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Electron-capture dissociation tandem mass spectrometry

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Electron capture dissociation (ECD) is a new fragmentation technique used in Fourier transform ion cyclotron resonance mass spectrometry and is complementary to traditional tandem mass spectrometry techniques. Disulfide bonds, normally stable to vibrational excitation, are preferentially cleaved in ECD. Fragmentation is fast and specific and labile post-translational modifications and non-covalent bonds often remain intact after backbone bond dissociation. ECD provides more extensive sequence coverage in polypeptides, and at higher electron energies even isoleucine and leucine are distinguishable. In biotechnology, the main area of ECD application is expected to be the top-down verification of DNA-predicted protein sequences, *de novo* sequencing, disulfide bond analysis and the combined top-down/bottom-up analysis of post-translational modifications.

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Abbreviations

ECD	electron capture dissociation
FTICR MS	Fourier transform ion cyclotron resonance mass spectrometry
HECD	hot ECD
MS/MS	tandem mass spectrometry
PTM	post-translational modification
REMMA	reconstructed molecular mass analysis

Introduction

Proteomics is a large scale and complex problem, as it includes the quantification of proteins (e.g. expression levels), the analysis of co-translational and post-translational modifications, and the study of protein–molecule interactions, as well as other aspects not found in genomics. As a result, there is an urgent need in proteomics for a sensitive high-throughput tool for protein characterization. Tandem mass spectrometry (MS/MS) can be such a tool, provided it is based on an efficient fragmentation technique.

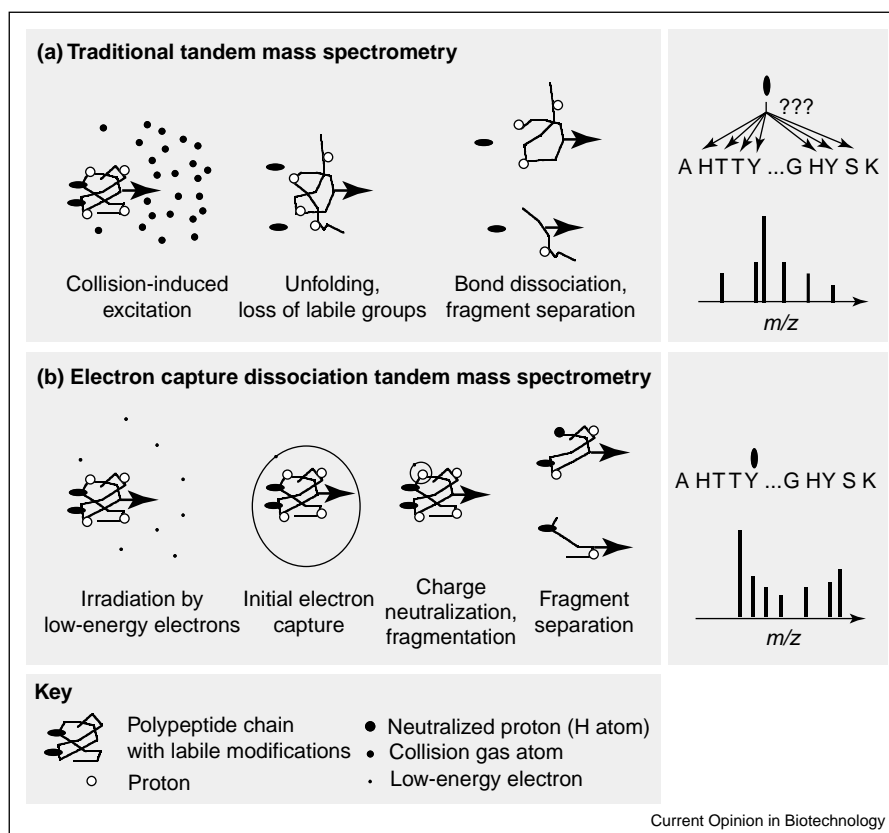
Traditional MS/MS employs vibrational (collision- or infrared-induced) excitation of gas-phase molecular ions,

and is known to provide information on both protein sequence and post-translational modifications (PTMs). Yet, the sequence information obtained is rarely so complete that *de novo* sequencing of a whole protein is possible. Furthermore, many PTMs are labile in the gas phase and the information on their position is lost during excitation. The alternative fragmentation technique of electron-capture dissociation (ECD) [1] is based on the dissociative recombination of multiply protonated polypeptide molecules with low-energy electrons.

