REACTIONS OF POLYPEPTIDE IONS WITH ELECTRONS IN THE GAS PHASE

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Received 8 October 2002; revised 19 January 2003; accepted 26 January 2003

Reactions of electrons in the energy range below 70 eV with polypeptide cations and anions are reviewed, as well as their applications for the structural analysis of polypeptides. At very low energies (≤0.1 eV), the major outcome is electron-capture dissociation (ECD) of S–S and backbone N–C_α bonds, leading to c′ and z· fragments. ECD is useful in sequencing and characterization of post-translational modifications (PTMs), because c′, z· fragmentation is abundant and the fragments usually retain labile groups. Electron capture at higher energies (3–13 eV) induces secondary fragmentation in radical z· fragments; this hot ECD (HECD) allows one to distinguish between the isomeric leucine and isoleucine residues. If a hot electron is not captured, then the induced electronic excitation converts internally into vibrational energy, resulting in fragmentation of the C(O)–N backbone bond (so-called EIEIO process). Above 9–10 eV, further ionization of n-charged cations occurs. If the formed (n + 1)+· cations capture electrons, then the C_α–C backbone bond is usually broken. For anions that collide with ≈20 eV electrons, the ejection of an electron leads to the creation of a radical positive charge (hole) that recombines internally with a negative charge. Such recombination leads to various backbone bond cleavages. This electron-detachment dissociation (EDD) is analogous to ECD for negative ions.
 Mass Spec Rev 22:57–77, 2003; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mas.10042
Keywords: *peptide fragmentation; electron ionization; electron capture dissociation; radical ions*

Contract grant sponsor: Danish National Research Councils; Contract grant numbers: STVF-0001242, SNF-51-00-0358, STVF-26-01-0058; Contract grant sponsor: INTAS; Contract grant numbers: 99-478, 99-647.
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I. INTRODUCTION

A. Challenges in the Gas-Phase Fragmentation of Polypeptides

Organic mass spectrometry has been historically based on electron-impact ionization (EI). As the interest of the scientific community has shifted from small organic molecules to biopolymers, the shortcomings of EI became apparent. The major drawbacks were the frequent absence of the molecular ion peak, complicated dissociation pattern, insufficient sensitivity for large molecules, and a very limited mass range. Despite partial success in volatilizing small polypeptides by derivatizing their functional groups and in rationalizing their fragmentation (Biemann, 1962), EI-based MS techniques remained largely inadequate for these molecules. It was, therefore, with a great enthusiasm that the advent of ''soft'' ionization techniques, especially the recent matrix-assisted laser desorption/ionization (MALDI, Karas & Hillenkamp, 1988) and electrospray ionization (ESI, Fenn et al., 1989), was met by the MS community. Electrons and polypeptides became separated, because the MS instrumentation devoted to studying polypeptide samples would no longer feature electron sources. For a while, it looked like the polypeptide/electron combination would never be of interest again. However, the discovery of electron-capture dissociation (ECD) (Zubarev, Kelleher, & McLafferty, 1998), showed that there is still a significant unused potential in electrons in application to biopolymers. ECD is a kind of ''soft'' fragmentation technique that breaks preferentially certain strong bonds (mainly S–S and N–C_α backbone bonds) while the fragments, even containing very labile bonds, often survive until detection. Exploration of the ECD properties led to the discovery of other ion–electron reactions, confirming the notion that mass spectrometry has not yet exhausted its fundamental basis. These reactions fragment polypeptides in a different way from ECD and conventional vibrational (collisional CAD or infrared IRMPD) excitation, and, therefore, yield additional structural information.

To be sure, ion–electron reactions that involve peptides and proteins are still rather rarely used in mass spectrometry laboratories. One of the reasons for that fact is that their instrumental realization is limited to Fourier transform ion cyclotron resonance (FT ICR) mass spectrometers. Making these reactions useful in routine analyses requires combined efforts of more than just a few research groups. One of the objectives of this review is to increase the awareness of the broad scientific community of the potential of ion–electron reactions in analytical applications, and to encourage other research groups to join this dynamic and rapidly developing field. In doing so, we intend to demonstrate how various ion–electron reactions can potentially contribute to addressing some of the challenges in mass spectrometry of peptides and proteins. Because only a few ion–electron reactions have been thoroughly studied, and not all conceivable reactions have been observed, there is plenty of fundamental research yet to be done. And finally, because the success of a technique often hinges on its instrumental realization, we would like to attract the attention of instrument developers to these reactions.

The ultimate goal of mass spectrometry of polypeptides is to achieve absolute sensitivity and specificity. Whereas first objective requires the ionization efficiency to approach unity, the second goal means obtaining a set of data that uniquely characterize the primary, secondary, and tertiary structure of the polypeptide, including the determination of the site and type of all post-translational modifications (PTMs). Primary structure (sequence) determination usually implies the cleavage of bonds between all residues. Determination of the position of a labile PTM demands that the backbone cleavage is ''soft;'' i.e., preferential despite the presence in the molecule of much more labile bonding. Another challenge is of quite a different nature. Because two amino acids, leucine and isoleucine, are constitutional isomers, distinguishing between them requires, in addition to primary backbone cleavage, secondary fragmentation of the side chains. On one hand, the determination of the secondary structure requires fragmentation sensitive to it, and to some extent preserving it. On the other hand, sequencing of large molecules is impossible without first breaking the weak bonding of the secondary structure. If these requirements sound contradictory, then it is because they are. It comes, therefore, as a surprise that ion–electron reactions with only two adjustable parameters—electron kinetic energy and flux—seem to be capable of meeting all these needs.

One of the ion–electron reactions, ECD, is more known than other reactions. Several reviews have been published that highlighted the ECD basic principles (Zubarev, 2000), history and mechanistic studies (Zubarev et al., 2002), as well as potential analytical applications to proteins (McLafferty et al., 2001) and peptides (Zubarev, 2002). Yet, other reactions may be more useful for specific applications and, therefore, deserve attention as well. Below, we describe the fundamental interactions that lie in the basis of all these reactions.

B. Ion–Electron Interactions

An electron interacting with a polyatomic cation engages in a range of reactions, depending upon its energy. At very low energies ($\leq$0.1 eV), the major outcome is electron capture (reduction of the cation), almost always dissociative:

$$[\mathrm{M}+n\mathrm{H}]^{n+}+\mathrm{e}^{-}\rightarrow\left([\mathrm{M}+n\mathrm{H}]^{(n-1)+\cdot}\right)_{\text{transient}}\rightarrow\text{fragments}. \quad (1)$$

At higher energies (a few electronvolts), the cross-section for electron capture is greatly reduced, and vibrational degrees of freedom can be effectively excited, which can result in vibration-induced dissociation:

$$[M + nH]^{n+} + e^- \rightarrow ([M + nH]^{n+})_{hot} + e^- \rightarrow \text{fragments} + e^-. \quad (2)$$

As the energy range of 5–7 eV is approached, electronic excitation becomes possible, which can result in either prompt dissociation:

$$[M + nH]^{n+} + e^- \rightarrow ([M + nH]^{n+})^* + e^- \rightarrow \text{fragments} + e^- \quad (3)$$

or in slower vibration-induced dissociation due to intramolecular energy conversion and redistribution:

$$[M + nH]^{n+} + e^- \rightarrow ([M + nH]^{n+})^* + e^- \rightarrow ([M + nH]^{n+})_{hot} + e^- \rightarrow \text{fragments} + e^-. \quad (4)$$

Electronic excitation can be followed by electron capture; in this case, a cascade of fragmentation reactions occurs:

$$[M + nH]^{n+} + e^- \rightarrow ([M + nH]^{(n-1)+\cdot})^*_{transient} \rightarrow \text{fragments} \rightarrow \text{secondary fragments}. \quad (5)$$

Above the ionization limit (>9 eV), ejection of an electron may take place, resulting in further ionization:

$$[M + nH]^{n+} + e^- \rightarrow [M + nH]^{(n+1)+\cdot} + 2e^-. \quad (6)$$

For anions, the same process leads to charge-state reduction (oxidation):

$$[M - nH]^{n-} + e^- \rightarrow [M - nH]^{(n-1)-\cdot} + 2e^-. \quad (7)$$

The excess of energy deposited by the electrons can also lead to electronic excitation and fragmentation:

$$[M - nH]^{n-} + e^- \rightarrow ([M - nH]^{(n-1)-\cdot})^*_{transient} + 2e^- \rightarrow \text{fragments} + e^-. \quad (8)$$

And, finally, more energetic electrons may cause double ionization and Coulombic explosion. Although the last reaction is yet to be observed for polypeptide ions, the application of reactions 1–8 (Equations 1–8) to polypeptides are described below.

C. Nomenclature

An interaction of electrons with polypeptides leads usually to the production of polypeptide radical ions. If we disregard the protons (or their absence) that are responsible for charging, then two forms of peptide radicals can be distinguished. *Hydrogen-deficient* radicals require the addition of a hydrogen atom to become polypeptide molecules, whereas *hydrogen-abundant* species possess one or more extra hydrogen atoms. For example, $[M + H]^{2+\cdot}$ is a hydrogen-deficient radical, and so is the radical cation $M^{+\cdot}$, whereas the species $[M + 2H]^{+\cdot}$ that are formed in ECD are hydrogen-abundant radical ions.

