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Lermyte, F, Valkenborg, D, Loo, JA et al. (1 more author) (2018) Radical solutions: Principles and application of electron-based dissociation in mass spectrometry-based analysis of protein structure. Mass Spectrometry Reviews, 37 (6). pp. 750-771. ISSN 0277-7037

https://doi.org/10.1002/mas.21560

Radical solutions: Principles and application of electron-based dissociation in mass spectrometry-based analysis of protein structure

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Short title: Electron-based dissociation in protein structure analysis

Keywords: electron transfer dissociation, electron capture dissociation, top-down fragmentation, noncovalent complex, protein folding

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Abstract

In recent years, electron capture (ECD) and electron transfer dissociation (ETD) have emerged as two of the most useful methods in mass spectrometry-based protein analysis, evidenced by a considerable and growing body of literature. In large part, the interest in these methods is due to their ability to induce backbone fragmentation with very little disruption of noncovalent interactions which allows inference of information regarding higher-order structure from the observed fragmentation behavior. Here, we review the evolution of electron-based dissociation methods, and pay particular attention to their application in 'native' mass spectrometry, their mechanism, determinants of fragmentation behavior, and recent developments in available instrumentation. Although we focus on the two most widely used methods – ECD and ETD – we also discuss the use of other ion/electron, ion/ion, and ion/neutral fragmentation methods, useful for interrogation of a range of classes of biomolecules in positive- and negative-ion mode, and speculate about how this exciting field might evolve in the coming years.

I. Introduction

I.A. Origins of tandem MS and collision-induced dissociation

Determination of the mass of a gas-phase ion has been possible since the initial cathode-ray experiments by Francis Aston and J.J. Thomson at the beginning of the 20th century (Thomson, 1913). However, it took considerably longer to appreciate not only that radical molecular ions – at the time mostly generated by electron ionization (EI) – often undergo rapid, unimolecular fragmentation in the gas phase, but that measurement of fragment masses could provide information on dissociation pathways, and as a result, ion structure (Mclafferty, 1959). It is interesting to observe that, fifty years later, there is a renewed interest in the dissociation pathways of metastable radical cations (Turecek& Julian, 2013), although the focus has since shifted to large biomolecules, which are generally not amenable to ionization *via* EI, but can be transferred into the gas phase with electrospray ionization (ESI) (Fenn et al., 1989).

A next critical step in the use of mass spectrometry for structure determination was the deliberate activation of gas-phase ions through collisions with an inert background gas, which leads to collision-induced dissociation or CID. One major advantage of this workflow is that fragmentation of an otherwise stable even-electron ion is possible, so that this method is compatible with a wide range of ionization techniques, including ESI. Performing the ion activation after *m/z* selection of the precursor to be fragmented gave rise to the now ubiquitous technique known as tandem MS (Burinsky et al., 1982; Jennings, 1968; Louris et al., 1987; Louris et al., 1985). It is easy to show that the maximum amount of kinetic energy that can be converted to internal vibrational energy per collision is strongly dependent on the relative masses of both collision partners so that, particularly for high-mass ions, many collisions are required to increase internal energy to the point where dissociation occurs (Brodbelt, 2016; Sleno& Volmer, 2004). It is generally assumed that there is sufficient time for the energy transferred to the analyte ion to redistribute among the various vibrational degrees of freedom, such that the process is thermodynamically, rather than kinetically, controlled (although a very few exceptions have been described (Carpenter, 2005; Turecek& McLafferty, 1984)).

This thermodynamic control implies that the weakest (non)covalent interactions will be destroyed preferentially at each point, and specifically for peptide and protein analysis, that higher-order structure (inasmuch as it is not stabilized by covalent disulfide bonds) will be disrupted first, followed by loss of labile post-translational modifications (PTMs), and only then will backbone dissociation – from which sequence information can be obtained – occur efficiently. If an extreme collision energy – typically several keV – is used, a greater variety of fragments is often observed, largely due to secondary fragmentation (Claeys et al., 1996; Medzihradszky& Burlingame, 1994). For peptides specifically, the increased energy leads to the observation of side-chain based *d*, *v*, and *w* fragments as well as loss of small neutral molecules (*e.g.* H_2O, NH_3), in addition to the initial (primary) *a*, *b*, and *y* fragments (Medzihradszky& Burlingame, 1994; Pittenauer& Allmaier, 2009). Historically, these high activation energies were often achieved with the use of tandem sector instruments, but they have been available for dissociation of singly charged ions on MALDI-TOF/TOF platforms for some time (Pittenauer& Allmaier, 2009). For multiply charged ions (such as those generated by ESI), similar laboratory-frame energies can be achieved with the use of a lower voltage difference of around 100 V, for instance on quadrupole/time-of-flight instruments. A similar mechanism underlies

dissociation *via* other so-called 'slow heating' methods, such as sustained off-resonance irradiation collision-induced dissociation (SORI-CID), infrared multiphoton dissociation (IRMPD), and blackbody infrared radiative dissociation (BIRD) (McLuckey& Goeringer, 1997). This thermodynamic control, therefore, limits the use of these techniques for the direct interrogation of higher-order structure and/or heavily post-translationally modified proteins. This restriction is particularly challenging in a top-down workflow, in which intact proteins are ionized and fragmented (compared to a bottom-up approach, which relies on enzymatic digestion prior to LC-MS^n analysis).

I.B. New fragmentation methods: UVPD, SID, and electron-based dissociation

For top-down analysis, native MS (Leney& Heck, 2017), and investigation of PTMs, there was thus a need for orthogonal dissociation methods that are selective for the backbone, without first destroying higher-order structure and/or energetically labile PTMs. An overview of commonly used dissociation techniques is shown in Table 1. One dissociation technique that shows backbone selectivity is ultraviolet photodissociation (UVPD), which can deposit the required energy with a single photon, and has been used for selective protein backbone dissociation in noncovalent assemblies (this results in retention of weakly bound ligands as well as structure-dependent preference for certain cleavage sites (Cammarata et al., 2015; Cammarata& Brodbelt, 2015; Cammarata et al., 2016; Morrison& Brodbelt, 2016a; Tamara et al., 2016)) as well as heavily post-translationally modified proteins. This technique will not be discussed in further detail here, but an excellent review was recently published by Brodbelt (Brodbelt, 2014).

Another dissociation method on a timescale that allows only limited energetic redistribution is surface-induced dissociation (SID) (Cooks et al., 1990). In this approach, rather than with background gas molecules, the precursor ions collide with a specially treated solid surface. Because the mass of this surface is many orders of magnitude greater than that of the ion, all of the kinetic energy is typically assumed to be converted into internal energy in a single collision, on a picosecond timescale. Research is ongoing, but this technique shows great promise for the structural analysis of protein complexes, in particular their subunit connectivity (Christen et al., 1998; Jones et al., 2006; Konijnenberg& Sobott, 2015; Meroueh& Hase, 2002; Wysocki et al., 2008; Yan et al., 2017; Zhou& Wysocki, 2014). Significantly more attention has gone to the development of electron-based dissociation methods though, and as such, the application of these techniques to protein structure analysis is better understood and will be the focus of the rest of this discussion.

In 1998, Zubarev, Kelleher, and McLafferty discovered that the 193 nm laser used in their UVPD experiments could also be employed to release low-energy photoelectrons from a metal surface, and that capture of said electrons by an even-electron protein ion formed by ESI resulted in the formation of radical charge-reduction products, as well as selective cleavage of the N-C(α) bond (with a minor secondary pathway that resulted in cleavage of the CO-C(α) bond) (Zubarev et al., 1998). It is trivial to see that only 19 of the 20 common amino acid residues are susceptible to this type of dissociation, because cleavage of the N-C(α) bond in proline results in two 'fragments' that remain bound by the pyrrolidine side chain. The selectivity for the N-C(α) bond observed in this process, known as electron capture dissociation (ECD), is remarkable, and there is still an ongoing debate about the precise reaction mechanism (Turecek& Julian, 2013; Zhurov et al., 2013). The most-often cited mechanisms are shown in Figure 1, and will be discussed in the next section.

II. Mechanism(s) of electron-based dissociation methods

II.A. Electron-capture dissociation (ECD)

II.A.1. Cornell mechanism

The original mechanism proposed by McLafferty and colleagues (Zubarev, et al., 1998) involves electron capture at a protonated backbone amide group. The resulting aminoketyl radical was proposed to dissociate via homolytic cleavage of the N-C(α) bond located on the C-terminal side of the radical. The N-terminal fragment has an enolimine functionality, which rapidly tautomerizes to a significantly more stable amide. It was already acknowledged in this first publication that a protonated backbone amide is fairly unlikely to occur, given that these are typically considered rather unfavorable protonation sites. This mechanism was refined (Breuker et al., 2004) to what is now commonly referred to as the 'Cornell' mechanism. In this mechanism, rather than a protonated amide, the reaction starts with a protonated amine (typically a lysine side chain), which is solvated by an intramolecular hydrogen bond to an amide carbonyl group. It is this charged group where electron capture is assumed to occur, which results in a hypervalent ammonium radical, hydrogen bonded to an amide carbonyl oxygen. From here, migration of a hydrogen radical to the carbonyl group - most likely through proton-coupled electron transfer (Turecek& Syrstad, 2003) - leads to the formation of the aminoketyl radical, and dissociation progresses as originally suggested (Zubarev, et al., 1998). Because it has been shown that the side chains of other residues beside lysine often act as protonation sites in the gas phase (Morrison& Brodbelt, 2016b; Schnier et al., 1995), it is conceivable that these interact with the backbone amide and provide the hydrogen radical. As a result, this group is labeled as the generic 'XH^{+}' in Figure 1. One advantage of the Cornell mechanism is its ability to explain the aforementioned minor pathway in which the backbone CO-C(α) bond is broken. If the radical ends up on a backbone nitrogen (which is unlikely for energetic reasons), then homolytic cleavage of the adjacent CO-C(α) bond located on the N-terminal side of this nitrogen atom is plausible, and leads to formation of an $a^{\bullet}/x$ fragment pair, the latter of which is assumed to undergo loss of (neutral) carbon monoxide, so that $a^{\bullet}/y$ fragments are observed in addition to the more abundant $c/z^{\bullet}$ fragments (Breuker, et al., 2004). In the Cornell mechanism, it is also assumed, given the significant (~6 eV) energy released as the electron is initially captured in a high-*n* Rydberg state, that dissociation occurs before this energy can be redistributed among the ion's vibrational degrees of freedom (similar to UVPD or SID, described previously). This non-ergodic mechanism is still often cited (Mentinova et al., 2013; Xu et al., 2011); however, several alternatives have been proposed over the years.