The mechanism of ECD is, as yet, poorly understood. In general terms, polypeptide polycations initially capture an electron in a high orbit, which is followed by charge neutralization, leading to an excited radical species that rapidly (within 10^{-11} s [1]) undergoes bond cleavage. The presence of a radical makes this fragmentation very bond-specific. Disulfide bonds and some other bonds (e.g. to halogen atoms) dissociate with the highest rates [2]. Backbone fragmentation of larger polypeptides is abundant, rather homogeneous and dominated by N–C $_{\alpha}$ bond cleavage, preferentially giving N-terminal l' fragments (the prime sign indicated H \bullet transfer to the fragment before the bond cleavage) and C-terminal $z\bullet$ fragments. This is in contrast to the more sequence-specific, vibration-induced CO–N backbone fragmentation that produces b and y' ions. Secondary losses from termini and sidechains occur, but usually with much lower abundance than the small neutral losses (H $_2$ O, NH $_3$, etc.) typically observed in traditional MS/MS. Intriguingly, ECD fragments have been found to retain labile PTM groups, such as in sulfation and γ -carboxylation (Figure 1) [3]. This early study encouraged analyses of other PTMs, including O-glycosylation [4], phosphorylation [5,6] and N-glycosylation [7], amongst others [8–11]. In all cases, the retention of labile groups in ECD was far superior than in traditional MS/MS.

Although ECD has been successfully applied to cyclic peptides, linear synthetic polymers, oligonucleotides, oligosaccharides, peptide nucleic acids and hyper-branched polymers, the most promising area of application remains that of linear polypeptides. The applicability of ECD in protein identification by limited fragmentation has been demonstrated [12 \bullet], but the inevitable loss of signal owing to partial charge reduction reduces the attractiveness of the technique for this task. Instead, ECD is advantageous in top-down sequence characterization, *de novo* sequencing, S–S bond analysis and PTM mapping. In most cases, ECD-derived information is complementary to that obtained using traditional MS/MS, so that their combined use is beneficial. For instance,

Figure 1



Comparison of (a) the traditional (collision-based) MS/MS and (b) ECD-based MS/MS techniques in the analysis of modified polypeptides. Whereas collisional excitation leads to polypeptide chain unfolding and losses of labile groups, ECD largely preserves the secondary structure and the labile groups (left panels). This facilitates the assignment of the sites of labile groups (right panels).

in traditional MS/MS the peptide bond N-terminal to proline residues is preferentially cleaved, whereas in ECD it is the only site that does not produce c' , z^* fragments.

Tandem mass spectrometry with electron capture dissociation

Instrumentation

So far, ECD has only been used in Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS). The reason for this is twofold. Firstly, although fragmentation proceeds at a very high rate (can be non-ergodic [1]) when the electron approaches the charge at a close distance, to ensure electron capture by most precursor ions usually requires at least several milliseconds [13]. This exceeds the residence time of ions in many types of mass spectrometers, including time-of-flight and quadrupole instruments. Secondly, ECD efficiency is highest for electron energies <1 eV [14], which are difficult to provide in, for example, quadrupole ion traps. Nevertheless, efforts are currently underway to implement ECD in these MS analysers.

Most commercial manufacturers of FTICR MS instruments provide an ECD option for both older models as well as for the newest hybrid instruments. The magnet strength does not seem to be an essential parameter for ECD: good spectra have been obtained with a small 3 tesla magnet [15]. The currently used electron source is based on an indirectly heated dispenser cathode [11,13]. The superiority of this source over a heated filament not only results from the larger electron current, but also from the much larger emitting surface area. This feature provides better overlap with the ion cloud as well as trapping of the precursor ions and fragments by the space charge of the electrons [13]. The cathode can be placed off-axis [14] or feature a central hole [16] to allow for an on-axis infrared laser beam for the combined use of ECD with vibrational excitation.

ECD efficiency and sensitivity

Because of the concomitant charge reduction, the sensitivity of ECD-based MS/MS is lower than in traditional MS/MS. Furthermore, not all precursor ions should be allowed to capture electrons to avoid excessive neutrali-

zation of the fragments [17]. The overall ECD efficiency for peptides is 20 to 50%, but can be higher for proteins. ECD using femtomole amounts of peptides has been reported [18].