Generally speaking, this classification is relative, because it depends upon the assumptions as to the chemistry of M. In this review, M is the ''conventional'' polypeptide molecule. But, the two kinds of radicals are different not only in origin but also in properties. For instance, they exhibit different fragmentation behavior upon vibrational (collisional or infrared) activation.

Hydrogen-deficient cations can be produced from neutral or protonated polypeptides by ionization. An alternative method of production of these species by fragmenting multiply charged peptide–metal complexes has been suggested by Siu's group (Chu et al., 2000, 2001). Hydrogen-deficient radical cations are unstable against intra-molecular hydrogen rearrangement, leading to distonic ions, where the charged sites and the radical site are separated, with the positive charge being due to protonation. The radical site in distonic ions often initiates dissociation.

Hydrogen-abundant radical n-charged cations are formed by electron capture from $(n + 1)$-protonated molecules. In these species, at least one hydrogen atom is bound more or less loosely (H· affinity to carbonyl oxygen is only 0.6 eV (Zubarev et al., 2002), whereas the typical C–H bond strength is >4 eV). The loosely bound hydrogen atom can be lost as a result of vibrational excitation. Theoretically, hydrogen-abundant radicals can be formed from protonated molecules by hydrogen-atom capture. So far, the attempts to observe this process experimentally have been unsuccessful. Demirev reported hydrogen-atom exchange but the absence of capture for ≈ 1 eV hydrogen atoms (Demirev, 2000); other groups confirmed this observation (Zubarev et al., 2002).

In the case of anions, only hydrogen-deficient $[M - nH]^{(n-1)-\cdot}$ polypeptide species have been observed so far. These species can be produced by the ejection of an electron from deprotonated molecules $[M - nH]^{n-}$ by energetic electrons (Zubarev et al., 2000).

In treating peptide fragmentation, we will follow the amended nomenclature (Kjeldsen et al., 2002), in which, like in the commonly used notation (Roepstorff & Fohlman, 1984; Biemann, 1988) backbone C_α–C, C–N, and N–C_α bond cleavages after the n-th residue in a k-peptide produce N-terminal a_n, b_n, and c_n fragments, respectively, as well as complementary to them the x_{k-n}, y_{k-n}, and z_{k-n}

products. However, unlike in other nomenclatures, homolytic cleavage of all bonds is implicitly assumed, and the presence of a radical and hydrogen-atom transfers are explicitly designated. Thus, homolytic cleavage of the N–C_α bond in M would yield $c\cdot$ and $z\cdot$ products, whereas hydrogen-atom transfer to the N-terminal fragment gives c' and z, and to the C-terminus c and z' species. The heterolytic peptide-bond cleavage that typically occurs in low-energy collisions leads in this nomenclature to b and y' products. Note that everything said so far applies to neutral species. The charge in ions is assumed to be due to either protonation (in cations) or deprotonation (in anions). The number of extra protons or deprotonation sites and thus the charge state of the fragments is given by the superscript; for example, doubly protonated c_{12}' fragment is designated as $c_{12}'^{2+}$.

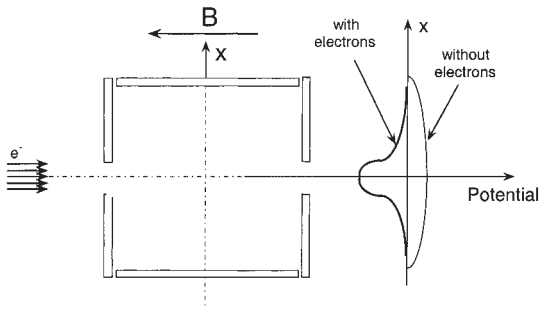

FIGURE 1. Radial distribution of the trapping electrostatic potential created by a broad and intense electron beam in a FT cell. (Reproduced from Tsybin et al., 2001 with permission. Copyright 2001 John Wiley & Sons, Ltd.)

II. INSTRUMENTAL REALIZATION AND APPLICATIONS

Ion–electron reactions are best realized in Fourier Transform mass spectrometry. The latter technique has been extensively reviewed (e.g., Marshall, Hendrickson, & Jackson, 1998). All commercial FT ICR instruments can, in principle, be used to implement these reactions. The size of the magnet is not believed to be a limitation: good data have been acquired with a 3 T magnet as the lowest (Polfer et al., 2002) and 9.4 T as the highest (Axelsson et al., 1999). The parallel strong magnetic and weak electrostatic fields that trap the ions inside the Penning cell of a FT ICR instrument do not change significantly the kinetic energy of the electrons in the region of ion–electron interaction, which permits long interaction times (minutes) and energy-resolved measurements. In contrast, in quadrupole ion traps, where the ions are confined by *rf* voltage applied to the ring electrode, the potential in the center is swinging with the frequency of the *rf* voltage, which is the main reason that prevented so far the implementation of ECD and other ion–electron reactions in these instruments.

In the first ECD experiments, low-energy electrons were produced by UV laser irradiation of the surrounding walls and electrodes. Later, the laser was replaced by a filament-based electron source (Zubarev, Kelleher, & McLafferty, 1998). Recently, it has been realized that these sources possess inherent limitations—the most serious of which is the narrow width of the electron beam (Haselmann et al., 2001). When an electron beam of much smaller diameter than the ion cloud interacts with this cloud, the rate of interaction is limited by ion mixing inside the cloud. The rate of interaction of an electron beam as broad as the ion cloud is, on the other hand, determined mainly by the electron density (Tsybin et al., 2001). Broad and intense electron beams can trap cations by creating a potential well for them (Donets, 1976), as illustrated in Figure 1. The trapped cations interact with electrons more efficiently than the free species that can escape from a narrow electron beam. If the trapped cation dissociates, then cationic products can become trapped as well, which increases the collection efficiency of fragmentation reactions. These considerations led to the introduction of dispenser cathodes as efficient electron sources for ion–electron reactions, which reduced the required irradiation time from seconds to milliseconds (Haselmann et al., 2001; Tsybin et al., 2001). As an example, Figure 2 shows a single-acquisition (''single scan'') ECD spectrum of substance P dications irradiated with electrons for only 5 ms. Such short irradiation times, in turn, permitted on-line use of HPLC in combination with ECD (Davidson & Frego, 2002; Palmblad et al., 2002). Palmblad et al. identified in a single HPLC run 75 tryptic peptides of the protein BSA, 15 of which gave ECD fragments during the chromatographic

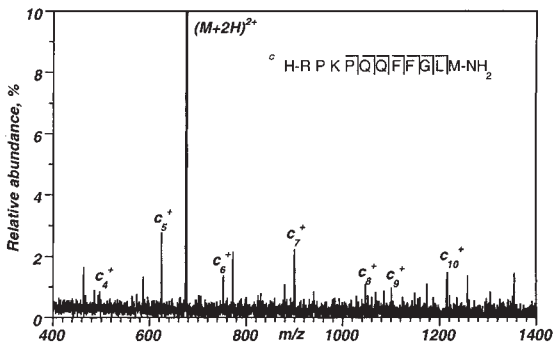

FIGURE 2. One-scan electron-capture dissociation (ECD) spectrum of dications of substance P obtained by 5 ms electron irradiation. (Reproduced from Tsybin et al., 2001 with permission. Copyright 2001 John Wiley & Sons, Ltd.)

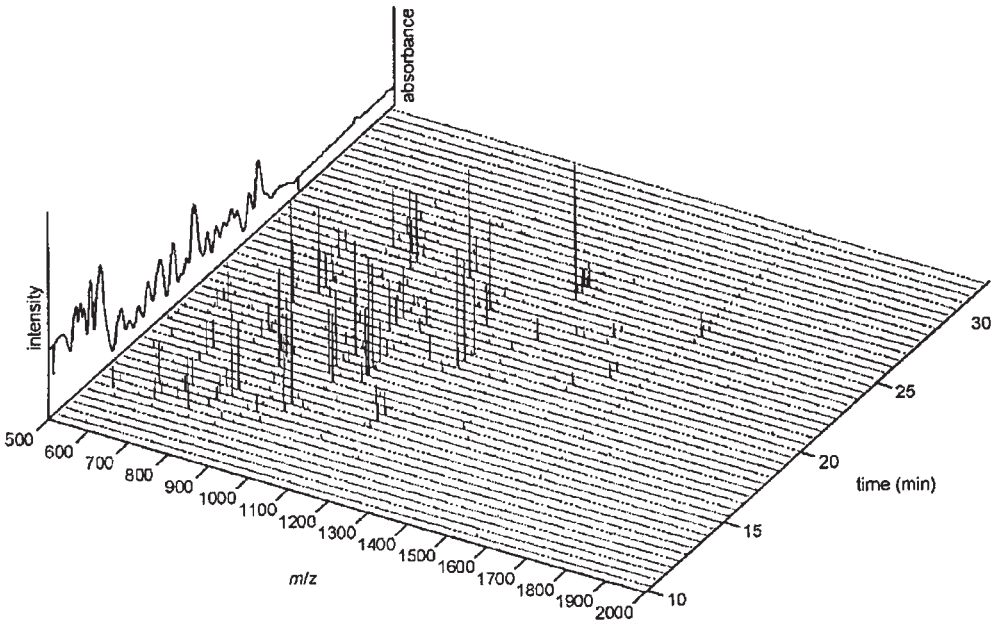

FIGURE 3. LC/FTICRMS/ECD data on a BSA tryptic digest without precursor-ion isolation. (Reproduced from Palmblad et al., 2002 with permission. Copyright 2002 John Wiley & Sons, Ltd.)

run (Fig. 3). Davidson and Frego fragmented by ECD pepsin digest peptides of cytochrome *c* separated with on-line micro-HPLC (Fig. 4). These achievements represent the first breakthroughs on the way to the implementation of ion–electron reactions in traditional proteomics studies.

