact subsequent to the ECD process. Additional fragmentation was observed (e.g., at lower m/z) by irradiation of protonated peptide ions with an IR laser (infrared multi-photon activation) followed by ECD. The low fluence laser irradiation deposited incremental kinetic energy to the ions yielding a higher degree of sequence coverage during the ECD process. This elegant approach was pioneered by McLafferty and co-workers [28], and has been referred to as the activated-ion electron capture dissociation (AI-ECD).

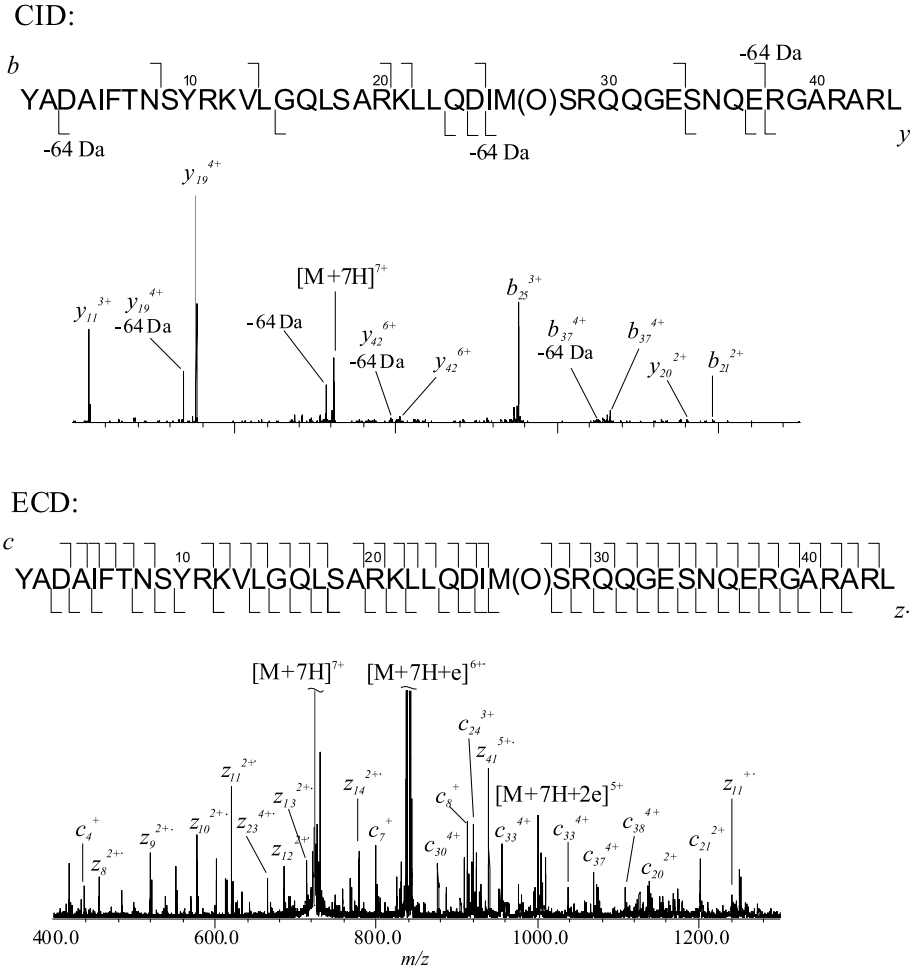

Fig. 3. Product maps indicating the cleavage sites in CID and ECD of the human growth hormone-releasing factor (GHRF) with their corresponding spectra.

Methionine oxidation

Methionine (Met) has a sulfur-containing side chain that is susceptible to oxidation via enzymatic or non-enzymatic pathways. The Met oxidation in proteins plays a prominent role in aging and age-related degenerative diseases. Recently, Guan et al. [29] demonstrated that the combined data from CID and ECD spectra can be used to rapidly detect and localize the Met-oxide (Met(O)) residues in a series of peptides, including the one with an internal disulfide bond.

A distinct fragmentation pathway in CID of Met(O)-containing peptides is the neutral loss of 64 Da from the precursor and/or product ions. The characteristic neutral loss of 64 Da corresponds to the ejection of methanesulfenic acid (CH_3SOH, 64 Da) from the side chain of Met(O). The loss of 64 Da is unique, in proteins and peptides, to Met(O), and is useful in differentiating between Met(O) and phenylalanine (both residues have the same nominal mass of 147 Da) in MS/MS peptide sequencing [30]. However, this low-energy pathway can further inhibit the sequence fragmentation by CID, especially for larger peptides. In fact, the complete MS/MS characterization of Met(O) using CID has so far been limited to small peptides, such as those from enzymatic digestion [30].