II.A.2. Utah-Washington mechanism

The main alternative to the Cornell mechanism is referred to as the 'Utah-Washington' mechanism (Chen& Turecek, 2006; Sawicka et al., 2003; Syrstad& Turecek, 2005; Turecek, 2003; Turecek et al., 2008), as two highly similar mechanisms were simultaneously proposed *circa* 2003 by the groups of Jack Simons (University of Utah) and Frantisek Tureček (University of Washington). In the Utah-Washington mechanism(s), as in the Cornell mechanism, the reaction starts with a carbonyl

group involved in an intramolecular hydrogen bond to a positive charge site. Unlike in the Cornell mechanism though, the main function of hydrogen bonding in this case is to lower the energy of the amide π^* (LUMO) orbital *via* Coulomb stabilization. It is in this orbital that electron capture occurs, to form a highly basic amide anion. At this point, a subtle difference between both mechanisms exists: In the 'Washington' variant, the amide anion at this point is neutralized by intramolecular proton transfer – usually assumed to come from a distant charged side chain – to lead to formation and dissociation of an aminoketyl radical, as described by the Cornell mechanism. In the 'Utah' variant, however, homolytic cleavage of the N-C(α) bond located on the C-terminal side of the amide occurs immediately, to result in an N-terminal enolimidate, which is neutralized by proton transfer, to lead immediately to the conventional amide structure for the *c* fragment, without the requirement for tautomerization.

Essentially, the main differences between the most commonly invoked ECD mechanisms are therefore: (1) the Cornell mechanism postulates electron capture at a positive charge site, which results in the formation of a hypervalent species and attack of a hydrogen atom on an adjacent backbone C=O bond, whereas the Utah-Washington mechanism assumes electron capture at an amide π^* orbital, which results in a zwitterion; (2) in a later step, the Utah mechanism assumes that backbone cleavage precedes proton transfer, while this order is reversed in the Washington mechanism. Evidence for the Utah-Washington mechanism is found in the ECD behavior of peptides where the only available charge carriers are arginine residues (Chen& Turecek, 2006). According to quantum chemical calculations, after capture of an electron by a charged arginine side chain (the first step in the Cornell mechanism), loss of the guanidinium group is favored over hydrogen radical migration. Because these peptides are experimentally found to dissociate via backbone N-C(α) cleavage, it would seem more likely that electrons in this case are captured elsewhere. However, it has also been found (Chamot-Rooke et al., 2007; Li et al., 2008) that, if peptides are modified so that all charge sites are fixed and no mobile protons are present, then backbone cleavage is significantly inhibited or even eliminated, and this behavior would at least seem at odds with the Utah mechanism.

II.A.3. Other proposed mechanisms

Another variant, commonly referred to as the 'nonlocal' mechanism, was proposed in 2006 by Zubarev and colleagues (Patriksson et al., 2006). In this mechanism, an NH– – –OC hydrogen bond is again required (with initial electron capture occurring at the nitrogen atom); however, the presence of a positive charge is not essential. Although this mechanism matches the periodic fragmentation behavior observed in α-helices in a number of studies (*vide infra*) (Ben Hamidane et al., 2009b; Breuker et al., 2002), Crizer and McLuckey have shown that methylation of the backbone amide nitrogens has little effect on electron transfer dissociation (ETD, *cf. infra*) of peptides, which casts doubt on the idea that hydrogen bonding that involves this nitrogen is needed for electron-based dissociation to occur (Crizer& McLuckey, 2009).

Mechanisms that assume cleavage on the N-terminal side of the amide group that the unpaired electron interacts with have also been proposed, initially in 2007 by Zubarev and colleagues (Savitski et al., 2007) and then in 2010 by Tureček and colleagues (Turecek et al., 2010). Some experimental evidence for this N-terminal cleavage was reported in 2009 by Tsybin and

colleagues (Ben Hamidane et al., 2009a); however, for small peptides at least, experimental (Ben Hamidane et al., 2010; Sargaeva et al., 2011) and computational (Turecek, 2003) research raised doubts about the feasibility of this mechanism. On the other hand, in several recent studies, Tsybin and colleagues used combined computational and experimental methods to argue that this 'enol' mechanism is in many cases thermodynamically and kinetically favored (Wodrich et al., 2014; Wodrich et al., 2012; Zhurov et al., 2014). Because the peptide conformation is important to determine reaction kinetics, it is possible that peptide size and amino acid composition – in particular the type(s) of residue that carry charge (Chen& Turecek, 2006; Xia et al., 2007) – play a role to determine which mechanism dominates, and it is clear that further research is required in this very active field of research.

In several studies, O'Connor and colleagues emphasize the radical nature of the $z^{\bullet}$ fragment formed by the initial N-C(α) cleavage in ECD, and argue this radical can, even with a low electron energy, react further in a 'cascade' of backbone cleavages, side chain losses, and/or loss of small neutrals such as $H^{\bullet}$, H_2O,… (Leymarie et al., 2003). Strong evidence for the occurrence of multiple bond cleavages is provided by the observation of fragments in ECD of cyclic peptides, such as gramicidin S and cyclosporin A (Leymarie, et al., 2003). With deuteration and resonant ejection of charge-reduced species, O'Connor and colleagues also showed significant hydrogen migration (to form '*c-1*' and '*z+1*' fragments, as will be discussed later) within intact, charge-reduced precursor ions (Lin et al., 2006; O'Connor et al., 2006b). This approach does not differentiate between hydrogen migration that occurs intramolecularly during a free-radical cascade or intermolecularly within a noncovalently bound *c*/*z* fragment complex though. Introduction of spin-trapping and fixed-charge modifications within peptide structures resulted in a significant reduction (in some cases complete elimination) of backbone cleavage, and was found to promote the loss of side chains (Belyayev et al., 2006; Li, et al., 2008). Although these observations are in agreement with the free-radical cascade mechanism for ECD, other commonly proposed mechanisms also require migration of hydrogen radicals or protons from acidic side chains, and could, therefore, also be expected to be inhibited by these modifications. Therefore, although strong evidence exists that these cascades do occur at least to some extent during low-energy ECD, it is at present unclear how common they are.

II.A.4. (Non-)ergodicity of ECD

Another important point of contention is the alleged non-ergodicity of ECD fragmentation; *i.e.,* the claim that bond cleavage occurs on a timescale that does not allow for energy redistribution over the ion's internal degrees of freedom (Jones et al., 2007; Laskin et al., 2007; Turecek, 2003; Zubarev, et al., 1998). Although this hypothesis was originally believed to be the only way to explain why the N-C(α), rather than the thermodynamically more labile (in the neutral, closed-shell peptide) amide bond, is cleaved (Breuker, et al., 2004; Zubarev, et al., 1998), quantum mechanical calculations have shown that the N-C(α) bond in the aminoketyl (or enolimidate) radical is actually thermodynamically very labile, and the energetic barrier for cleavage extremely low, such that dissociation occurs rapidly in thermalized ions and the non-ergodic hypothesis does not need to be invoked (Laskin, et al., 2007; Turecek, 2003). Indeed, with the use of secondary dissociation pathways as kinetic 'thermometers', Pepin and Tureček managed to estimate how the excess (*i.e.,* not consumed during backbone

dissociation) cation/electron recombination energy is divided between the *c* and *z* fragments in ECD fragmentation of peptides, and found that this distribution is proportional to the fragments' number of vibrational degrees of freedom (Pepin& Turecek, 2015). These results strongly suggest that the ECD process is ergodic after all, and raise the question of why fragmentation patterns so closely match higher-order structure, and why labile PTMs and even noncovalently bound ligands are often retained. To (partially) answer this question, it is worth noting that full distribution of the few eV recombination energy over all degrees of freedom of a peptide or protein results in only a minute increase in ion 'temperature', compared to the dozens or hundreds of eV transferred in 'slow heating' methods such as CID. As such, the ergodic hypothesis is not actually at odds with the observed preservation of PTMs and structure.

Despite the controversy on the details of the ECD mechanism, what is certain is that this technique allows backbone cleavage without first annihilating the higher-order structure, and in fact, the resulting *c* and *z* fragments often remain bound noncovalently, and necessitate the use of mild supplemental (vibrational) activation to induce fragment release (Geels et al., 2006; Horn et al., 2000). A useful indicator for the survival of products that represent "fragment complexes," often referred to as 'ECnoD (electron capture without dissociation) survive is the use of fragment isotope patterns: As mentioned, migration of a hydrogen radical from the *c*- to the *z*-fragment is often observed within these complexes, and results in radical '*c-1*' and even-electron '*z+1*' ions (O'Connor, et al., 2006b; Tsybin et al., 2007). Observation of the fragment mass shifts with and without moderate vibrational activation of ions prior to or concomitant with ECD, therefore, allows convenient distinction of N- and C-terminal fragments (Tsybin, et al., 2007).

II.B. Electron-transfer dissociation (ETD)

In 2004, Hunt and colleagues achieved ECD-like dissociation by allowing ESI-generated peptide and protein cations to react with radical anions in an ion trap. In this type of experiment, the radical anion, rather than a cathode, serves as a source of low-energy electrons, and this technique is, therefore, known as electron-transfer dissociation (Syka et al., 2004). ECD and ETD are largely comparable, although it has been suggested that differences in internal energy and angular momentum transfer could lead to a slightly different branching ratio between available reaction pathways (Mentinova, et al., 2013). Additionally, the presence of the anion introduces an additional reaction pathway not available in ECD; namely, transfer of a proton from the protein/peptide to the ETD reagent (Gunawardena et al., 2005; McLuckey& Stephenson, 1998; Pitteri& McLuckey, 2005). This process is generally referred to as the proton-transfer reaction (PTR) and results in formation of an even-electron, charge-reduced analyte and a neutral radical reagent. It was also proposed by Tureček and colleagues (Moss et al., 2011) that a cation's dipole moment can 'guide' an electron to preferential cleavage sites in ECD, whereas the site of electron incorporation by the analyte is (partly) determined by relative position and orientation of reagent and analyte in ETD (and thus more random due to thermal motion). Nonetheless, these two dissociation techniques are often collectively referred to as ExD methods. A timeline that shows some of the highlights in the spectacular development of ECD and ETD since 1998 is shown in Figure 2. As in ECD, fragments in ETD often fail to separate and are detected as ETnoD products if the internal energy of ions is not increased either prior to, or following, the ETD process (Lermyte et al., 2014; Lermyte& Sobott,

2015), particularly in peptides or proteins in which histidine residues act as charge carriers (Xia, et al., 2007). Concomitant collisional activation is difficult to achieve in practice, because the increase in kinetic energy results in increased relative velocity of the protein and ETD reagent anion, to cause a sharp decrease in reaction rate (proportional to $v_{relative}^{-4}$) (McLuckey& Stephenson, 1998) for electron transfer. Concomitant activation via IR laser irradiation is perhaps a better alternative, as this has been shown to result in enhanced fragment yield and (by reduction of fragment isotope distortion *via* hydrogen radical migration) more confident identification in ETD of protein cations and anions (Ledvina et al., 2009; Riley et al., 2015a; Riley et al., 2015b).