Among other achievements, one should name the remarkable work of McLafferty and co-workers, who pursue the ''top-down'' approach of the characterization of intact proteins (e.g., McLafferty et al., 1999). In their ''activated ion ECD'' (AI ECD) technique (Horn, Ge, & McLafferty, 2000), the precursor ions of $\leq$45 kDa molecules are first vibrationally excited to unfold the tertiary structure and to break intra-molecular weak bonding, after which (or simultaneously) irradiation with electrons takes place (Fig. 5). Although it remains to be shown that labile PTMs survive AI ECD, the amount of sequence information that this technique produces in a single ECD spectrum is truly impressive (Sze et al., 2002).

Most of the literature published so far on ion–electron reactions concern either proof-of-principle, fundamental, or instrumental issues. However, reports on applications to biologically relevant problems have also started to appear. McLafferty and co-workers characterized deviations in protein sequences from those predicted by DNA analysis (Ge et al., 2002). Fenselau and colleagues applied ECD for characterization of biomarkers from spores of *Bacillus cereus* T (Demirev, Ramirez, & Fenselau, 2001). Costello and co-workers characterized with ECD and CAD a proline-rich protein PRP-3 (Leymarie et al., 2002). Undoubtedly, many more application reports are either in press or in preparation.

III. ELECTRON-CAPTURE DISSOCIATION

A. Electron Capture

When an electron approaches a cation, both species experience mutual Coulombic attraction. For this attraction to result in capture, the cation has to dissipate the excess of energy within a very short time interval, $<10^{-14}$ s (Bardsley & Biondi, 1970). Of course, the electron by itself cannot dissipate energy because of the absence of internal degrees of freedom. The atomic movements in the ion could absorb the excess of energy, but are too slow ($\approx 10^{-13}$ s). Radiative dissipation (emission of a photon) is another possibility, but it typically proceeds at an even slower pace ($\geq 10^{-9}$ s). Because of the absence of available channels, the recombination rates of atomic cations, even multiply charged, are slow. For polyatomic cations, there is a convenient outcome, prompt dissociation that stabilizes the electron within 10^{-16}–10^{-15} s (Bardsley & Biondi, 1970). In this case, electron capture and bond cleavage appear to be one concerted process. Such dissociation is known as dissociative recombination (DR), and it proceeds at a very high rate, exceeding the rate of ion-molecule reactions by orders of magnitude. The experimentally

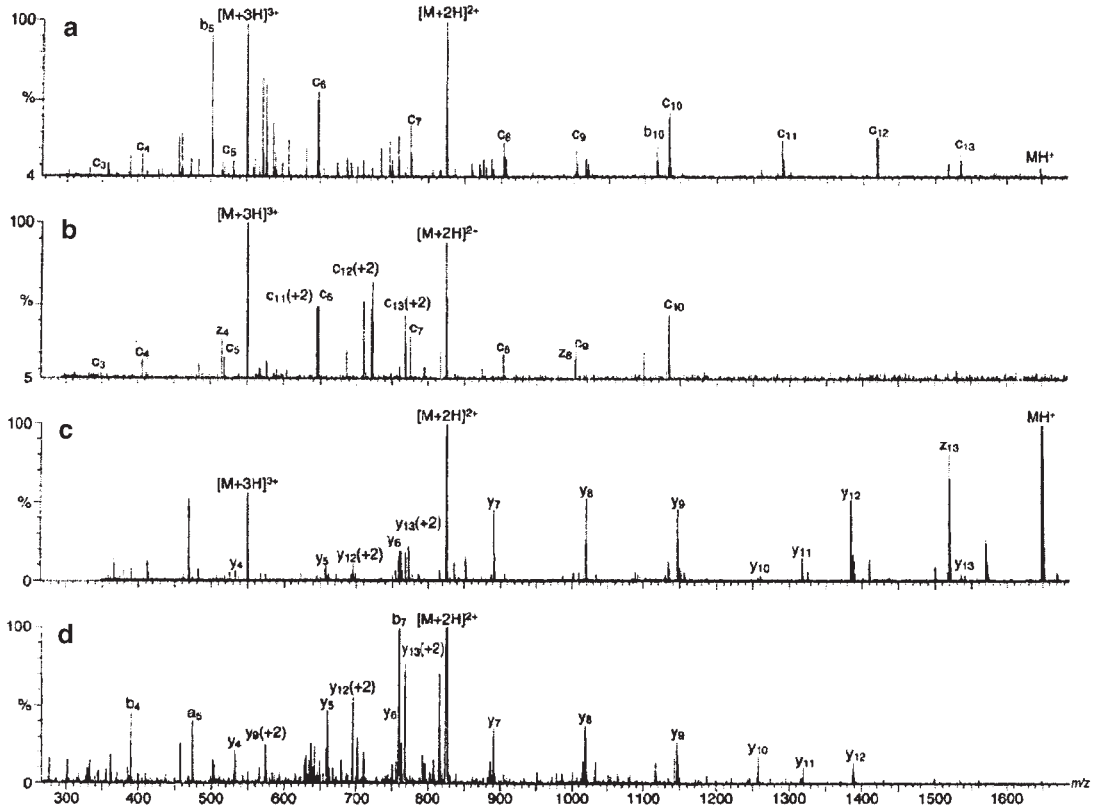

FIGURE 4. Spectra of peptide 81–94 of cytochrome *c* obtained with: (**a**) μHPLC/FTMS/ECD without precursor-ion isolation; (**b**) the same with isolation of 3 + precursor ion; (**c**) μHPLC/CAD/FTMS with nozzle-skimmer vibrational excitation in the ion source without precursor-ion isolation; (**d**) LC/MS/MS, using a triple quadrupole instrument with CAD. (Reproduced from Davidson & Frego, 2002 with permission. Copyright 2002 John Wiley & Sons, Ltd.)

observed high rates do not contradict the possibility that the electron may be captured first on a high Rydberg orbit, which may have a lifetime considerably longer than 10^{-15} s (so-called indirect DR). What is important in this context is that, even in this case, the bond-cleavage mechanism appears to be mostly electronic, and that it proceeds faster than atomic vibrations and rearrangements. As a result, statistical theories cannot account for the observed branching ratios (e.g., Andersen et al., 1996).

It is still an open question whether ECD is a variant of DR. Of several dozens of cationic species that have been studied in DR experiments, no one can be viewed as a reasonable model of multiply protonated peptides. Still, some parallels can be drawn. For instance, DR of protonated hydrocarbon molecules seems to occur through the neutralization of the protonated site, involve rupture of a single bond, and release of a hydrogen atom or a small group involved in protonation (Mitchell & Rebrion-Rowe, 1997). Alternative to DR mechanisms of ECD, more appealing to chemical intuition, have also been proposed.

B. N–C_α Bond Cleavage

The striking feature of ECD spectra of peptide polycations is the absence of the *b* and *y*′ ions that are familiar from collisional and infrared activation mass spectra, and the presence instead of *c*′ and *z*· fragment species. Although *b* and *y*′ ions originate from the dissociation of the peptide (amide) bond, *c*′ and *z*· ions arise from the cleavage of the N–C_α (amine) backbone bonds. The reason for the absence (or low abundance) of the peptide-bond cleavage in ECD is believed to be due to two circumstances. First, the peptide-bond fragmentation occurs through intermediates that require relatively long times (up to microseconds) to form (Tsaprailis et al., 1999). These times are so long compared to the rate of intra-molecular vibrational energy redistribution (IVR), that peptide-bond fragmentation is almost always ergodic. On the contrary, ECD is believed to occur faster than 10^{-12} s, which allows only direct bond scission to happen. Perhaps more importantly, the fragmenting species in ECD are not even-electron ions, as in vibrational excitation, but hydrogen-abundant radical cations.