CID of the Met-oxidized human growth hormone-releasing factor (GHRF) only yields limited backbone fragmentation (Fig. 3, top-panel), with 12 out of total 43 inter-residue bonds being cleaved. The most abundant product ions (b_{25}^{3+}, b_{37}^{4+}, y_{11}^{3+}, and y_{19}^{4+}) arise from the cleavages at the C-terminal sides of aspartic acid and glutamic acid residues, the favorable cleavage sites in CID. The peaks corresponding to the loss of

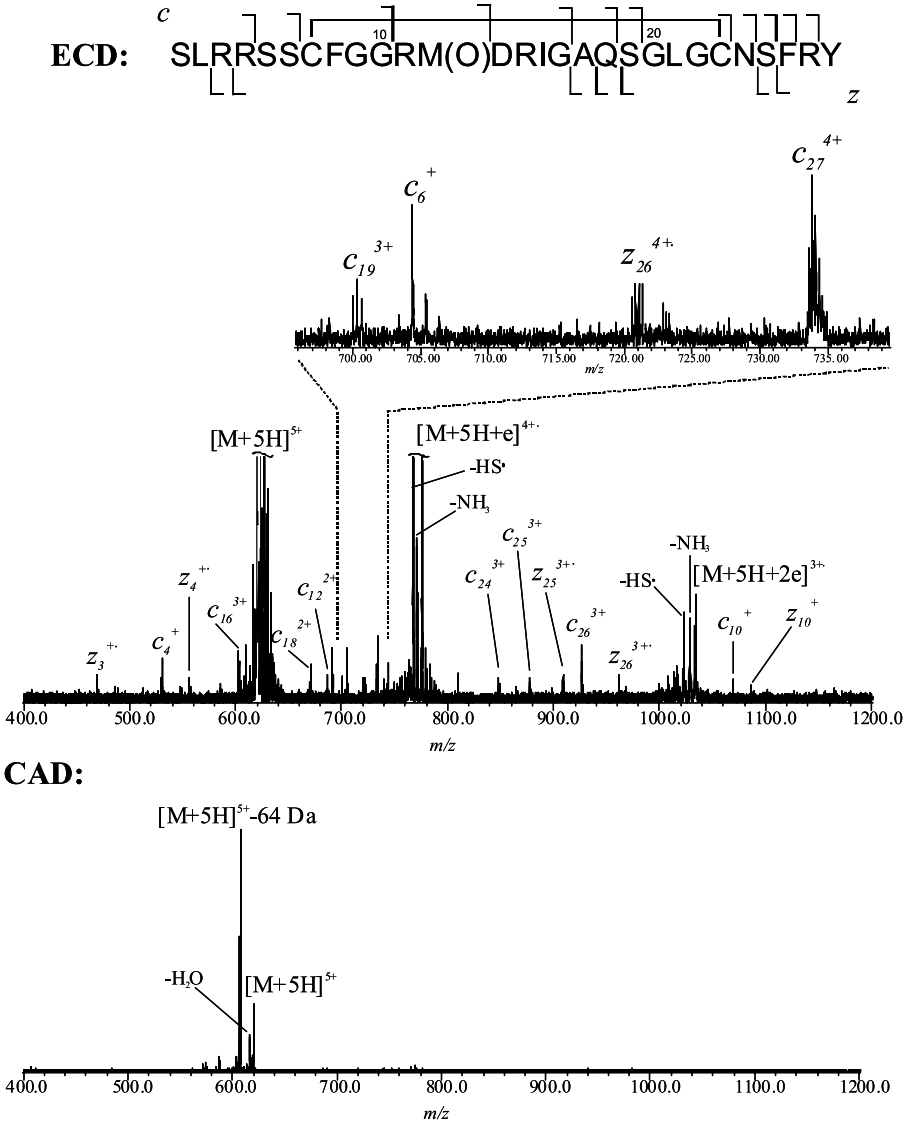

Fig. 4. CID and ECD spectra of $[M+5H]^{5+}$ of ANP(O) with its corresponding product ion map. The isotopic resolution as shown in the zoom view of the ECD spectrum is crucial in assignment of the charge states and identification the product ions.