III. Instrumentation

III.A. ECD implementations

Generally, efficient ECD requires sufficient overlap of peptide/protein cations and low-energy electrons to allow significant interaction. In practice, this overlap is most conveniently achieved by storage of ions in a Penning trap, and nearly (although some exceptions have been reported (Baba et al., 2004; Ding& Brancia, 2006; Silivra et al., 2005)) all ECD studies have consequently been performed on Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometers (Figure 3A). Soon after the initial observation of photoelectron-driven ECD by McLafferty and colleagues, the efficiency of the process was dramatically improved by the use of directly (Axelsson et al., 1999; Zubarev, et al., 1998) and indirectly (Haselmann et al., 2001; Tsybin et al., 2001) heated cathodes for electron injection. Recently, however, attempts have been made to implement ECD on quadrupole/time-of-flight instruments. A first such effort is an atmospheric pressure ECD source (Robb et al., 2014a; Robb et al., 2014b), which works by introduction of a dopant gas (typically acetone) into the electrospray chamber. A photoionization lamp ionizes the dopant gas, and dissociation is effected by capture of the resulting photoelectrons by the analyte cations (Figure 3B). Backbone cleavage thus occurs prior to entry into the vacuum of the mass spectrometer, and without precursor *m/z* selection the fragments are rather difficult to assign to a particular structure. This method takes full advantage of the capability of ECD to induce backbone cleavage without destruction of higher-order structure though, because noncovalently bound *c/z* fragment complexes (*i.e.,* ECnoD products) can be preserved, selected in the quadrupole, and made to dissociate with low-energy CID. Another more conventional approach is the development of a miniature (*ca.* 8 cm length) electromagnetostatic ECD cell (Figure 3C), which was successfully mounted within triple-quadrupole instruments (G6460, Agilent Technologies, Santa Clara, CA, USA, as well as Finnigan TSQ700, Thermo Fisher Scientific, Waltham, MA, USA), and quadrupole/time-of-flight instruments (QSTAR XL, Applied Biosystems, as well as ultrOTOF-Q, Bruker Daltonics, Billerica, MA, USA) (Voinov et al., 2009; Voinov et al., 2014; Voinov et al., 2008; Voinov et al., 2011; Voinov et al., 2015b). Recently, this cell has also been used to perform electron-induced dissociation, a technique that will be discussed in detail in section IV.A (Voinov et al., 2015a).

III.B. ETD implementations

Comparatively, ETD is more straightforward to implement, because the only requirement is a significant overlap of a cation and anion 'cloud'. In practice, this overlap has mostly been achieved with charge-sign independent ion trapping; *e.g.,* in a quadrupole ion trap (Figure 3D) or linear trap quadrupole (Coon et al., 2005b; Pitteri et al., 2005; Syka, et al., 2004). Depending on the precise implementation, these can either be used as standalone mass spectrometers (Hartmer et al., 2008; Sobott et al., 2009), or integrated in a hybrid instrument; *e.g.,* linear trap quadrupole (LTQ)/Orbitrap (Figure 3E) (McAlister et al., 2007). Note that, in Figure 3E, the chemical ionization source is mounted on the rear of the mass spectrometer, as in the initial implementation of ETD on an LTQ/Orbitrap instrument. The Easy-ETD source (used in the Orbitrap Fusion), however, allows the reagent anions to be generated in the first differentially pumped region of the instrument, so that they follow the same path to the ion/ion reaction region as the analyte cations (Earley et al., 2013). More recently, it has been found that implementation of the ETD reaction in either the higher-energy collisional dissociation (HCD) cell, or a specially designed multi-dissociation reaction cell (MDC) – which both have a higher charge capacity than the LTQ – results in higher reaction rates and more confident identifications in top-down and bottom-up workflows (Riley et al., 2016; Rose et al., 2013).

More recently, a new ETD implementation has been commercialized by Waters (Wilmslow, UK), in which the overlap of both ion clouds is not achieved by simultaneous trapping, but rather by transmission of a cation 'beam' through a cloud of radical anions (Williams et al., 2010). The reaction is implemented within a travelling-wave ion guide (Figure 3F) located between both mass analyzers in a hybrid quadrupole/time-of-flight instrument (Synapt G2, later also adopted in the G2-S and G2-Si models). The ion-mobility capabilities of this instrument (IM cell located immediately downstream of the ETD cell) allow global structural characterization with an orthogonal method, as well as conformation- or charge state-selective fragmentation, again through dissociation of noncovalent fragment complexes (Lermyte et al., 2015b), and interrogation of the structure of charge-reduced species (Laszlo et al., 2016; Lermyte et al., 2015a; Lermyte et al., 2017). Bruker (Bremen, Germany) has also implemented ETD on a quadrupole/time-of-flight instrument (maXis HD; Figure 3G), also by placing the reaction cell between both mass analyzers (Hartmer et al., 2009). This implementation routinely achieves a mass resolution of 40,000 or more, with mass accuracy in the low-ppm range (Fornelli et al., 2013), which makes this instrument particularly attractive in a top-down proteomics context.

IV. Other electron-based gas-phase dissociation techniques

IV.A. Techniques based on higher electron energy: 'hot' ECD, EED, and EID

Over the years, a number of alternative dissociation techniques based on the introduction of an unpaired electron have been developed for biomolecular analysis. For an overview, we refer again to Table 1. Although these methods are generally neither as far developed, nor as commonly used as ECD or ETD, a few of them will be briefly discussed in this section.

In 'hot electron' ECD (Kjeldsen et al., 2002; Kjeldsen et al., 2003; Williams et al., 2009), rather than thermalized electrons, peptide or protein cations are irradiated with electrons that possess kinetic energies of around 6-10 eV. Interestingly, the transition between a 'normal' and 'hot' ECD regime is not, as one might expect, continuous, but a 2-3 eV 'gap' exists between the two, in which

the cross-section for electron capture and subsequent dissociation is minimal (Kjeldsen, et al., 2002). The main difference between the observed fragmentation behavior in 'normal' and 'hot' ECD is the far greater abundance of secondary (*w*-type) fragments that result from further unimolecular dissociation of radical $a^{\bullet}$ and $z^{\bullet}$ fragments (the aforementioned 'free radical cascade') . Cooper and colleagues exploited this secondary fragmentation in order to distinguish variants of the hemoglobin β subunit in which residue 54 was either leucine or isoleucine, as secondary fragmentation results in the loss of an isopropyl (43 Da) or ethyl (29 Da) radical, respectively (Williams, et al., 2009). Budnik and Zubarev irradiated $[M+H]^{+}$ peptide ions with electrons that possessed kinetic energies up to 70 eV (Budnik& Zubarev, 2000), which resulted in electron ejection from the cations and led to the formation of $[M+H]^{2+\bullet}$ radicals. Interestingly, it was reported in this work that no fragmentation occurred; the authors rationalized this result by postulating that the reaction cross section for dissociation of the ions is maximized when the incoming electron has a significantly lower energy (below 9 eV) than used here, in accordance with earlier reports from the EI literature (Cody& Freiser, 1979).

An alternative approach, also developed by Zubarev and colleagues, did generate fragments, and deserves mention here (Nielsen et al., 2000; Nielsen et al., 2003). In this technique, named electronic-excitation dissociation (EED), $[M+H]^{+}$ ions generated with matrix-assisted laser desorption/ionization (MALDI) were irradiated with high-energy (>10 eV) electrons, which induces loss of a second, thermalized electron from the analyte and the formation of an intact $[M+H]^{2+\bullet}$ radical. The slow, ejected electrons were then reflected back at, and absorbed by, these hydrogen-deficient radicals. Unlike the $[M+H]^{+}$ precursor, absorption of a single electron by these doubly charged radicals does not neutralize all charge, and thus does not prevent the detection of fragments. This experiment led to ECD-like fragmentation and formation of predominantly *a*, *c*, and *z* fragments. In later experiments by Zubarev and colleagues, the use of electrons with kinetic energies around 40 eV resulted in fragmentation of singly and multiply charged proteins and peptides without reflection of ejected, thermalized electrons (Fung et al., 2009). This technique is referred to as 'electron-ionization dissociation'. Due to mechanistic similarities, 'electron-induced dissociation' has been proposed (Lioe& O'Hair, 2007) as a generic term for high-energy electron-based dissociation methods – specifically electron-ionization dissociation, electronic-excitation dissociation, and an older technique (not discussed in detail here) known as 'electron impact excitation of ions from organics' (EIEIO) (Cody& Freiser, 1979; Cody& Freiser, 1987).

IV.B. Use of ion/neutral electron transfer: ECID

Ion/neutral collisions can also induce ECD-like fragmentation, in a method called electron capture-induced dissociation (ECID) (Chakraborty et al., 2006; Holm et al., 2007; Hvelplund et al., 2007; Hvelplund et al., 2003a; Hvelplund et al., 2003b; Liu et al., 2004; Moss, et al., 2011). In this approach, multiply charged $[M+nH]^{n+}$ cations are accelerated to high (~100 keV) kinetic energies and made to collide with an electrically neutral 'target' in the gas phase. Na, Cs, and C_{60} have all been used successfully as targets. The collision results in transfer of an electron from the neutral species to the cation, to lead to the formation of $[M+nH]^{(n-1)+\bullet}$ radicals, which exhibit ECD-like fragmentation patterns.

IV.C. Techniques for anion analysis: EDD, niECD, NETD, and EPD

A number of radical-based techniques for fragmentation of biomolecular anions (*e.g.,* acidic peptides, nucleic acids, etc.) in the gas phase have also been developed. Irradiation of anions with moderate- or high-energy electrons induces ejection of an electron, and the resulting radical subsequently undergoes unimolecular dissociation. However, in contrast to the behavior of cations, the main fragmentation pathway for peptides in this case leads to cleavage of the CO-C(α) bond, and the formation of *a* and *x* fragments. This technique is called electron-detachment dissociation (EDD) (Adamson& Hakansson, 2007b; Anusiewicz et al., 2005; Budnik et al., 2001; Ganisl et al., 2011; Kalli& Hakansson, 2007; Kjeldsen et al., 2005; Taucher& Breuker, 2012; Yang et al., 2005), and an ion/ion equivalent exists in which loss of an electron by the analyte anion is effected by interaction with a reagent cation rather than an electron beam. Appropriately, this ion/ion reaction is called negative electron-transfer dissociation (NETD) (Coon et al., 2005a; Crizer et al., 2009; Huzarska et al., 2010; McAlister et al., 2012; Rumachik et al., 2012). A third method to induce loss of an electron from a gas-phase anion is the absorption of one or more photons. This method, which leads to similar fragmentation as EDD and NETD, is known as electron-photodetachment dissociation or EPD (Antoine et al., 2007; Gabelica et al., 2006; Joly et al., 2008; Larraillet et al., 2009; Larraillet et al., 2010). Although this technique is still very novel, promising results have been reported in the analysis of negatively charged oligonucleotides, peptides, proteins, oligosaccharides, lipids, and synthetic polymers. EPD itself, as well as these applications, have recently been the subject of an excellent review, to which the reader is directed for further information (Antoine et al., 2014).