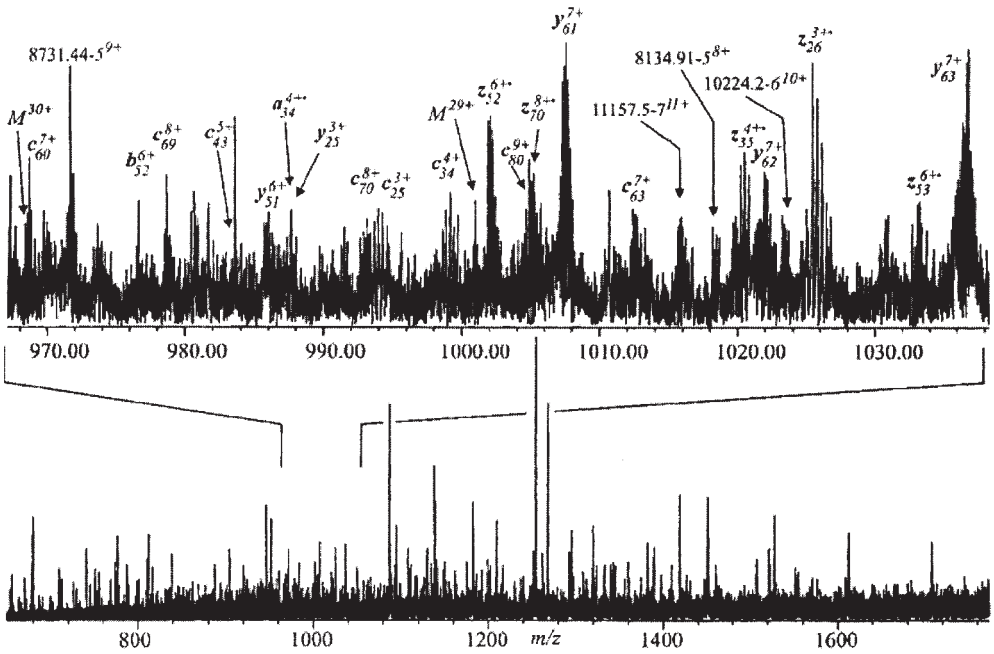

FIGURE 5. Activated ion ECD (AI ECD) spectrum (50 scans) of carbonic anhydrase B (29 kDa) without precursor-ion isolation. (Reproduced from Horn, Ge, & McLafferty, 2000 with permission. Copyright 2000 National Academy of Sciences.)

The presence of a radical site changes the strengths of the adjacent bonds. Thus radical-site-induced cleavage following the capture of the excess hydrogen atom by the carbonyl oxygen has been proposed as an explanation for N–C_α bond fragmentation (Zubarev et al., 1999):

$$\begin{aligned} R_1\text{–}C(O)\text{–}NH\text{–}R_2 + H\cdot &\leftrightarrow R_1\text{–}\cdot C(OH)\text{–}NH\text{–}R_2 \\ &\rightarrow R_1\text{–}C(OH)\text{=}NH + \cdot R_2. \end{aligned} \quad (9)$$

High-level calculations showed that it takes only 19 kJ/mol to produce CH_3–C(OH)=NH and $\cdot CH_3$ fragments from the CH_3–$\cdot$C(OH)–NH–CH_3 radical (Zubarev et al., 2002). This energy is small compared to the available recombination energy between 400 and 700 kJ/mol. Yet the presence of a 123 ± 5 kJ/mol barrier for N–C_α bond cleavage found in the calculations poses a certain difficulty, due to the competition with the alternative channel—the desorption of the hydrogen atom from the carbonyl group. This competition has been studied by a simplified (ergodic) theoretical model that showed that, in order to proceed with a higher rate than H$\cdot$ desorption, the N–C_α bond cleavage should occur faster than 10^{-12} s (Zubarev et al., 1999). This model, in turn, requires concentration of almost all recombination energy in just a few bonds around the cleavage site. In a larger molecule, such a concentration of energy is unlikely and contradicts the ergodic conditions assumed for that model.

Because of the complication caused by the large barrier, an alternative mechanism has been suggested within the hydrogen-atom model. In this mechanism, hydrogen atom ''bounces'' of the carbonyl oxygen atom without capture (or with reversible capture) and attacks instead the backbone nitrogen atoms, forming a transient hypervalent species. Calculations show that these species are unstable (as has been famously predicted by McLafferty (1986)) and fragment without a barrier, giving $z\cdot$ and isomeric c' fragments (Zubarev et al., 2002):

$$\begin{aligned} R_1\text{–}C(O)\text{–}NH\text{–}R_2 + H\cdot &\rightarrow R_1\text{–}C(O)\text{–}(\cdot HNH)\text{–}R_2 \\ &\rightarrow R_1\text{–}C(O)\text{–}NH_2 + \cdot R_2. \end{aligned} \quad (10)$$

This fragmentation is by 32 kJ/mol exothermic, in contrast to the endothermic cleavage in Equation 9. However, energetic considerations alone cannot be used to distinguish between alternative mechanisms of N–C_α bond cleavage. The best hope of getting an insight into the processes involved rests with high-level molecular dynamics simulations. As for the experiments performed so far, they invariably showed that an extra hydrogen atom is present in one of the fragments, which implies hydrogen rearrangement. What remains unclear is whether this rearrangement occurred before or after N–C_α bond scission.

Another possible outcome of the H· attack on the backbone nitrogen is the amide bond cleavage, leading to y' fragments and unstable $b\cdot$ species that further fragment to CO and $a\cdot$ ions. This suggestion explains the presence of abundant $a\cdot$ species in some ECD spectra, although N–C_α fragmentation always dominates. *Ab initio* calculations showed that the CO loss is by 71 kJ/mol endothermic (Zubarev et al., 2002), and yet stable $b\cdot$ ions have not been observed in ECD of polypeptides. ECD of multiply charged *b* ions prepared by vibrational excitation of multiply protonated peptides often gives abundant CO losses, resulting in $a\cdot$ species (Haselmann et al., 2001). The relative importance of the $a\cdot$, y' channel in ECD increases with the level of vibrational excitation of the precursor ions. Given the earlier suggestion that fragmenting peptides can exist, upon peptide-bond cleavage, as charge-bound complexes of *b* and y' species (Polce, Ren, & Wesdemiotis, 2000), one can speculate that the $a\cdot$, y' channel in ECD may be due to such complexes. This suggestion requires, however, experimental verification.

C. S–S Bond Reduction

If mono- and disulfide-bonds are present in a polypeptide molecule, then fragments due to the rupture of these bonds are prominent in ECD mass spectra (Zubarev et al., 1999). This feature allows for an easy determination of the S–S bond presence and position (Fig. 6), which is especially useful given that typical conditions of low-energy collision excitation do not lead to cleavage of these bonds. The hot hydrogen-atom mechanism suggests that H· attacks disulfides that have even higher affinity to hydrogen atom than carbonyl groups. H· attachment to disulfides is irreversible and leads to rapid dissociation:

$$R_1\text{–}S\text{–}S\text{–}R_2 + e^- \rightarrow R_1\text{–}SH + \cdot S\text{–}R_2. \qquad (11)$$

The $\cdot S\text{–}R_2$ fragments are rarely observed in mass spectra because of their high reactivity. The radical site can induce backbone cleavage, such as in ECD of vasopressin dications (Zubarev et al., 1999). The ·S radical can also attack other disulfides if present in the molecule, leading to the separation of A- and B-chains in insulin (Zubarev et al., 1999). The latter process is facilitated by vibrational excitation of the molecule.

As in the case of the N–C_α bond cleavage, capture of an electron to a high-*n* Rydberg state with subsequent direct electron-induced S–S bond cleavage has been suggested as an alternative to the hot hydrogen mechanism (Zubarev et al., 1999). Further experiments, notably energy-resolved ECD, should answer the question, which

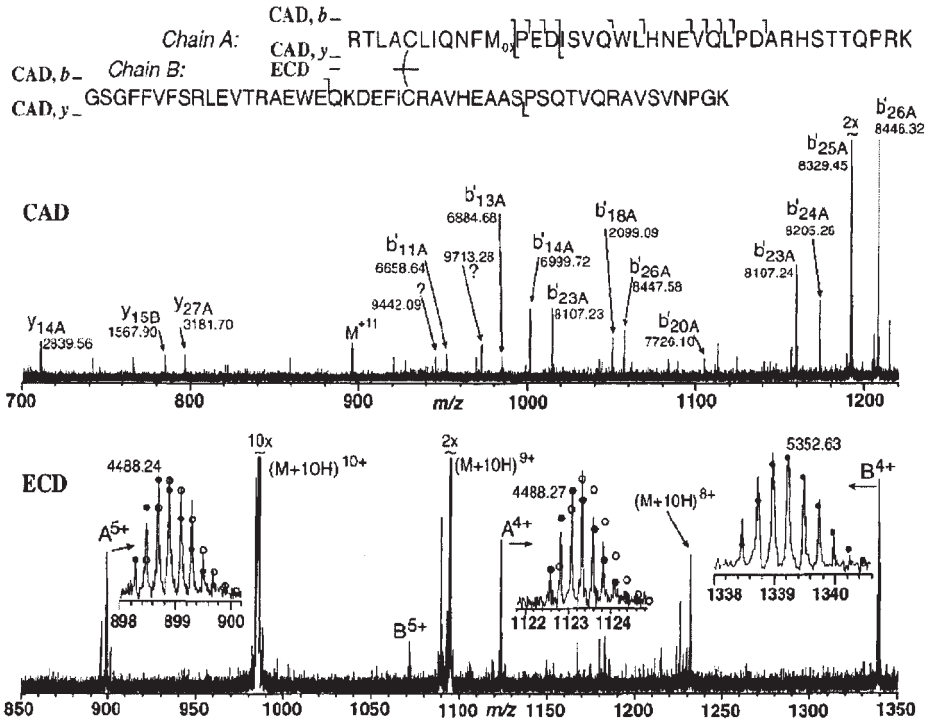

FIGURE 6. Comparison of CAD and ECD spectra of 10+ ions of disulfide-linked peptides 135–172 and 175–222 of Fcε3-4 of human IgE. Note that, whereas CAD produced backbone dissociation, ECD gave S–S bond cleavage. (Reproduced from Zubarev et al., 1999 with permission. Copyright 1999 American Chemical Society.)

of the mechanisms prevails. Again, the dominance of the fragments with an extra hydrogen atom renders the hot hydrogen-atom mechanism empirically adequate.