Applications of tandem mass spectrometry with electron capture dissociation

De novo sequencing

For sequenced genomes, the tasks of protein identification and DNA sequence verification require only limited protein sequencing. This can be done with ECD alone [19] or, more conveniently, in combination with traditional MS/MS [20^{••}]. In proteins and some peptides, non-covalent bonding in secondary structures reduces the number of observed ECD cleavages. Secondary structure can be destabilized by applying limited vibrational excitation, either by collisions ('activated-ion ECD' [19]), infrared irradiation or collisions with <15 eV electrons ('plasma ECD' [21]).

The *de novo* sequencing of proteins is mostly reserved for unknown genomes. The task not only requires cleavage within each pair of residues, but the isomeric leucine and isoleucine residues also need to be distinguished. Increasing the electron energy above 3 eV (hot ECD, HECD) promotes secondary sidechain losses from the radical z^{\bullet} ions (and more rare a^{\bullet} ions) to produce even-electron w (d) [22[•]] and low-abundance u [23] ions. The losses are $\bullet\text{CH}(\text{CH}_3)_2$ (43 Da) from leucine and $\bullet\text{CH}_2\text{CH}_3$ (29 Da) from isoleucine. Complete sequencing and determination of 23 out of 25 Xle residues in peptides comprising a 15 kDa bovine milk protein PP3 has been achieved by HECD [22[•]]. The two remaining leucine/isoleucine residues were at the N termini and could be identified by other techniques.

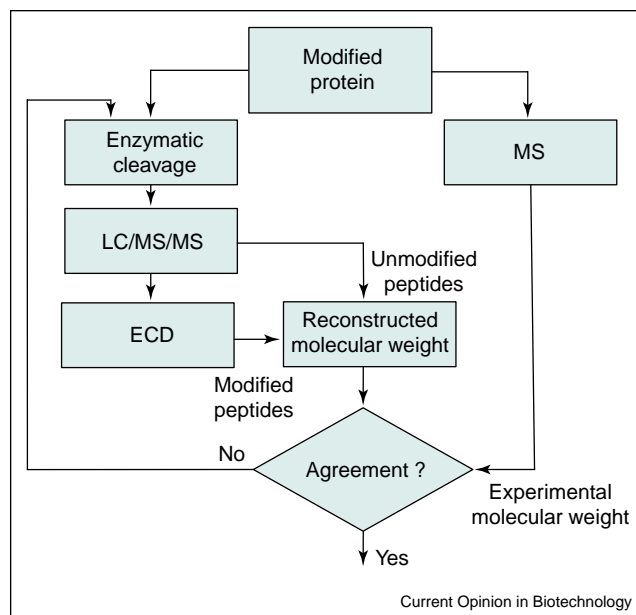
Disulfide bond analysis

The vibrational activation of S–S bound peptides dimers does not usually rupture disulfide bonds but leads instead to backbone cleavages in respective polypeptide chains, which may complicate the analysis. At the same time, the capture of an electron preferentially cleaves one disulfide bond [2]. This feature can be used for the determination of S–S bond order or for the analysis of synthetic disulfide-bound peptide nanostructures [24]. Vibrational excitation before or after electron capture can result in the rupture of another S–S bond (e.g. in separating the A and B chains in bovine insulin [2]). Through radical-site initiated secondary reactions, ECD also cleaves the backbone inside internal loops produced by intramolecular disulfide bonds [2].

The determination of post-translational modifications

Plasma ECD of intact bovine β -casein could identify the positions of all five phosphorylation sites [21]. The same could be achieved more routinely by enzymatic cleavage and immobilized metal affinity chromatography (IMAC)

Figure 2



A simplified workflow diagram of the combined top-down/bottom-up approach [25^{••}] utilizing both traditional MS/MS in combination with liquid chromatography (LC/MS/MS) as well as ECD for the analysis of PTMs in proteins. This procedure can provide the basis for a fully automated, high-throughput technique for PTM mapping.

isolation of phosphopeptides [5]. In human osteocalcin, the positions of both γ -carboxylated residues have been identified using ECD MS/MS despite the presence of an internal S–S bond [10].