Finally, it was recently reported by Håkansson and colleagues that lowering the electron energy in EDD to around 5 eV actually results in electron capture by singly or multiply charged peptide anions generated by ESI (Hersberger& Hakansson, 2012; Yoo et al., 2011). This process is obviously fairly inefficient due to a high Coulomb barrier, and irradiation times of 10 to 20 seconds are typically required. Interestingly, negatively charged *c*- and *z*-fragments were detected in these experiments, suggesting that these peptide anions are zwitterionic in nature, and that electron-capture actually occurs at a positive charge site. Further development of this technique, called niECD (negative ion electron-capture dissociation) is required; however, this method has the potential of becoming a powerful tool for studying the so-called acidic proteome, since these (*e.g.* highly phosphorylated/sulfated) proteins often do not ionize efficiently in positive-mode ESI.

Although not a dissociation method for ESI-generated biomolecular ions, it is worth mentioning here that there are many mechanistic similarities between electron-based dissociation methods and MALDI in-source decay (ISD) (Asakawa, 2016). Like ECD/ETD, this process leads to extensive cleavage of N-C(α) bonds in peptides, as well as formation of *w* fragments (as in 'hot' ECD) (Asakawa et al., 2013). This fragmentation is thought to proceed through formation of an aminoketyl radical within the MALDI plume (Knochenmuss& Zenobi, 2003).

While there is thus a growing 'toolbox' for electron-based dissociation of both biomolecular cations and anions, the rest of the current discussion will focus on ECD and ETD fragmentation of peptides and (native) proteins in positive ionization mode.

V. Determinants of ExD fragmentation behavior of peptides

V.A. Residue selectivity and effect of precursor charge state

As mentioned previously, the main drawback of 'slow heating' methods in tandem MS is the distribution of the gained internal energy over all vibrational degrees of freedom of a peptide or protein, which results in preferential dissociation at a limited number of thermodynamically labile sites, often N-terminal to proline (Bleiholder et al., 2011; Vaisar& Urban, 1996). In contrast, statistical analysis of the ETD fragmentation patterns of a large number of peptides showed much less bias, with only a mild preference for cleavage N-terminal to K, and C-terminal to E and R residues (Li et al., 2011c). The precursor charge state plays a much more significant role than amino acid composition, however, because fragmentation yield and sequence coverage have been shown to correlate with the charge 'density' (*i.e.,* charge/residue ratio) (Good et al., 2007). Later, it was confirmed that, for small tryptic peptides, ETD of triply charged precursors resulted in more efficient fragmentation compared to doubly charged species (Chalkley et al., 2010). Interestingly, in this work, doubly charged precursors yielded significant *z+1* fragments, whereas if the triply charged ion was subjected to ETD, the intensity of *z+1* fragments is decreased significantly in favor of regular *z* ions, which suggests a slightly higher-energy process and somewhat more rapid separation of the ETD fragments (Tsybin, et al., 2007).

Another important factor to determine the appearance of an ETD spectrum is the ratio of analyte cations to reagent anions (Good, et al., 2007). In particular, an overabundance of cations will result in the observation of primarily non-dissociative charge reduction (Lermyte, et al., 2015b; Lermyte et al., 2015c), whereas an excess of anions can lead to multiple reaction steps, including neutralization of reaction products, which prevents their detection (Good& Coon, 2006). Use of a very short or very long reaction time has a similar effect to a very low or high anion/cation ratio, respectively. A reduction of either anion concentration (usually in significant excess, so that it can be considered part of a 'pseudo-first-order' rate constant) or reaction time results in a smaller number of cation/anion interaction events (*i.e.* proton or electron transfer); therefore, it is not unexpected that they have a similar effect on the appearance of the ETD spectrum. Two explanations for why this results in a spectrum dominated by charge reduction immediately come to mind (and it is likely that both contribute). First of all, the majority of cation/anion interaction events do not lead to ETD and therefore, a large portion of the fragments observed in a typical ETD spectrum are the result of a series of several such events. Second of all, due to the fairly uniform cleavage observed in ETD, fragment intensity is distributed over a large number of species, whereas only a handful of charge-reduced species exist, which results in a greater intensity and signal-to-noise ratio for the latter, especially if the total number of reaction product ions (ETD and charge reduction) is limited. Recently, Coon and colleagues introduced a calibration routine to rapidly optimize the reaction time and number of reagent anions to maximize information in a shotgun proteomics experiment (Rose et al., 2015). Because this method only requires limited user expertise, it could become a significant step forward in the successful implementation of ETD in high-throughput, bottom-up proteomics workflows, which today are still heavily reliant on collisional activation.

V.B. Effect of higher-order (secondary) structure

Even on the peptide level, it has been shown that secondary structure plays an important and often dominant role to determine the observed ExD fragmentation pattern. For instance, ECD of α-helical

peptides, produced by enzymatic digestion of myoglobin, resulted in fragments that exhibit a periodic intensity, with maxima spaced three to four residues apart (Ben Hamidane, et al., 2009b). A different example of an α-helical peptide from the same study is shown in Figure 4. This periodic product-ion abundance is, of course, reminiscent of the 3.6-residue periodicity that is characteristic for an α-helix in solution. Hudgins, Simons, and colleagues have studied disulfide-linked α-helical peptides both experimentally and by *ab initio* computational methods and found that, while cleavage of the disulfide bond dominated, it is indeed plausible for an unpaired electron or a hydrogen radical to propagate via the hydrogen bond network (Sawicka, et al., 2003; Skurski et al., 2007). The apparent preference for a specific 'face' of the helix can be rationalized by solvation of a charged group; for instance, the N-terminal amine. Similarly, in ECD of some 15,000 peptides of different lengths (represented as *n*), derived from human and *E. coli* cell lysate, the z_{n-4} fragment was found to usually be the most abundant by Zubarev and colleagues, to indicate preferential cleavage C-terminal to the fourth residue (Savitski et al., 2006). This observation is consistent with an N-terminal charge that is solvated intramolecularly in an α-helix-like motif in the gas phase. It has also been shown that ion activation with IR laser prior to ECD leads to increased sequence coverage and fragmentation efficiency in peptides (Lin et al., 2008). Similarly, ECD of substance P and gramicidin S in an ICR cell cooled to 86 K resulted in significantly less fragmentation than at 313 K (Mihalca et al., 2004). In both cases, increased conformational heterogeneity and reduced stabilization of higher-order (secondary) structure by hydrogen bonding were posited to account for the increased fragmentation observed in ions that possessed more internal energy. Cooper and colleagues recently expanded this type of work beyond the analysis of secondary structure only, and used a combination of ECD, ion mobility, and molecular dynamics simulations to investigate the nature of intramolecular interactions within six related pentadecapeptides (Kim et al., 2015). Due to the presence of tertiary and quaternary structure in native proteins and complexes, their behavior in ExD experiments is, of course, even more complex than that of peptides, and will be discussed in a later section. In the next section, we will briefly consider a different area where ECD and ETD have played a significant role, namely the analysis of post-translational modifications.

VI. ExD fragmentation of peptides and intact proteins for bottom-up and top-down analysis of post-translational modifications

Although not the focus of this review, the use of ExD methods for protein sequencing is important enough to elaborate on. In particular, the selectivity for cleavage of the N-C(α) bond makes these methods ideally suited for analysis of extensively post-translationally modified proteins, particularly as side reactions such as cyclizations and formation of internal fragments – which all lead to difficult-to-interpret background signals – are also less prevalent than in CID. The fairly equal probability for cleavage at 19 of the 20 common amino acid residues (proline, as mentioned, is the obvious exception) provides advantages for top-down analysis of intact proteins, because spectra are not dominated by a handful of preferentially formed fragments. This potential was already understood in the early days of ExD, and initial efforts focused mainly on the characterization of glycoproteins and histone PTMs, which have consistently remained important application areas (Coon, et al., 2005b; Garcia et al., 2007; Hakansson et al., 2001; Mirgorodskaya et al., 1999). In a similar vein, 74 isoforms of the human histone H4 subunit were identified in embryonic stem cells with ETD in 2008 (Phanstiel et al., 2008). The first pilot project of the Consortium for Top-Down Proteomics, a broad international

collaboration that works toward the development of high-throughput methods for intact protein analysis, also focused on histone proteoforms (Dang et al., 2014; Smith& Kelleher, 2013), and ExD methods dominated here as well, to demonstrate that they have gained importance since their initial development. In a similar vein, electron-based dissociation has also been essential in the work of Ge and colleagues, who, over the last decade, have studied protein phosphorylation with the aim to improve understanding of the molecular mechanism(s) of cardiovascular disease (Ayaz-Guner et al., 2009; Ge et al., 2009; Nelson et al., 2010; Peng et al., 2014; Zabrouskov et al., 2008; Zhang et al., 2011b).

Much research has been performed by Tsybin and colleagues at the Ecole Polytechnique Fédérale de Lausanne (EPFL). This research includes the use of ETD for rapid top-down identification of clinically significant hemoglobin variants on QTOF (Graca et al., 2015) as well as ion trap instruments (Graca et al., 2012). Also, this group has made significant efforts in another area, where post-translational modifications are highly biologically relevant; namely, the top- and middle-down analysis of monoclonal antibodies (Fornelli et al., 2014; Fornelli et al., 2012; Srzentic et al., 2014; Tsybin et al., 2011). In this work, it was consistently found that sequence coverage is significantly improved if disulfide bonds are reduced first, because they can prevent fragment separation and have a very high electron affinity, whereas capture at these sites does not generally result in backbone cleavage (Ganisl& Breuker, 2012; Simons, 2010; Zubarev et al., 2000).

O'Connor and colleagues have shown that distinction between aspartic/isoaspartic and glutamic/γ-glutamic acid residues is fairly straightforward with ECD and ETD, because the isomerization products show unique peaks due to the presence of C(β) in the backbone (Cournoyer et al., 2005; Li et al., 2010; O'Connor et al., 2006a). This approach also allows detection of deamidation of asparagine/glutamine residues. Supplemental activation facilitates cleavage of the C(α)-C(β) backbone bond, particularly if ETD is used (Chan et al., 2010). Activation (with an infrared laser) prior to ECD has also proven effective, and, by variation of the laser power, this approach has been used to construct melting curves for each residue in the terminal regions of calmodulin and β_2 microglobulin (Soulby et al., 2015). It was shown in this study that deamidation leads to a reduction in melting temperature, likely due to increased flexibility and local weakening of the hydrogen bond network. Paradoxically, overall sequence coverage was reduced in deamidated proteins; the authors rationalize this result by proposing that a strengthening of the 'global' salt bridge network, due to replacement of neutral with negatively charged side chains, occurred. The O'Connor group has also used ECD to study artificially induced covalent protein modifications, specifically binding of Pt- and Ir-based anticancer drugs, and showed that this technique outperforms slow-heating methods such as CID and IRMPD (Li et al., 2011a; Li et al., 2011b; Qi et al., 2013).