D. Dissociation of Strong Bonds in the Presence of Weak Bonding

One of the biggest challenges in mass spectrometric structure determination of polypeptides is the cleavage of backbone bonds in the presence of much weaker bonding, such as hydrogen bonding or a link to a PTM group. If the energy deposited during excitation is statistically distributed over all available degrees of freedom, then many bonds in the species compete for dissociation, with weaker bonds having much greater chance for rupture. Only if a large excess of energy is deposited, and the frequency factor of the stronger bond is greater than that of the weaker bond, can the stronger bond fragment at a higher rate. But even then, weak bond fragmentation cannot be totally avoided, especially if the time of observation is long (seconds) as in FT ICR. This factor is because the alternative channels for dissipation of the deposited energy are very slow. For instance, at typical FT ICR conditions, the cooling of excited molecular ions by either collisions or infrared radiation takes tens of milliseconds to seconds (Dunbar, 1992; Schnier et al., 1996). Therefore, ''clean'' fragmentation of strong bonds without dissociation of weaker linkage is a formidable task in FT ICR. If this task is to be achieved by energy deposition, then three conditions need to be met: (i) excess of energy should only slightly exceed the amount required for the desired bond rupture, (ii) deposition of energy should occur very close to the bond to be broken, and (iii) bond dissociation must proceed faster than the energy redistribution over all degrees of freedom. The latter condition is known as the non-ergodic process.

Non-ergodic cleavage in ECD has been predicted by F.W. McLafferty in the following passage: ''By using a multiply charged ion such as $H_2N–R–CON^+H_2–R'–CO–N^+H_2–R''–CON^+H_2–RCOOH$, formation from one of its charge sites of a hypervalent species, $H_2N–R–CON^+H_2–R'–CO–NH_2–R''–CON^+H_2–RCOOH$ should cause non-ergodic cleavage with relatively high efficiency at the sites of highest proton affinity, producing $H_2N–R–CON^+H_2–R'–CO\cdot + H_2N–R''–CON^+H_2–RCOOH$ as odd- and even-electron, respectively, ionic products'' (McLafferty, 1986). This remarkable prediction contains such important features of ECD as (i) neutralization at the protonation site (supported by the observations of small losses in ECD (Cooper et al., 2002)); (ii) $a\cdot$, y' backbone cleavage (Zubarev et al., 1999, 2000); and (iii) non-ergodic fragmentation. The last has been a subject of a heated debate since its postulation in the first ECD publication.

What is the evidence to support the non-ergodic mechanism? Firstly, cleavage of the strong $N–C_\alpha$ bonds on the FT ICR timescale in large (up to 45 kDa (Ge et al., 2002)) species requires the deposition of significant amounts of energy (tens of electronvolt) if the energy is to be statistically distributed over all degrees of freedom. In ECD, the energy release is $\leq$7 eV, which corresponds to just a millielectronvolt energy excess if randomized over thousands of degrees of freedom. Secondly, the already-mentioned RRKM calculations made by Carpenter supported non-RRKM timescales ($<10^{-12}$ s) and excess energy concentration in just a few bonds. But, perhaps the most convincing argument is the broad range of observations that $N–C_\alpha$ cleavage is preferred even in species that contain much more labile bonds, in stark contrast with vibrational activation that leads to their facile cleavage. The labile bonds can be the links to post-translationally added groups, such as in phosphorylation (Shi et al., 2000; Stensballe et al., 2000), *N*-glycosylation (Håkansson et al., 2001), γ-carboxylation (Kelleher et al., 1999; Niiranen et al., 2002), *O*-glycosylation (Mirgorodskaya, Roepstorff, & Zubarev, 1999; Haselmann et al., 2001), and sulfation (Kelleher et al., 1999; Haselmann et al., 2001). As an example, Figure 7 demonstrates the application of the activated-ion ECD technique for β-casein molecular ions that contain five phosphorylation sites, of which one has been identified, and three more are restricted to four possible sites (Shi et al., 2000). Figure 8 demonstrates the ECD spectrum of MUC1 peptide O-glycosylated by six N-acetylgalactoseamine (GalNAc) groups, of which four have been identified and two more restricted to a cluster of two residues (Haselmann et al., 2001).

Arguably, the determination of PTMs is the most-promising ECD application. Gamma-carboxylation, *O*-glycosylation, and sulfation are especially labile modifications that pose a great difficulty in the analysis by conventional tandem MS techniques. But the ECD ''softness'' is not limited to labile covalent bonds—even weak bonding survives the $N–C_\alpha$ bond cleavage. The preservation of intra-molecular weak bonding was first demonstrated on bovine ubiquitin (Zubarev et al., 2000), whose low charge states (6+ through 8+) preserve in the gas-phase folded tertiary structure. ECD of these tight conformers is inefficient in terms of backbone cleavages, and produces a significant amount of reduced species $[M + nH]^{(n-1)+\cdot}$. Upon even slight collisional or infrared activation, these species fall apart into *c* and *z*-type ions. The sites of the cleavages are complementary to those observed in the direct ECD of these charge states. The amount of *c*- and *z*-fragments in direct ECD showed a temperature dependence, which was used to derive information, including melting temperatures and melting enthalpy (Fig. 9), on the gas-phase conformers of ubiquitin cations (Breuker et al., 2002a). Higher charge states of

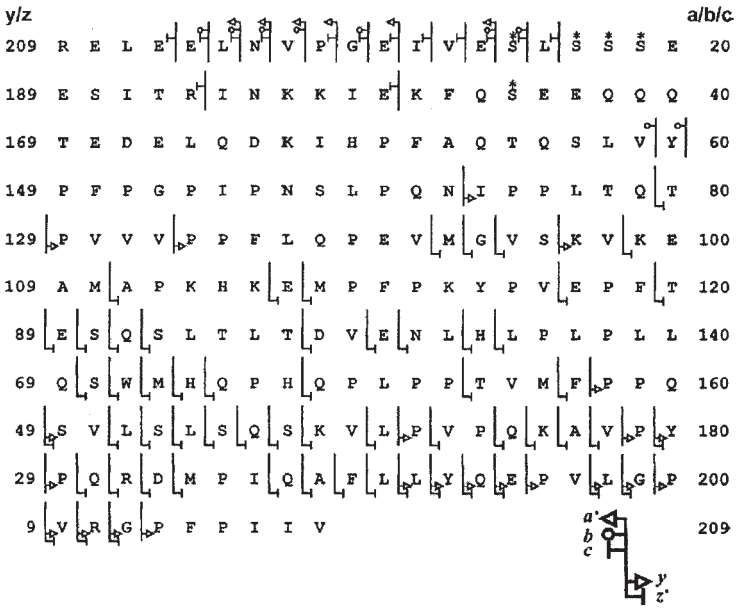

FIGURE 7. Fragmentation map of AI ECD of β-casein derived by the automatic data interpretation program THRASH. (Reproduced from Shi et al., 2000 with permission. Copyright 2000 American Chemical Society.)

ubiquitin that exist in the gas phase in an unfolded state showed no or little temperature-dependence, consistent with non-ergodic N–C_α bond cleavage. ECD was also used to detect the presence of folding intermediates of cytochrome *c* gas-phase cations; the fragmentation pattern of 15+ ions was also preserved until 130°C (Horn et al., 2001). Finally, ECD showed potential for determination of gas-phase structure of peptide–drug and peptide–peptide complexes (Haselmann et al., 2002b). The unique ability of ECD to preserve weak bonding will undoubtedly play an important role in future studies of gas-phase polypeptide structures.

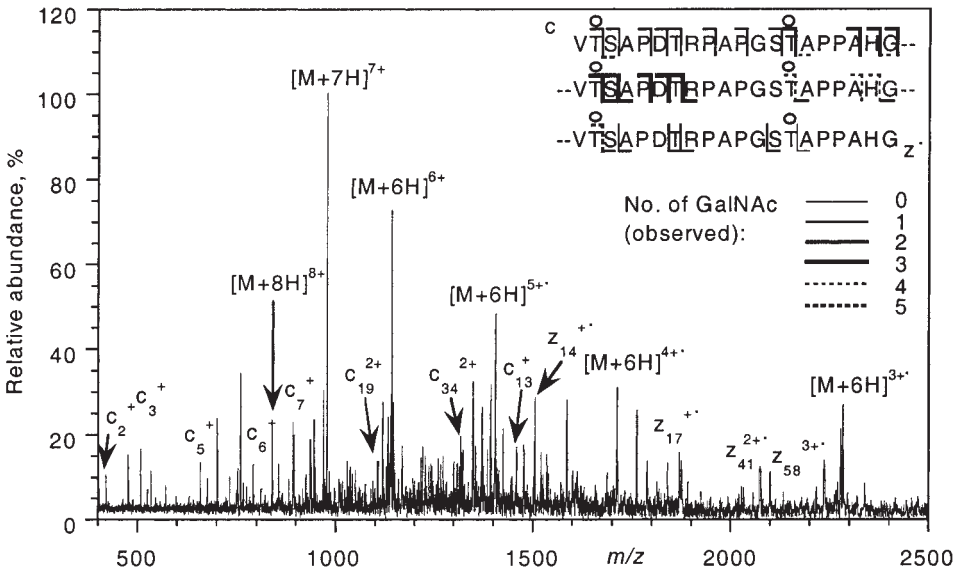

FIGURE 8. ECD mass spectrum (200 scans) of the O-glycosylated 60-residue human MUC1 peptide. (Reproduced from Haselmann et al., 2001 with permission. Copyright 2001 American Chemical Society.)