Top-down characterization using ECD with ion activation [20^{••}] is the approach of choice for the analysis of PTMs in relatively robust and homogeneous proteins. If plasma ECD or activated-ion ECD proves too harsh for labile PTM groups, a combined top-down/bottom-up approach is favoured. Figure 2 shows a simplified workflow diagram of a variant of this approach termed REconstructed Molecular Mass Analysis (REMMA [25^{••}]). The molecular weight of the intact protein is obtained by MS and the protein is digested by one or more enzymes. The products are then chromatographically separated and analysed by MS. MS/MS of unmodified peptides reveals the protein identity, as in the standard proteomics approach. The appearance of a 'strange' peptide mass triggers ECD, which either identifies the peptide as contamination or miscleavage, or identifies the PTM position and, in many cases, the structure. When full sequence coverage is obtained (a feature not found in other strategies), the combined mass of all fragments can be compared to the measured molecular weight. An agreement between the two values means that the PTM mapping is complete.

REMMA has been tested in the off-line, manual interpretation regime on the PP3 protein, which is naturally modified to a molecular weight >19 000 (i.e. >25% by weight). All known modifications (multiple phosphorylations, N- and O-glycosylations) were found and a previously unreported O-glycosylation at Ser60 was discovered. The challenge now is to make the data analysis, decision-making and instrument-tuning processes fully automated. Achieving this should reduce the time of analysis of one protein form to less than 24 h (i.e. 10–100 times faster than traditional approaches). These advances should make the previously unattainable task of mapping all forms of human proteins a realistic goal.

Potential for analysis of weakly bound gas-phase structures

Several studies have shown that weak, non-covalent bonds can survive ECD [26*,27]. Besides the determination of thermodynamic parameters of various gas-phase protein conformers [28], this feature is expected to be useful in revealing gas-phase structures of protein complexes. One should, however, bear in mind that protonation and proton solvation can cause strong side effects. Charge solvation can alter the secondary structure, while coulombic repulsion between the charges can destabilize it. If the solution-phase structure can be transferred to the gas phase, it is likely to be preserved for relatively low charge states, in which the charges are usually localized in well-defined sites. This can reduce the number of backbone cleavages produced by ECD.

Several groups have used ECD in analyses of H/D exchange in backbone amides. Although both theoretical expectations and early results indicated greatly reduced H/D scrambling compared with vibrational excitation, the ECD results obtained so far have been inconclusive. This means that, besides straightforward ECD applications, attention should be paid to less direct approaches (e.g. vibrational excitation of the reduced molecular species).

Analysis of negative ions

Although there are as many acidic proteins in nature as there are basic ones, the vast majority of mass spectrometric investigations on polypeptides are performed in the positive ion mode. This can be explained by the fact that the fragmentation of polypeptide cations is usually easier to rationalize. But, positive mass spectra alone rarely yield 100% protein sequence coverage. Moreover, such important PTMs as phosphorylation, sulfation and γ -carboxylation are more stable in anions than in cations.

Direct ECD of anions has not been achieved, probably because of the coulombic repulsion between the electron and the negative charge. However, a fragmentation process analogous to ECD but applicable to anions has been found. Electron detachment dissociation (EDD [29]) uses the same instrumentation as ECD, but employs electrons

accelerated to >11 eV. EDD gives rise to a variety of backbone fragments, including a , c , z and x ions that are capable of retaining, for example, sulfation and phosphorylation groups [29].

Conclusions

The role of ECD, and other ion-electron reactions, in tandem mass spectrometry is expected to increase rapidly in the near future, particularly when novel hybrid FTICR instruments will make the technique more robust and easily available. With improvements in ion transmission and isolation, the reduced sensitivity of ECD will no longer be an obstacle. Rapid PTM mapping is expected to be the main application in protein research [30*], with *de novo* sequencing, disulfide bond analysis and DNA-predicted sequence verification also being important areas.

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 The REMMA procedure applied to the bovine milk PP3 protein containing 25% modifications by weight yielded all known modifications (five phosphorylations, two O-glycosylations and one N-glycosylation), as well as the previously unreported NeuNAc-Hex-[NeuNAc]HexNAc group O-linked to Ser60. It is argued that the approach can serve as a basis for high-throughput, high-sensitivity PTM characterization of biological important proteins.
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This recent review discusses the importance of high-sensitivity PTM analysis for providing insight into biological function. The potential of novel mass spectrometric peptide sequencing approaches, including ECD, for mapping modification sites is discussed. It is predicted that ECD in combination with novel hybrid FTICR instruments will provide characterization of labile modifications with a sensitivity and speed approaching standard mass-spectrometric peptide sequencing.