Hydrogen-deuterium exchange (HDX) is another method by which proteins are chemically modified, and has proven highly useful for structural protein analysis. This is because the rate of exchange (of backbone amide hydrogen) is higher in regions of the protein that possess greater solvent accessibility and local backbone flexibility (Wei et al., 2014). If proteolytic digestion is performed under low-pH/low-temperature conditions that inhibit (back-)exchange, deuterium incorporation can be quantified for each peptide, and provide information about the relative degree of protection in different regions of the protein sequence. To increase the 'resolution' of this technique to the single-residue level, gas-phase fragmentation (*i.e.*, tandem MS) is required. CID (and, by extension, other slow-heating methods) are not ideal for this, as they promote

intramolecular gas-phase 'scrambling' (*i.e.*, migration) of the deuterium labels (Jorgensen et al., 2005; Wei, et al., 2014). ECD and ETD have, however, been successfully used to induce fragmentation after HDX, and have been shown to allow residue-specific localization of deuterium incorporation, without significant 'scrambling' (Abzalimov et al., 2009; Kaltashov et al., 2009; Pan et al., 2008; Pan et al., 2009; Rand et al., 2008; Rand et al., 2011; Rand et al., 2009; Zehl et al., 2008).

Finally, ECD and ETD have proven to be so little disruptive toward peptide and protein structure that in addition to weak covalent bonds, even noncovalent interactions often survive the process. As a result, dissociation of protein-ligand complexes will often result in the detection of fragments that are still bound to the ligand, to allow identification of, *e.g.,* a ligand-binding site (Goth et al., 2016; Jackson et al., 2009; Konijnenberg et al., 2016; Xie et al., 2006; Yin& Loo, 2010), residues involved in a specific peptide/peptide interaction (Muller et al., 2014), and mapping of a protein/peptide interface (Clarke et al., 2011). The preservation of noncovalent interactions during the ExD process implies that higher-order protein structure should also survive, and have some effect on the observed dissociation pattern. Because this phenomenon is a highly important one, which has been the subject of many investigations over the past 15 years, the next section is devoted to the use of ExD methods in this context.

VII. ExD for interrogation of protein higher-order structure

VII.A. Initial efforts: ExD for characterization of secondary structure and salt bridge patterns of small monomeric proteins

The observation that ECD could induce backbone cleavage without significantly disrupting higher-order structure led McLafferty and colleagues to investigate the effect of gas-phase protein conformation on ECD fragmentation behavior in the early 2000s (Breuker, et al., 2002; Horn et al., 2001; Oh et al., 2002). For this research, they focused on the analysis of relatively small proteins such as ubiquitin and cytochrome c. Because ECD causes extensive backbone cleavage in these proteins even in their folded state, they could infer information about noncovalent interactions based on which fragments were and were not released. In these landmark papers, several factors such as protein charge state, ion activation (either by blackbody radiation, collisional activation, or laser irradiation), and storage time were investigated, and their effects on gas-phase unfolding and refolding were quantified. In particular, a 3- to 4-residue periodicity in fragment intensity distributions was observed, and suggested significant occurrence of α-helices in the gas-phase structure of ubiquitin, particularly at high charge states. Lack of observed fragmentation in certain regions was assumed to correlate with the gas-phase salt-bridge pattern, and different patterns were proposed to occur in different charge states, as summarized in Figure 5 (Oh, et al., 2002).

Later, Breuker and colleagues performed similar work with a different model system, the three-helix bundle protein KIX. As with ubiquitin and cytochrome c previously, they investigated the effect of charge state, collisional activation, and storage time on the ECD fragmentation pattern. In this way, they showed that KIX likely retains most of its native structure in the gas phase on a time scale of at least several seconds, and also demonstrated that the three helices in this structure have a different stability, which correlates very well with salt bridge density within each helix (Breuker et al., 2011a; Schennach et al., 2016).

This pioneering work has some caveats from a native MS/structural biology point of view, because the proteins analyzed in these studies were almost always sprayed from acidified solutions that often also contained a significant amount of organic solvent. Specifically, for ubiquitin experiments, charge states greater than 8+ were sprayed from solutions containing more than 10% organic solvent and at least 1% (formic or acetic) acid; solutions with less than 10% organic solvent, but containing 1% acid were used to generate 7+ and 8+ ions; and 5+ and 6+ ions were sprayed from 1% aqueous NH_4OH (resulting in a non-physiological pH slightly above 10). For cytochrome c, solutions contained 50% methanol and 3% acetic acid. For experiments with KIX, acidified solutions containing 20% methanol were used to generate charge states 7 – 12, whereas higher charge states were generated by the use of 50% methanol (the authors do, however, acknowledge that the latter solvent system is denaturing). One can, therefore, question to what extent the proteins' gas-phase conformations are still closely associated with known solution structures. Specifically for ubiquitin, the most popular model system in these studies, it is known that these solution conditions cause a significant proportion of the protein to adopt the semi-extended, largely α-helical 'A' state, rather than the native 'N' state (Brutscher et al., 1997). These non-native solution conditions, along with the fairly harsh interface and relatively long (ca. 1 s) gas-phase ion storage time common in ECD on FTICR instruments might, to some extent, help explain why Vachet and colleagues, who performed ETD of charge states of ubiquitin generated by electrospray from a purely aqueous solution (Zhang et al., 2014; Zhang& Vachet, 2017), found that the native salt-bridge pattern best explained the observed fragmentation, and indicated greater retention of the native structure in the gas phase than usually reported (Zhang et al., 2013a). And more recent work suggests even more importance to salt-bridges on the gas phase dissociation of native proteins (Loo& Loo, 2016). Nevertheless, it is hard to overestimate the importance of the early work carried out in the McLafferty laboratory for our present understanding of electron-based dissociation methods in structural biology.

In 2003, Breuker and McLafferty developed a technique they named 'native ECD' (NECD) (Breuker, 2006; Breuker& McLafferty, 2003; Breuker& McLafferty, 2005). In these experiments, a 100 µM (1.2 mg/mL) aqueous solution (pH = 5.5) of cytochrome c was subjected to ESI, which resulted in the formation of noncovalent dimers during the spray process (Smith& Lightwahl, 1993; Smith et al., 1992). Asymmetric charge partitioning (Schwartz et al., 1995) due to in-source dissociation of these dimers (within the heated capillary through which ions enter the mass spectrometer) introduced a radical site in the protein chain, without the introduction of external electrons *via* a cathode. Because concomitant reduction of Fe(III) to Fe(II) was observed, the protein's heme group was believed to be involved in this process. This hypothesis was corroborated by the observation that cleavage was preferred in residues known to interact noncovalently with the heme group. To the best of our knowledge, no reports of NECD appeared in the literature for the following decade, and it was only very recently that Kelleher and colleagues extended this approach to the 490 kDa ferritin 24mer (Skinner et al., 2017). In the past decade, continued experimental and computational work by Breuker and McLafferty has provided a more detailed understanding of the evolution of the structure of small proteins in the gas phase, on a timescale that ranges from picoseconds to minutes (Breuker et al., 2011b; Breuker& McLafferty, 2008; McLafferty et al., 2010; Schennach& Breuker, 2014; Schennach& Breuker, 2015; Schennach, et al., 2016; Skinner et al., 2013; Skinner et al., 2012; Steinberg et al., 2007; Steinberg et al., 2008). Their model, which represents a stepwise structural evolution, is summarized in Figure 6. It should be noted that both the gas-phase stability of the native structure, as well as the timescale of structural rearrangements, depends on a

number of factors. Proteins and complexes that are larger and/or mainly stabilized by electrostatic interactions (for instance, the KIX protein discussed previously) are expected to retain much of their native structure for a longer time – sufficient to study this structure by mass spectrometry. In contrast, smaller proteins, proteins stabilized primarily by the hydrophobic effect (which is absent in the gas phase), and intrinsically disordered proteins, will likely undergo rapid structural changes in the gas phase.

VII.B. New frontiers: Combination of ExD and native MS for structural analysis of large noncovalent complexes

With the increased use of native mass spectrometry for the characterization of large protein complexes (Benesch et al., 2007; Loo, 1997; Sobott& Robinson, 2002), there has been considerable interest in the application of ECD/ETD to native protein structures and complexes (not to be confused with 'native ECD' as developed by Breuker and McLafferty) in the last five years. Several landmark papers that used ECD (using FTICR) in this context were published by Gross and colleagues (Cui et al., 2015; Jones et al., 2013; Zhang et al., 2010; Zhang et al., 2013b; Zhang et al., 2011a; Zhang et al., 2016). In these studies, as in the small-protein work by the McLafferty group, it was assumed that backbone cleavage occurs throughout the sequence, and that selectivity in the observed fragmentation pattern is due to the different propensities of protein regions to unfold in the gas phase. The crystallographic B factor (a measure for local dynamics or flexibility) was used as a proxy for this propensity. Interestingly, in one of these studies, it was found that ECD fragmentation within an antibody-antigen complex occurred most readily in parts of the sequence that – due to high flexibility – are not resolved in X-ray crystallography (Zhang, et al., 2016).

With ETD on a quadrupole/time-of-flight instrument for the same purpose (Lermyte, et al., 2014; Lermyte& Sobott, 2015), several differences with the work by Gross and colleagues were observed: First of all, under the gentlest experimentally accessible conditions, almost no fragmentation of the alcohol dehydrogenase (ADH) tetramer was apparent. Instead, significant additional collisional activation was required. Activation of the ions prior to entry of the ion into the ETD reaction cell (Lermyte& Sobott, 2015) resulted in fragmentation patterns that matched the literature ECD results obtained on FTICR instruments, as shown in Figure 7.

Serendipitously, three independent studies were published over a period of two months in 2015; all showed stepwise collisional activation of the hemoglobin tetramer prior to ExD fragmentation (Cui, et al., 2015; Lermyte& Sobott, 2015; Zhang et al., 2015). Interestingly, similar observations were made in all cases, and fragmentation in the first 20 to 25 amino acid residues in the α– and β-subunits was observed at the lowest voltages required for transmission. Fragmentation up to around residue 35 required intermediate activation, and large fragments (up to c_{58} for the α and c_{68} for the β chain) were reported at high levels of 'pre-heating' in the ECD studies. Interestingly, in all these studies, the 'boundaries' between the different regions in which fragmentation is observed at increasing energy levels, correspond quite well to the N-terminal ends of α-helices in the crystal structure. This behavior indicates that this type of 'activated ion' ExD can be used to monitor the initial gas-phase unfolding steps of subunits within large, noncovalent protein complexes.