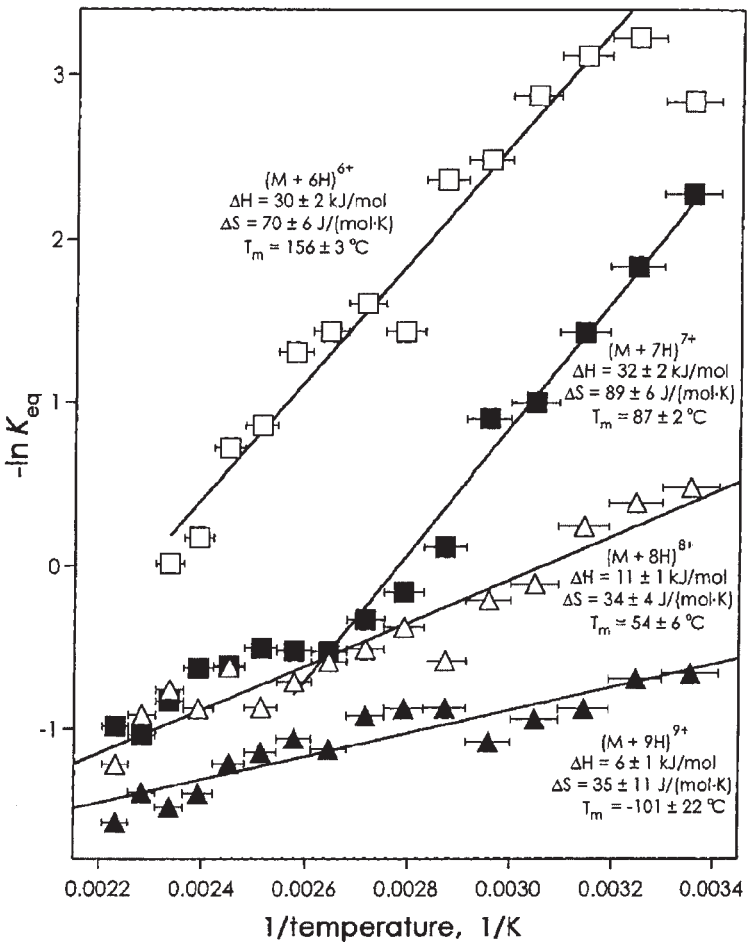

FIGURE 9. van't Hoff plot and changes of enthalpy ΔH and entropy ΔS derived from the ECD data on ubiquitin ions. (Reproduced from Breuker et al., 2002a with permission. Copyright 2002 American Chemical Society.)

E. Frequencies of N–C_α Bond Cleavage

Reliable automatic sequence determination in polypeptides requires the cleavage of all inter-residue bonds (Horn et al., 2001). In solving this task, ECD is doing much better than the conventional fragmentation techniques based on vibrational excitation (Zubarev, Kelleher, & McLafferty, 1998; Kruger et al., 1999a,b). Only the N–C_α bond cleavage N-terminal to proline is not observed in ECD spectra because of the tertiary nature of the backbone nitrogen in that residue. Luckily, the same site is the favorite position of C–N bond cleavage in vibrational excitation (Yu et al., 1993). This factor makes ECD mass spectra complementary to those produced by conventional activation techniques.

Yet even cleavage of all inter-residue bonds cannot guarantee full sequence determination, because some N-terminal and C-terminal fragments may have very similar masses, or the mass difference between them may be arbitrarily close to the mass of an amino acid. Either of these two situations can upset the sequencing algorithm (Budnik et al., 2002a). It has been suggested that the fragment *abundances* could provide the much-needed additional sequence-related information. For these abundances to be useful, clear rules must exist to predict the relative abundances of the fragments based on the sequence. Ideally, the fragment abundance (which is proportional to the cleavage probability or frequency) should be a function of only the *local* sequence (i.e., neighboring amino acid residues) and independent of more distant groups. In this ideal case, in which fragmentation is called reducible, the rules predicting the fragmentation can be based on the average relative abundances of fragments with the same local sequence. Compared to vibrational excitation, where not only the total amount of internal energy and the balance of protons and basic sites (e.g., Dongre,

Somogyi, & Wysocki, 1996), but also the secondary structure influence the fragmentation pattern, ECD gives far more reducible spectra (Budnik et al., 2002a). This result is despite the fact that ECD cleavage frequencies vary in a smaller range than those in vibrational excitation techniques (Kruger et al., 1999a). The dependence of ECD cleavage frequencies on the local sequence is illustrated in Figure 10 for three variants of bradykinin. Despite the fixed number of protons (dications were used in all three cases), and different basicities, the fragmentation patterns correlate very well. The reason for this correlation and the factors that determine the ECD cleavage abundances are not totally clear, although these factors are likely to include the distribution along the backbone of the positive charge and hydrogen-atom affinities. For instance, the high hydrogen-atom affinity of the phenylalanine residue appears to account for the enhanced cleavage next to it, consistent with the hydrogen rearrangement mechanism. The good repeatability of the average ECD cleavage abundances is due to the fact that the recombination energy is determined mostly by the Coulombic attraction between the polycation and the electron, and thus is largely condition- and instrument-independent.

IV. ELECTRONIC EXCITATION

A. Hot-Electron-Capture Dissociation (HECD)

DR cross-sections of small cations often exhibit a local maximum at the electron kinetic energy of $\approx$10 eV (Larsson, Mitchell, & Schneider, 2000). The presence of this maximum can be explained by electronic excitation prior to electron capture. Because fragmentation upon electron capture is fast, one of the fragments of the polyatomic cation may still be in an electronically excited state, which later relaxes either radiatively or via inter-system conversion. In the latter case, the excess of energy may cause secondary fragmentation. This mechanism is implied for HECD of polypeptide polycations. The greatest utility of HECD is the ability to distinguish the isomeric leucine and isoleucine residues. Xle (Leu or Ile) identification is required for protein *de novo* sequencing and is helpful in protein identification. Because 16% of all amino acids found in natural proteins are either Leu and Ile (with even greater abundance in the important trans-membrane proteins), peptide sequences longer than three residues are likely to contain at least one Xle residue. Until recently, the Xle mass spectrometric identification has been performed only by high-energy collisions, the technique no longer available on most modern mass spectrometers.

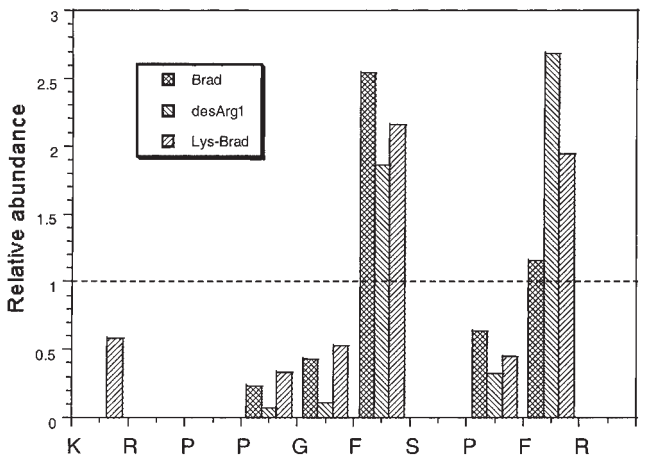

FIGURE 10. Comparison of relative ECD fragment abundances for bradykinin, des-[Arg1]-bradykinin and Lys-bradykinin dications. Note the pattern preservation despite the change in basicity. (Reproduced from Budnik et al., 2002a, with permission. Copyright 2002 Elsevier Science.)

In HECD, where polypeptide polycations capture 3–13 eV electrons, the excess of energy goes into secondary fragmentation (Kjeldsen et al., 2002). Generally, many more cleavages of different types are observed in HECD than in conventional ECD, with a smaller average mass of the fragments. Because radical ionic fragments are generally less stable than even-electron ions, secondary fragmentation of $z\cdot$ ions leads in HECD to even-electron w ions. The $\approx$1 eV endothermic cleavage between the β- and γ-carbons in the leucine and isoleucine side chains removes 43 and 29 Da, respectively, which distinguishes between these residues (Fig. 11).