CH_3SOH (64 Da) from $[M+7H]^{7+}$ and three product ions (y_{19}^{4+}, y_{42}^{6+}, and b_{37}^{4+}) are observed. In contrast, ECD offers far more extensive backbone fragmentation (Fig. 3, bottom-panel), with 41 of the total 43 inter-residue bonds being cleaved. Of the c, $z^{\cdot}$ fragment ions, 32 are complementary pairs whose masses sum up to that of a molecular ion (no loss of 64 Da is observed). The position of Met(O)-27 can be unequivocally determined by the two complementary pairs of ECD product ions ($c_{26}^{3+}/z_{18}^{2+\cdot}$ and $c_{27}^{3+}/z_{17}^{2+\cdot}$). The product maps of CID and ECD are summarized in Fig. 3 [33], which provide compelling evidence in support of the complementary nature of the two dissociation techniques. Furthermore, a combination of ECD and CID offers an "easy-to-interpret" spectra for rapid identification and localization of the Met(O) residues in polypeptides. The characteristic elimination of CH_3SOH (64 Da) in CID serves as a signature tag for the presence of Met(O) in peptides. ECD then offers extensive backbone fragmentation without detaching the labile side chain, to allow for the direct localization of the Met(O) residues.

The human atrial natriuretic peptide (ANP) is a 28 amino acid peptide with a 17 amino acid ring closed by a disulfide bond between Cys-7 and Cys-23. The predominant fragmentation pathway in CID of ANP(O) is the loss of CH_3SOH (64 Da) from the molecular ion $[M+5H]^{5+}$ (Fig. 4) [29]. No appreciable backbone fragmentation or disulfide bond cleavage is observed in the CID spectrum. It is well known that disulfide bonds are generally resistant to cleavage by low-energy CID [31]; thus, reduction of the disulfide bonds with dithiothreitol followed by –SH alkylation is an obligatory step prior to MS/MS analysis. ECD of ANP, however, resulted in 14 backbone cleavages, including six from cleavages within the cyclic structure. The product ions from within the cyclic structure, c_{10}, c_{12}, c_{16}, c_{18}, c_{19}, z_{10}, z_{11}, and z_{12}, arise from ECD cleavage of two bonds: a backbone amine bond and a disulfide bond (Fig. 4) [29]. None of the Met(O)-containing products (c_{12}, c_{16}, c_{18}, and c_{19}) is accompanied by the loss of CH_3SOH (64 Da). The ability to cleave disulfide bonds is a distinctive feature of ECD. In fact, the disulfide bond cleavage is favored over backbone bond cleavage in ECD [21,32,33]. The mechanism for the disulfide bond cleavage by ECD is still under investigation. It has been suggested by McLafferty and co-workers that disulfide bond cleavage is likely due to the high affinity of the -S-S- site for the $H^{\cdot}$ atom (from neutralization of a proton by electron capture), to form a hypervalent intermediate that rapidly leads to the dissociation of the disulfide bond [32,33]. The gas-phase disulfide bond cleavage by ECD holds the promise of direct sequencing of disulfide-containing peptides by MS/MS, thus eliminating the need for time- and mate-