Besides the absence of fragmentation in low-energy ETD of ADH, it was also noted that, if collisional activation is only applied downstream of electron transfer, and every effort is thus made to ensure that the most native-like gas phase conformation interacts with the anion, then fragments are less abundant and the observed fragmentation pattern generally matches surface accessibility better than backbone flexibility (Lermyte, et al., 2014; Lermyte& Sobott, 2015). Under the assumption – made in all commonly cited ExD mechanisms – that backbone cleavage occurs very rapidly and requires no additional activation of vibrational modes, this behavior would indicate that there is, in these cases, a preference for cleavage at the exposed surface, which leads to noncovalently bound *c*- and *z*-fragments which must be separated by gentle collisional activation.

Intuitively, this surface-selectivity may seem to be at odds with what is known about ExD fragmentation, based both on the various proposed mechanisms, as well as the aforementioned small-protein work by McLafferty and colleagues. However, the discrepancy is not as fundamental as one might initially think, if we assume that rather than surface *per se*, ETD might be selective for dissociation near charge sites, in particular protonated side chains. These are known to be located primarily at the exposed surface in protein ions generated by native ESI (Kaltashov& Mohimen, 2005; Schnier, et al., 1995). Indeed, the possibility of charge-site selective fragmentation has been raised by the McLafferty group (Breuker, et al., 2002; Skinner, et al., 2013), and is not only compatible, but even expected in the Cornell mechanism, as it proceeds through creation of a hydrogen radical by neutralization of a positive charge site. So is this an indication that ETD proceeds *via* this mechanism in this case? Let us consider the main alternative model, the Utah-Washington mechanism. If we assume that electrons, generated by a cathode discharge, are 'blind' to the presence of charged side chains, and only interact with amide π^* orbitals, we would indeed expect deeper penetration into the protein core, possible following some type of Beer-Lambert attenuation law. In this case, the fragmentation pattern would be determined by ease of fragment release, *i.e.* flexibility, as is for instance observed in native UVPD (Cammarata& Brodbelt, 2015). In the Utah-Washington mechanism however, electron absorption is greatly facilitated if the amide forms a hydrogen bond to a positively charged group, as the resulting Coulomb stabilization significantly lowers the energy of the amide π^* (LUMO) orbital. As such, a preference for cleavage near charge sites is not unexpected (see Figure 8).

Furthermore, it has been suggested by Jack Simons, in whose group the Utah mechanism was developed, that rather than direct absorption of an electron by an amide π^* orbital, the electron might actually be captured in a high-*n* Rydberg state by a positively charged group, as in the Cornell mechanism (Simons, 2010). In contrast to the Cornell mechanism, however, the reaction according to Simons then proceeds by intramolecular transfer of this electron to an amide functionality. What is particularly intriguing about this possibility, is that the maximum range over which this electron transfer can occur efficiently, depends on the principal quantum number of the aforementioned Rydberg state, which in turn depends on the energy of the incoming electron. As such, it is conceivable that (low-energy) ETD would be more selective for cleavage near charge sites than 'normal' ECD using thermalized electrons, which in turn would be more charge site-selective than 'hot electron' ECD. The fact that no surface selectivity is found in ExD of small proteins is easily explained by the fact that no true protected 'core' region exists here (*i.e.* these proteins are 'all surface'), which has been confirmed by calculation of surface accessibility per residue in the crystal structure (Fraczkiewicz& Braun, 1998; Lermyte& Sobott, 2015). Clearly, more work is required to definitively address how each proposed mechanism directly relates to the data from ExD of native

proteins; however, we can conclude that the charge- and/or surface-selectivity in ExD of native proteins and complexes can be compatible with all previously published ExD experiments and proposed mechanisms.

VIII. Future perspectives

The past decade has witnessed a tremendous evolution of electron-based dissociation methods, and one may be forgiven for occasionally forgetting that the first of these methods, ECD, was developed nearly twenty years ago. Undoubtedly, this evolution will continue over the next years, driven in large part by the ever-increasing availability and affordability of ECD and ETD, either as an integral part of commercial mass spectrometers, or as easily added modules. It was predicted (Coon, 2009) nearly a decade ago that electron- and collision-induced dissociation methods would become roughly equally important for sequence analysis in the 21st century, and today, we seem to be at the brink of this prediction becoming a reality in many proteomics laboratories, particularly in top-down approaches. Because ECD and ETD of peptide cations are almost commonplace, it is an open question whether increasing interest in the analysis of the acidic proteome, nucleic acids, and other classes of biopolymers that are more conveniently ionized in negative mode, will result in either EDD, niECD, NETD, and/or EPD becoming more popular, or the development of methods for more efficient positive-mode ECD and/or ETD of these analytes. NETD has recently been implemented, although not commercialized, on an LTQ/Orbitrap instrument (McAlister, et al., 2012), and led to improved fragmentation of acidic peptides; however, some publications report ECD/ETD of oligosaccharides (Adamson& Hakansson, 2007a; Huang et al., 2014; Liu& Hakansson, 2011; Zhao et al., 2008; Zhou& Hakansson, 2013) and nucleic acids (Hakansson et al., 2003; Schultz& Hakansson, 2004; Smith& Brodbelt, 2009) in positive-ion mode, and both avenues are still open for future development.

Not only will we see an evolution in these (nearly) completely new application areas, but significant improvements are still being made in 'normal' positive mode ECD/ETD. Annihilation of noncovalent interactions (present due to either gas-phase refolding or – in native MS – higher-order structure in solution) prior to or concomitant with ETD shows great promise to improve fragmentation efficiency (Ledvina, et al., 2009; Lermyte& Sobott, 2015; Lermyte& Sobott, 2017; Riley, et al., 2015a; Riley, et al., 2015b), and will likely become more common over the next years in studies that aim to maximize sequence coverage. Conversely, in 'native' ExD, focus will shift toward larger, more challenging systems than the small monomeric proteins that have heretofore received most attention. This evolution is already apparent, with several recent reports of ECD and ETD of large noncovalent complexes (Cui, et al., 2015; Lermyte, et al., 2014; Lermyte& Sobott, 2015; Li et al., 2017a; Li et al., 2014a; Li et al., 2014b; Zhang, et al., 2010; Zhang, et al., 2013b; Zhang, et al., 2011a) and highly dynamic proteins (Harvey et al., 2014; Harvey et al., 2015; Phillips et al., 2015). In this context, the specifics of the mechanism will surely continue to be investigated, and more sophisticated experimental methods such as interrogation of radical intermediates via action spectroscopy might provide more insight (Nguyen et al., 2015). Very recently, a report was published that suggested that EID might deserve more attention than it has so far received in the context of native top-down MS (Li et al., 2017b). In this work, the extra electron energy was able to induce sufficient disruption of noncovalent interactions to significantly increase sequence coverage

compared to ECD, while still being sensitive to higher-order structure. One important class of protein that so far has proven nearly impervious to 'native' electron-based fragmentation are membrane proteins. Robinson and colleagues have pioneered the MS-measurement of membrane protein complexes (Barrera et al., 2008; Zhou et al., 2011), and thus it is likely that ExD activation of membrane proteins will be demonstrated. Because this type of experiment could provide valuable insights into protein-protein and protein-lipid interactions in these biologically highly significant proteins, it is expected that this problem will receive considerable attention from many researchers in the near future.

As befits these techniques that use the gas-phase behavior of odd-electron species, the advances currently being made in many laboratories across the world will undoubtedly take electron-based dissociation into radically new and exciting directions over the next years.

Acknowledgements

The authors are grateful to Peter O'Connor (University of Warwick) for valuable discussions regarding mechanistic aspects of ExD. F.L. thanks the Research Foundation – Flanders (FWO) for funding a PhD fellowship and the Engineering and Physical Sciences Research Council (EP/N033191/1) for postdoctoral funding. Financial support from the Hercules Foundation – Flanders is gratefully acknowledged. J.A.L. acknowledges support from the US National Institutes of Health (R01GM103479) and the US Department of Energy (UCLA/DOE Institute for Genomics and Proteomics; DE-FC03-02ER63421). All authors thank the reviewers for their valuable comments.

Name	Fragmentation induced by	Polarity	Typical instrument(s)	Main fragments (proteins)
CID	Collisions	+/-	Trap/QTOF	*b*/*y*
keV CID	Collisions	+/-	MALDI-TOF/TOF	*a*/*b*/*y* (*d*, *v*, *w*)
SORI-CID	Collisions	+/-	FTICR	*b*/*y*
IRMPD	Multiple photons	+/-	FTICR	*b*/*y*
BIRD	Blackbody radiation	+/-	FTICR	*b*/*y*
UVPD	Single photon	+/-	FTICR/LTQ-Orbitrap*	*a*/*x* (*c*/*z*, *b*/*y*)
SID	Surface	+/-	QTOF/FTICR	*b*/*y*
ECD	Electron addition	+	FTICR	*c*/*z*
ETD	Electron addition	+	Trap/QTOF	*c*/*z*
'Hot' ECD	Electron addition	+	FTICR	*c*/*z*
ECID	Electron addition	+	Sector	*c*/*z*
EID	Electron addition	+	FTICR	*c*/*z*
EED	Electron migration	+	FTICR	*a*/*x*, *c*/*z*
EDD	Electron loss	-	FTICR	*a*/*x*
niECD	Electron addition	-	FTICR	*c*/*z*
EPD	Electron loss	-	Trap	*a*/*x*
NETD	Electron loss	-	Trap/LTQ-Orbitrap*	*a*/*x*

**Note: the dissociation is carried out in the linear ion trap rather than the actual Orbitrap in these hybrid instruments (see Figure 3)*

Table 1. Common dissociation techniques used in biomolecular mass spectrometry. 'Slow heating' methods are shown in the top five rows; electron-mediated fragmentation approaches in the bottom ten.