In principle, N-terminal d-ions should form from $a\cdot$ fragments by a similar, but somewhat less endothermic, mechanism (Kjeldsen et al., 2002). However, no abundant d ions have been observed in the HECD of most polypeptides, likely due to the smaller abundance of the precursor $a\cdot$ ions compared to $z\cdot$ ions. For instance, for eight overlapping peptides of the PP3 bovine milk protein that contains totally 25 Xle residues, HECD has positively identified 20 Xle residues exclusively through w ions. The identities of three more Xle residues could be guessed correctly by the absence of the alternative fragment (Kjeldsen, Sørensen, & Zubarev, 2003). The fact that, in two cases, HECD failed to produce w fragments limits the analytical utility of this technique in view of the absence or low abundance of the complementary d fragments. Another obstacle on the way to reliable *de novo* sequencing of proteins is the existence of two nearly isobaric amino acids, lysine (128.095 Da) and glutamine (128.059 Da). w and d ions formed by side-chain losses from these residues have identical masses.

By hindsight, the 1 eV required for $z\cdot \rightarrow w$ conversion should also be available in the conventional ECD. Indeed, previously unreported in ECD w fragments were found for the same PP3 digest peptides, even at $<$1 eV electron

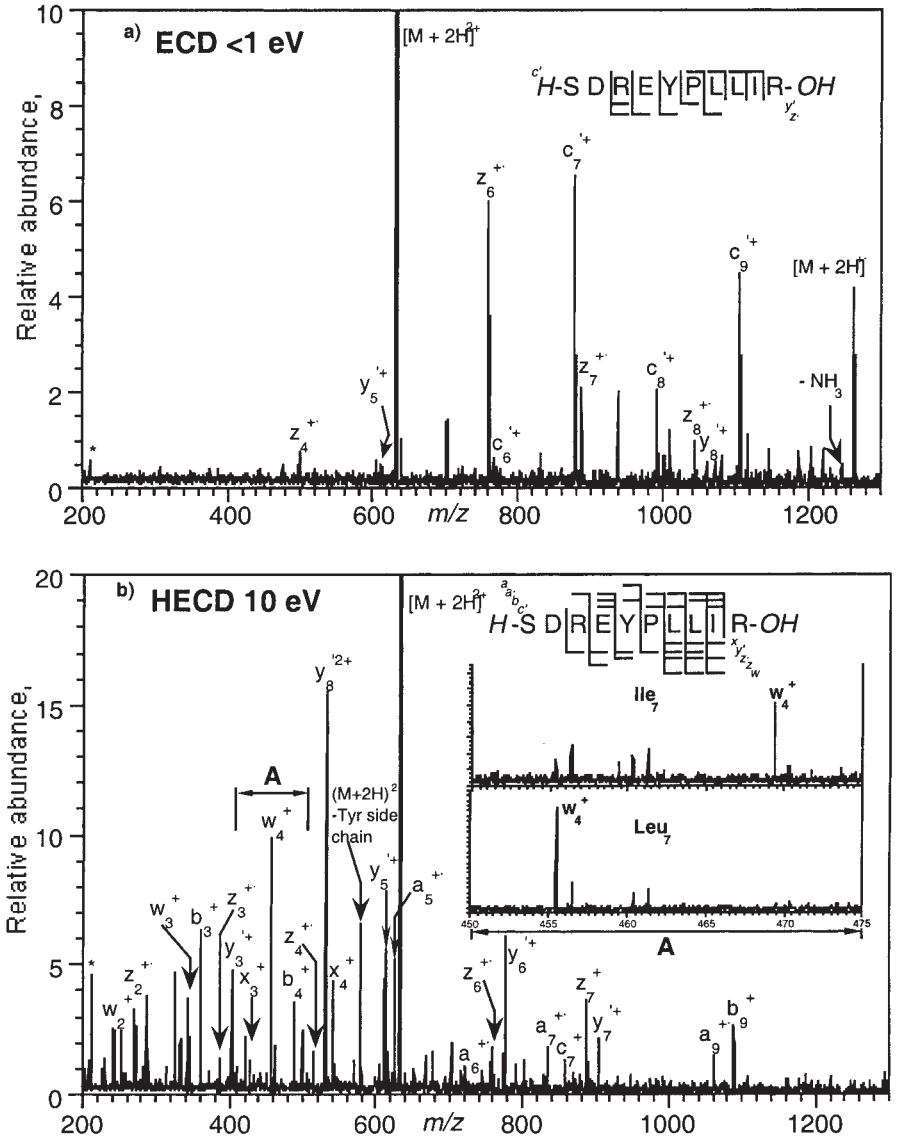

FIGURE 11. Mass spectra of ECD (**top**) and hot ECD (HECD) (**bottom**) of the peptide SRP and its Ile7 variant (inset). Note the presence in the HECD spectrum of abundant *w* ions that identify the nature of all three Xle (Ile or Leu) residues. (Reproduced from Kjeldsen et al., 2002 with permission. Copyright 2002 Elsevier Science.)

energy (Kjeldsen, Sørensen, & Zubarev, 2003). Understandably, the abundances were lower than in HECD, which still makes the latter technique at least twice as efficient for Xle characterization as conventional ECD.

B. Electronic Excitation Dissociation (EED)

Because ECD requires multiply protonated precursor ions, the use of this technique is limited to ESI-produced polycations. Even for dications, the ECD efficiency is reduced because one of the fragments is necessarily neutral and, therefore, escapes the detection by mass spectrometry. The reduced efficiency reflects in lower sensitivity for smaller peptides, which is a serious handicap for proteomics applications. This limitation can be removed if the charge state of cations can be increased *before* electron capture. Post-ionization of cations (so-called tandem ionization, TI) by energetic electrons is possible (Zubarev et al., 2000). The term post-ionization is used here to highlight the fact that, although ESI and MALDI both produce even-electron species, TI yields multiply charged radicals. For instance, TI of MALDI-produced ions gives

hydrogen-deficient species $[M+H]^{2+\cdot}$ (compare these ions with the radical cations $M^{+\cdot}$ generated from neutrals by the conventional EI). The multiply charged radical cations can now capture electrons, which is a process exothermic by the ionization energy of the protonated molecules $[M+H]^{+}$:

$$[M+H]^{2+\cdot} + e^{-} \rightarrow [M+H]^{+*}. \qquad (12)$$

The combination of TI and consecutive electron capture is equivalent to electronic excitation of the precursor $[M+H]^{+}$ ions (Nielsen et al., 2000, 2002). This result is why the subsequent fragmentation in this EED produces a pattern that is similar to that in UV photodissociation. For example, Figure 12 shows the EED spectrum of substance P protonated molecules produced by MALDI and irradiated by 17 eV electrons with subsequent capture of low-energy electrons. The spectrum is dominated by a series of *a* and *a*· ions, as in the UV photodissociation spectrum (Barbacci & Russell, 1999). Compared to the latter, the EED spectrum shows no ammonia losses from the fragments. This result can be explained by the limited amount of the deposited energy in EED, because capture of only one electron gives rise to the fragment mass spectrum (capture of two electrons results in full neutralization). In photodissociation, consecutive absorption of several photons may very well occur, which results in a broad distribution of internal ionic energies before fragmentation.

The absence of severe secondary losses in EED raises an expectation that the potential analytical utility of the technique is in the determination of the sites of PTMs in MALDI-produced peptide ions. An increase in the EED efficiency is, however, required for the technique to be practically useful, because EED is a combination of two as yet rather inefficient processes.

Note one more important difference between ECD and EED in addition to the higher exothermicity of the latter. Although the fragmenting species in ECD are hydrogen-abundant radical cations, those in EED are protonated molecules, albeit electronically excited. This difference is why the EED fragmentation is very different from that in ECD, and contains much fewer *c*- and *z*-type ions. The exact mechanism of *a* ion production upon electronic excitation is not known. It is, however, likely that *a* ions originate from secondary fragmentation of *b* species that result from peptide-bond cleavage.

V. VIBRATIONAL EXCITATION

A. Vibrational Excitation of Even-Electron Species

A peptide bond is the weakest link in the polypeptide backbone, and its dissociation is dominating under vibrational excitation. In gas-phase polypeptides, protonation at side chains of basic amino acid residues, as well as the

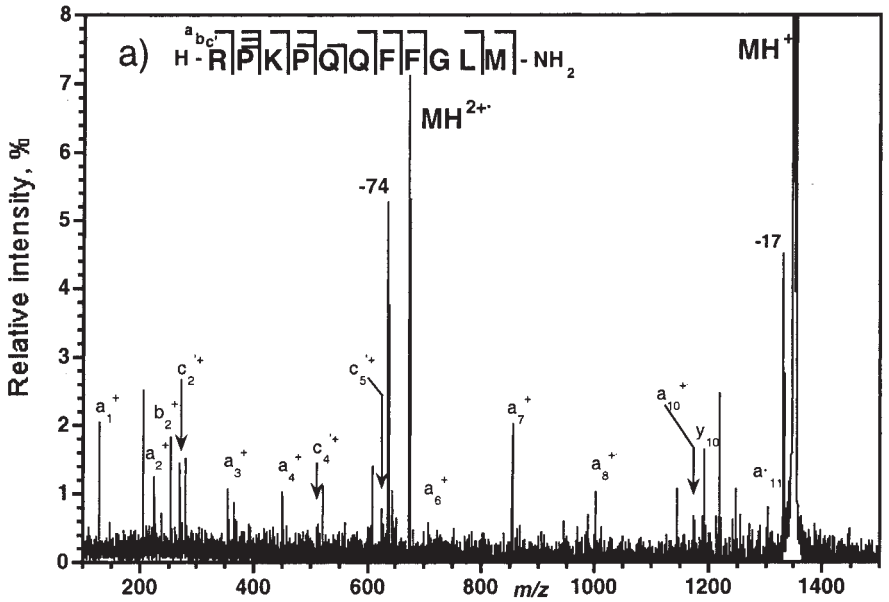

FIGURE 12. Electronic excitation dissociation (EED) spectrum of substance P, monocations of which were produced by MALDI and irradiated by 17 eV electrons. This result was followed by the capture by the formed radical dications of reflected low-energy electrons. (Reproduced from Nielsen et al., 2002 with permission. Copyright 2002 Elsevier Science.)