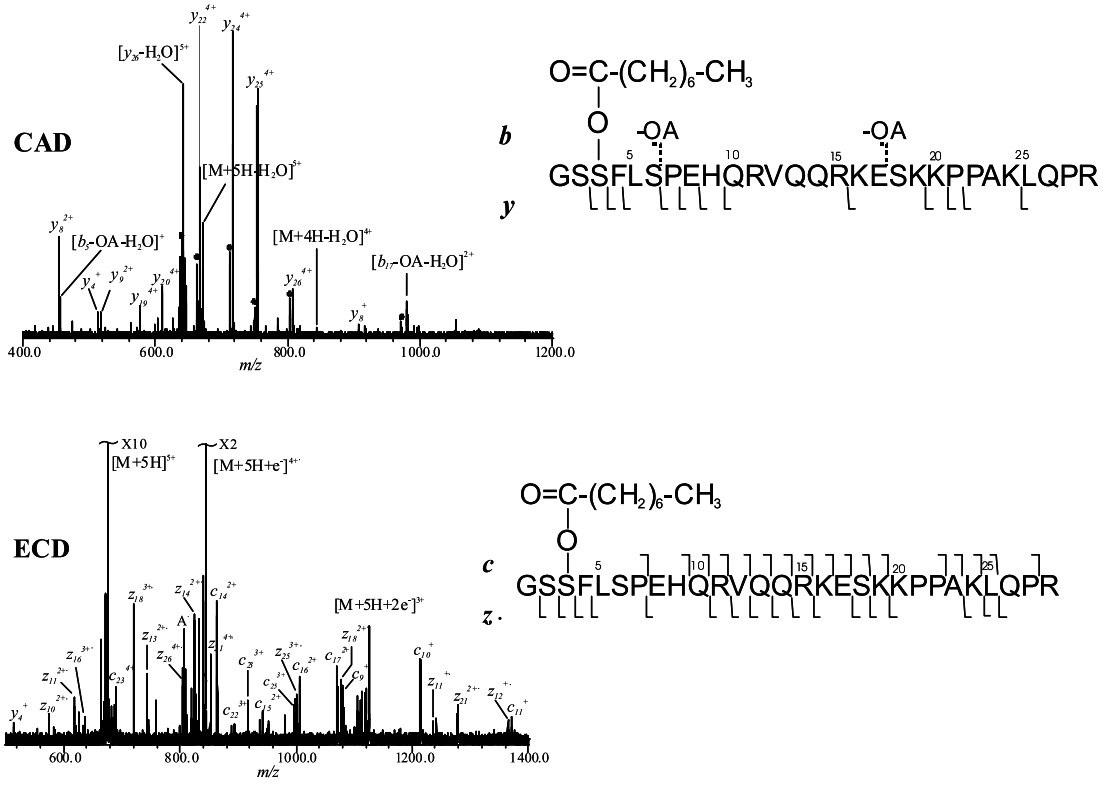

Fig. 5. CID and ECD spectra of $[M+5H]^{5+}$ of ghrelin with its corresponding product ion map. The dots in the top-panel denote signals corresponding to loss of a water molecule. OA is the abbreviation for octanoic acid.

rial-consuming procedures of disulfide reduction and –SH alkylation.

Acylation

Ghrelin is a post-translationally acylated peptide hormone that has an unusual C8:0 acylation (octanoic acid modification) at the Ser-3 residue. The ester-linked C8:0 fatty acyl moiety is essential for the activities of ghrelin, which include growth hormone secretion, feeding regulation, and energy homeostasis.

CID of ghrelin $[M+5H]^{5+}$ yielded two *b*- and 12 *y*-fragments (Fig. 5, top-panel), cleaving 12 inter-residue bonds, far fewer than the 21 inter-residue bonds cleaved by ECD. The comparison of backbone cleavages effected by ECD and CID of ghrelin $[M+5H]^{5+}$ is shown in Fig. 5. The major CID product ions (y_{22}–y_{26} ion series) of ghrelin and desacyl ghrelin are produced from fragmentations near the N-terminal region [34]. In comparison, the backbone fragmentation by ECD is far less selective; variations in ECD product ion abundance are much less significant than those in the CID spectrum. Many of the CID fragments are accompanied by structurally uninformative H_2O losses, which are absent in the ECD spectra. Among the three backbone fragments (b_5, b_{17}, and y_{26}) that contain Ser-3, the ester-linked octanoyl group is retained only on y_{26}, but completely lost from b_5 and b_{17} ions. These neutral losses (H_2O and the C8:0 fatty acid) from the backbone fragments are undesirable and severely complicate MS/MS data interpretation [34].

Fig. 5 (bottom-panel) is the ECD spectrum of $[M+5H]^{5+}$ of ghrelin. The observed 17 *c* and 17 $z^{\bullet}$ ions correspond to the cleavage of 21 out of 23 possible backbone amide bonds. The N-terminal sides of four prolines are not considered because they are not susceptible to cleavage by ECD due to the cyclic structure of proline (i.e., the peptide backbone would still be linked via the proline side chain even if the backbone imide-N to C bond is cleaved). The mass difference of 213 Da between ${z_{25}}^{3+\bullet}$ (m/z 999.5) and ${z_{264}}^{+\bullet}$ (m/z 803.2) corresponds to the mass of a C8:0 acylated serine, which permits the localization of the acylation site at Ser-3. The ester-linked C8:0 fatty acyl group is retained on all backbone fragment ions (*c* and $z^{\bullet}$) that originally contained the acylated Ser-3, with each of these fragments having a mass shift of 126 Da from the corresponding fragment produced from des-octanoyl ghrelin (data not shown) [34]. The retention of the ester-linked C8:0 acyl group on the backbone fragments during the ECD process supports the non-ergodic mechanism [13], and is in agreement with previous ECD results on other labile post-translational modifications. The ECD spectra of ghrelin and des-octanoyl ghrelin are very similar with respect to the backbone fragmentation, perhaps implying that there is little interaction between the peptide backbone and the hydrophobic C8:0 acyl chain. The current results demonstrate the applicability of ECD for characterizing an acylated peptide. ECD/FTMS provides sufficient structural information for identifying the chemical nature and determining the site of the fatty acid modification in ghrelin. ECD offers more extensive sequence fragmentation than CAD and preserves the C8:0 fatty acyl group on the backbone sequence fragment ions (*c* and $z^{\bullet}$ ions), allowing for direct localization of the acylation site.

Conclusions

Electron capture dissociation mass spectrometry is rapidly becoming a popular and complementary technique to the conventional CID process. The detection and localization of PTMs using a combination of high resolution mass spectrometry (e.g., FTMS) and ECD have ameliorated a number of difficulties associated with the conventional CID [35]. Additional applications of ECD mass spectrometry could involve automated de novo sequencing of proteins in a "top-down" approach [36] as well as characterization of protein-drug covalent adducts [37].

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