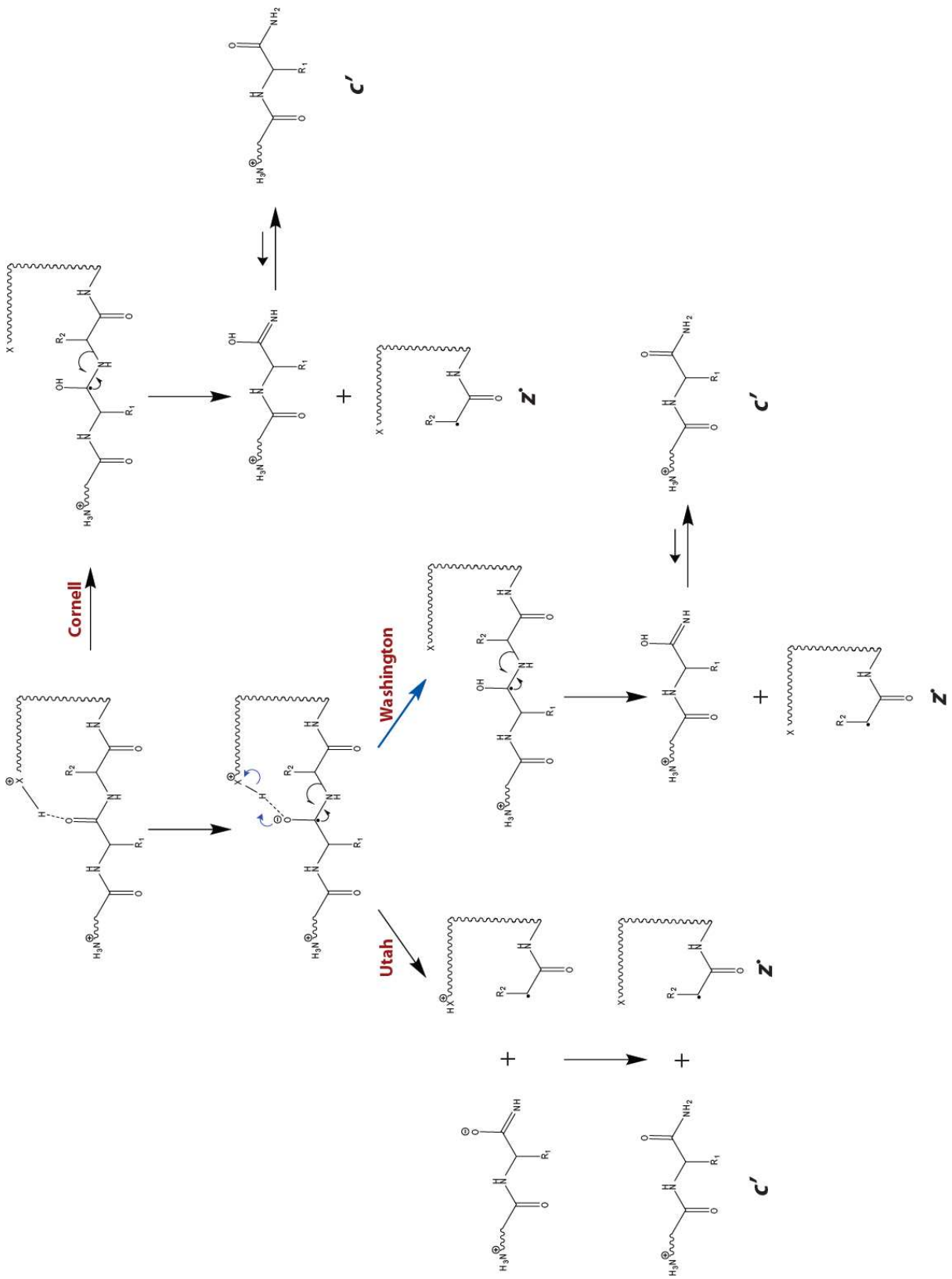

Figure 1. Cornell and Utah-Washington mechanism for ECD.

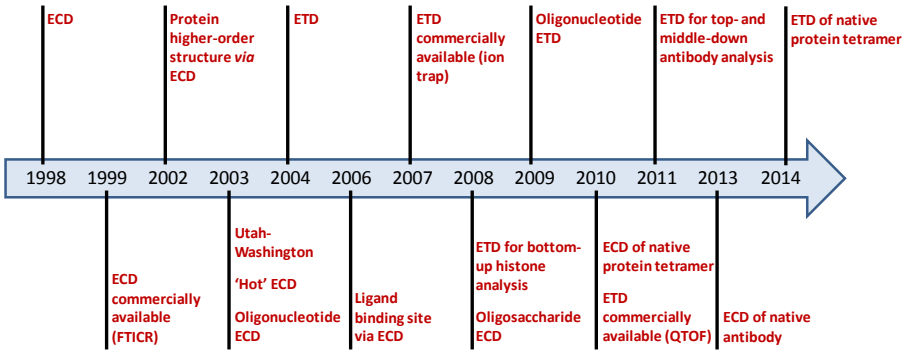

Figure 2. Timeline of 'milestones' in the development of ECD and ETD.

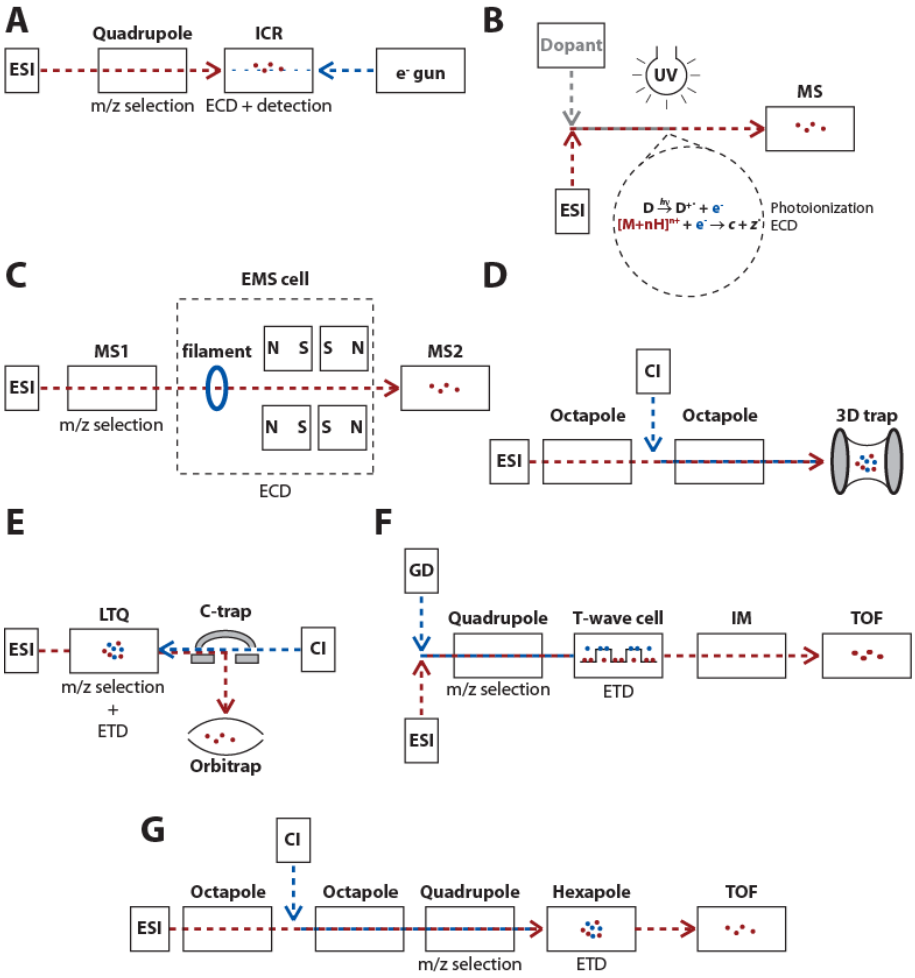

Figure 3. Selected implementations of ECD and ETD. (A) ECD on FTICR; (B) atmospheric pressure ECD (Waters); (C) electromagnetostatic (EMS) cell; (D) ETD on 3D ion trap (*e.g.* Bruker AmaZon); (E) ETD on LTQ/Orbitrap; (F) ETD within T-wave device (*e.g.* Waters Synapt); (G) ETD on QTOF (*e.g.* Bruker maXis). Abbreviations used: UV – ultraviolet; CI – chemical ionization; LTQ – linear trap quadrupole; GD – glow discharge.

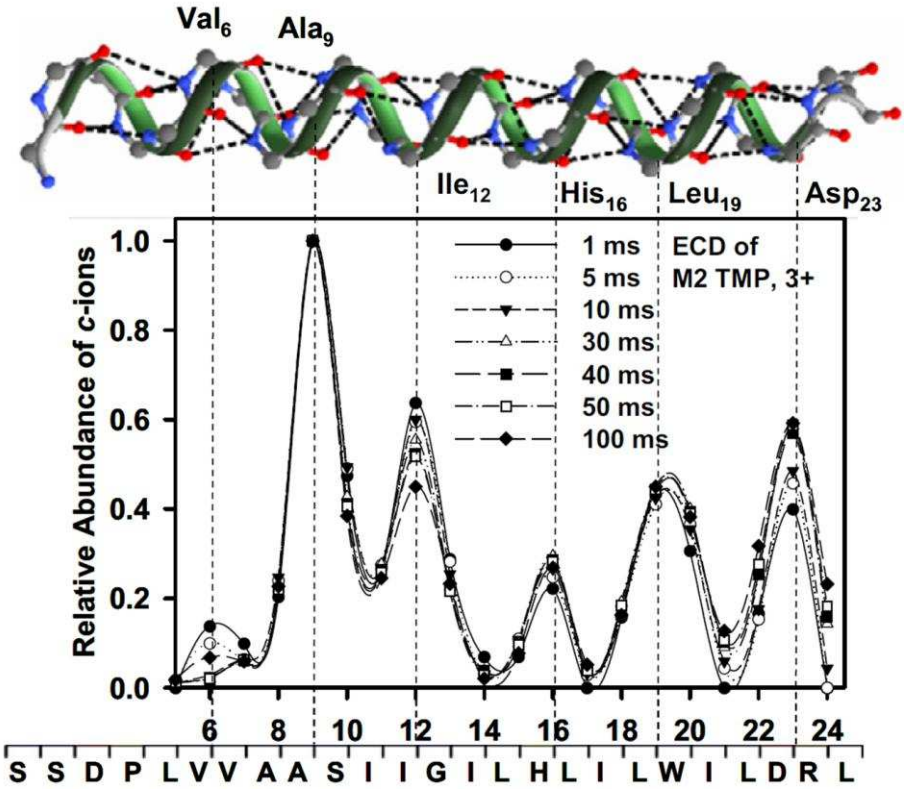

Figure 4. Periodic product-ion abundance in the ECD spectrum of a 3+ α-helical transmembrane domain of the influenza virus A membrane protein M2. Adapted with permission from (Ben Hamidane, et al., 2009b).

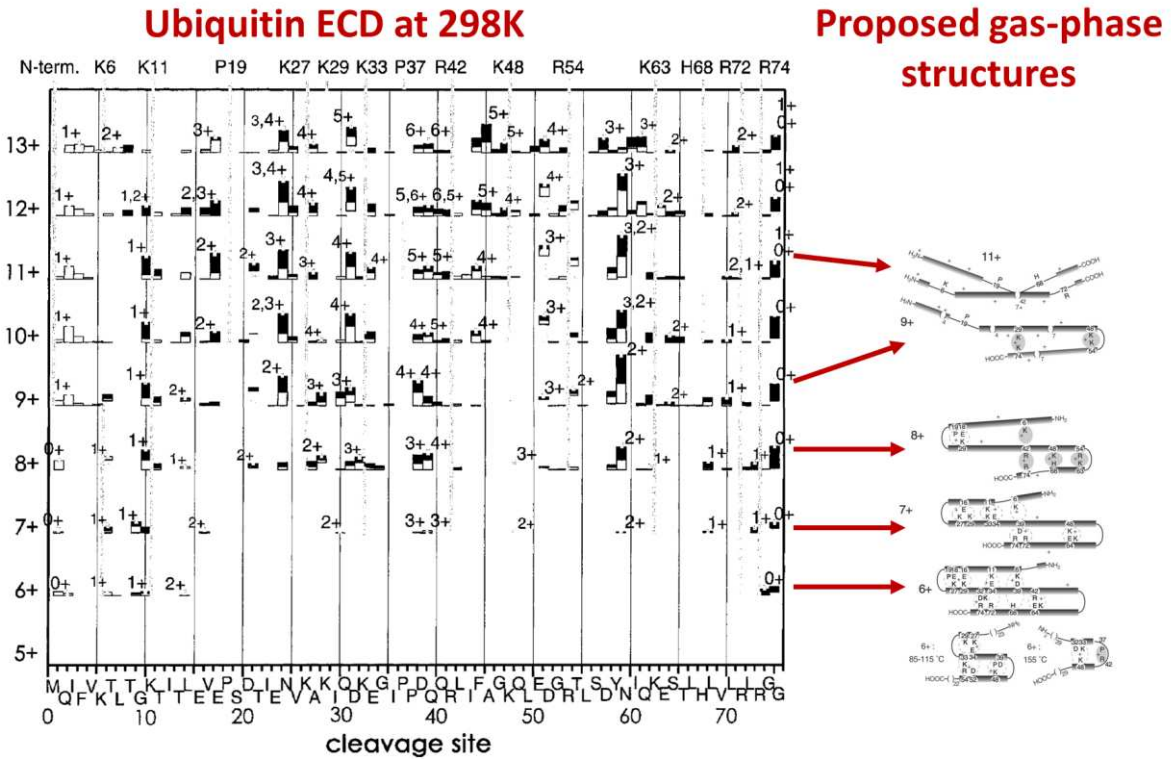

Figure 5. ECD as a structural probe for different ubiquitin charge states. Fragment intensities per cleavage site for charge states between 6+ and 13+ are shown on the left (black segments – *c*-fragments, open segments – *z*-fragments, grey fragments – *a*- and *y*-fragments), whereas the right-hand side shows proposed gas-phase structures with salt bridge patterns that account for regions that display low or no dissociation. Adapted with permission from (Breuker, et al., 2002) (Copyright (2002) American Chemical Society) and (Oh, et al., 2002) (Copyright (2002) National Academy of Sciences).

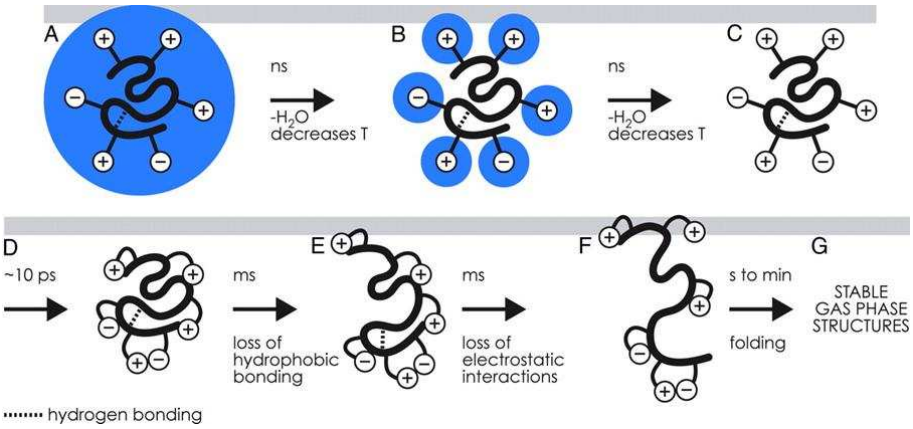

Figure 6. Stepwise evolution after ESI of the structure of a globular protein (*e.g.*, cytochrome c, ubiquitin). (A) Native protein covered with a monolayer of H_2O, followed by (B) nanosecond H_2O loss and concomitant cooling. (C) Exterior ionic functionalities lose hydration and rapidly (about 10 picoseconds) collapse. (D) The exterior-collapsed "near-native" protein, subsequently undergoes thermal re-equilibration, *via* (E) millisecond loss of hydrophobic bonding, and (F) millisecond loss of electrostatic interactions. (G) Formation of new noncovalent bonds occurs in seconds, and ultimately leads to stabilization to conformers that represent gas-phase energy minima in minutes. Reprinted with permission from (Breuker& McLafferty, 2008) (Copyright (2008) National Academy of Sciences).

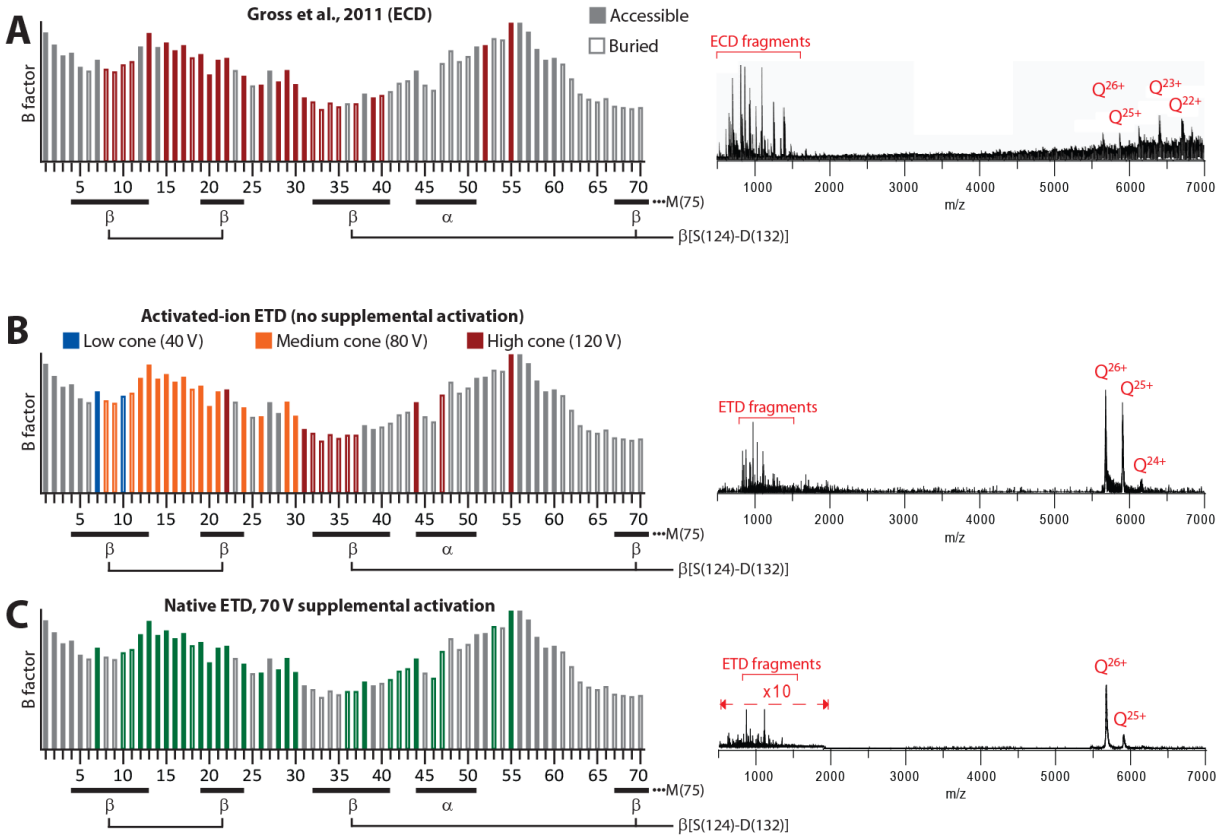

Figure 7. Fragmentation patterns observed in ECD and ETD of the native ADH tetramer. (A) FTICR-ECD with the entire native charge state distribution subjected to ECD; (B) QTOF-ETD of the 26+ tetramer with varying degrees of pre-ETD collisional activation and no supplemental activation (only additional fragments colored for higher cone voltages, *i.e.,* fragments observed with 40 or 80 V pre-heating are generally also found with 120 V); (C), as in (B), but with minimal pre-ETD voltages and 70 V of supplemental activation. Panel (A) is based on data published in (Zhang, et al., 2011a) (Copyright (2011) American Chemical Society); data to generate panels (B) and (C) is found in (Lermyte& Sobott, 2015). Crystallographic B factor is shown on the vertical axis. Horizontal axis only shows the first 70 (of 347) N-terminal residues for clarity (no fragments from further along the sequence were observed). Panels on the right shown the corresponding ExD spectra (note the 10-fold magnification of the region that contains ETD fragments in Panel C), with the result of 120 V of pre-heating shown in Panel B.

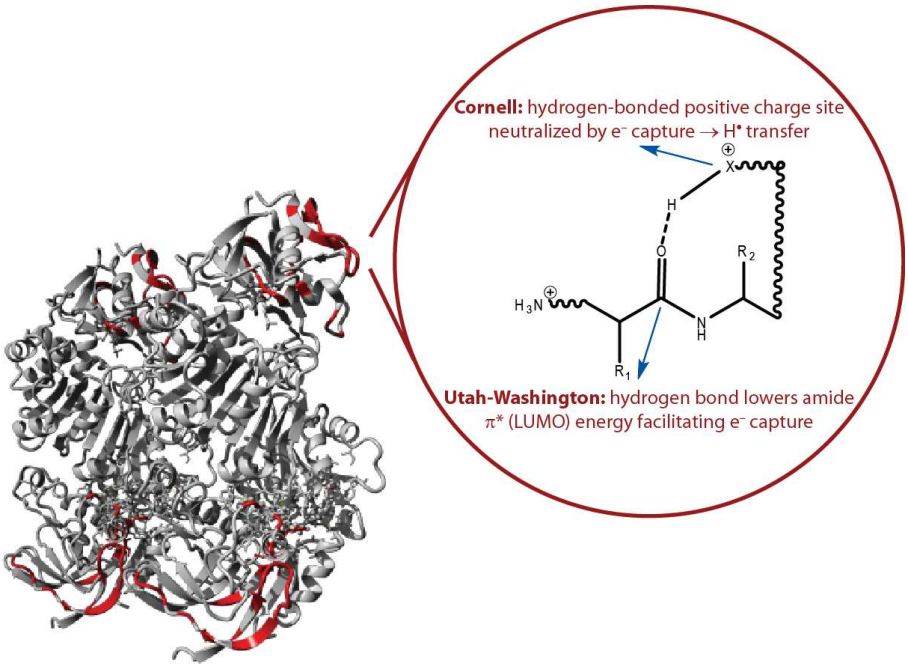

Figure 8. Crystal structure of the ADH tetramer (Protein Data Bank accession code 4W6Z) with ETD cleavage sites observed with minimal pre-ETD voltages and 70 V of supplemental activation (as in Figure 7C) shown in red. Inset shows how cleavage near charge sites (mostly found on the exposed surface) is expected in both the Cornell and Utah-Washington mechanisms for ExD.

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