N-terminus, is accompanied by charge solvation (charge-sharing) on backbone carbonyl oxygen atoms. This charge-sharing makes the peptide bond *stronger* than in the neutral case. Vibrational excitation leads to the shift of the proton to the less basic backbone nitrogen. As a result of that shift, the C(O)–N bond (peptide bond) becomes much longer (Rodriquez et al., 2001). Heterolytic cleavage of the weakened peptide bond is the lowest-energy channel, and it results in b and y' fragments. Although y' ions are just shorter peptides, b ions are likely to exist as protonated oxozolones (vanDongen et al., 1996). ECD experiments on doubly charged b ions provide evidence of the presence, in some cases, of the acylium ion structure (Haselmann, Budnik, & Zubarev, 2000).

Cody & Freiser (1979) introduced electron-impact excitation of ions from organics (EIEIO), a technique in which molecular ions are irradiated by electrons with energies typically below the ionization energy of the corresponding neutral molecules but sufficient for electronic excitation:

$$AB^+ + e^- \rightarrow [AB]^{+*} + e^- \rightarrow A + B^+ + e^-. \qquad (13)$$

The similarity of the EIEIO fragmentation patterns to those in CAD indicated that the intra-molecular energy conversion and distribution occurs prior to dissociation. The EIEIO efficiency was approximately 10% (Cody & Freiser, 1987). Wang & McLafferty (1990) implemented the EIEIO technique with a 500 μs pulse of 70 eV electrons for the cyclic peptide gramicidin S, and reported an 85% efficiency. The production of radical dications has not been reported, although these ions should have been formed under 70 eV irradiation. The possible explanation for this result is the presence in the EIEIO spectrum of gramicidin S of an abundant fragment at m/z 571; i.e., the same as for dications.

Because EIEIO did not produce analytically different information from that in CAD and IRMPD, its only advantage was the absence of the collisional gas. Repeatability of spectra being an issue, the technique has not become widely used.

B. Vibrational Excitation of Radical Species

1. Hydrogen-Atom Loss

In the simplest case, vibrational excitation of hydrogen-abundant radical cations $[M + nH]^{(n-1)+\cdot}$ leads to hydrogen-atom release. Because even-electron polypeptide cations do not easily lose hydrogen atoms, such a release testifies to the presence in the radical cations of loosely bound hydrogen atom, consistent with the hot hydrogen-atom mechanism. H· loss occurs under normal ECD conditions from reduced molecular species, and is especially abundant for smaller species. The McLafferty group studied this loss for 8.6 kDa bovine ubiquitin (Breuker et al., 2002b). Using the unique resolving power of FT ICR MS, they used as precursors single isolated isotopomers of the $[M + nH]^{n+}$ ions, and monitored relative intensities of reduced species with and without hydrogen loss. It was found that, although lower charge states (7+ and 8+) that retained in the gas phase their tertiary structure mostly kept their extra hydrogen atom, half of the highly charged (13+) and unfolded species lost H·. This result was rationalized through the presence in 13+ of the secondary structure (α-helix) that makes the carbonyl oxygen unavailable for proton solvation; the backbone nitrogen atom was proposed to be the prime charge solvation site. This result, suggested the researchers, reduced the energy release near the N–C_α bond and thus favored the low-energy H· loss compared to the c', $z\cdot$ cleavage. A possibility of recombination of c, $z\cdot$ species in α-helical 13+ species with subsequent H· loss was also suggested. A more straightforward explanation of the charge effect on the H· loss appears to be the higher exothermicity of electron capture by 13+ compared to 7+. The increase in exothermicity is equal to the decrease of the proton affinity; i.e., 2–3 eV. Because the recombination energy in the absence of immediate cleavage is eventually converted to vibrational energy, more exothermic processes should promote desorption of loosely bound H· atoms. Vibrational excitation of $[M + 13H]^{12+\cdot}$ led mainly to H· desorption with subsequent b, y' cleavage of even-electron species, which is consistent with both explanations. Charge states from 9+ to 12+ yielded similar results, whereas $[M + 7H]^{6+\cdot}$ and $[M8H]^{7+\cdot}$ ions gave upon vibrational excitation abundant c', $c\cdot$ and z', $z\cdot$ ions, which is an evidence for the N–C_α bond cleavage without dissociation of the tertiary structure. The loosely bound products of the N–C_α bond cleavage separated under very low excitation levels: only $\approx$10% of the IR laser power required for dissociation of $[M + 7H]^{6+\cdot}$ ubiquitin ions (Breuker et al., 2002a). The intensity of the reduced species obtained from higher charge states is usually lower, consistent with the absence of the tertiary structure and higher exothermicity of electron capture. These reduced species are also more stable, although less stable than even-electron species: sustained off-resonance irradiation (SORI) CAD of the mixture of $10+'\cdot$ and 10+ ubiquitin ions changed their ratio from 2:1 to 1:2 (Breuker et al., 2002a). The depletion of the $n+'\cdot$ species was not only due to hydrogen-atom loss, but also due to the loss of radical groups with masses 17, 44/45, and 59/60. Such losses from reduced molecular species frequently occur also in direct ECD (Cooper et al., 2002; Haselmann et al., 2002c).

The retention of the hydrogen atom by reduced species is more prominent for polypeptides than for other biopolymers. For instance, no evidence of such a retention

has been observed for polysaccharides and poly(ethylene glycol) (PEG) chains that contain up to 100 monomers (Cerda et al., 1999, 2001a, 2001b).

The above discussion concerns positive ions. As for the hydrogen-deficient radical anions produced by the ejection of an electron from deprotonated molecules, they show a propensity towards facile losses of small groups, typically CO_2 (Zubarev et al., 2000).

VI. ELECTRON DETACHMENT

A. Ionization

Although MALDI and ESI removed the urgent need for alternative means of ionization for large molecules, there are still analytical situations when the availability of an alternative ionization technique is beneficial. Because radical peptide cations fragment differently from even-electron species, their fragmentation may complement the sequence information obtained by traditional methods, using MALDI- and ESI-generated even-electron species. Furthermore, increasing the charge state of cations is expected to make them less stable and, therefore, more susceptible to fragmentation, as well as to increase the detection efficiency of the fragments. These considerations motivated the search of a technique for (post)-ionization of large polypeptide molecules and molecular ions in the gas phase.

Until recently, the conventional wisdom was skeptical on the possibility of ionizing large polypeptides by removing an electron. This opinion was supported by the experimentally observed universal decrease in the ionization efficiency, whether one used electron impact, single-photon or multi-photon ionization (Schlag, Grotemeyer, & Levine, 1992). However, these experiments could not tell whether the problem was in ionizing large molecules or merely in volatilizing them in intact form. In a Penning cell of a FT ICR instrument, large polypeptide ions can be stored for hours, giving the opportunity to separate these two effects. Irradiation of polypeptide ions with electrons can proceed for minutes, increasing the chance of ionization, as opposed to microsecond residence times in conventional EI sources that result in $\approx 1\%$ ionization efficiency. Long irradiation times have another benefit: the molecules that became, upon electron-impact, electronically excited relax to the ground state before the next impact, which decreases the chance of their fragmentation prior to ionization.

Direct experiments showed that ionization of polypeptide cations by approximately 20 eV electrons is feasible, and that most of the formed $(n+1)$-charged radical cations survive the process (Budnik & Zubarev, 2000). Post-ionization by EI of cations produced by MALDI and ESI has been dubbed tandem ionization TI (Zubarev, Budnik, & Nielsen, 2000). MALDI/EI and ESI/EI were used to determine the ionization threshold of singly and multiply charged polypeptides, as illustrated in Figure 13 (Budnik et al., 2002b). An empirical formula has been deduced to describe the ionization energy of polypeptide cations as a function of the charge state z:

$$\mathrm{IE}(z) = 9.8 + 1.1 \times z \pm 0.5\,\mathrm{eV}. \qquad (14)$$

The average IE increase of 1.1 eV per charge was attributed to Coulombic repulsion. The deduced ionization energy of a neutral polypeptide molecule, 9.8 ± 0.3 eV, was consistent with the literature expectation for vertical ionization (Campbell et al., 1992).

With TI, singly charged peptides up to 3.5 kDa and multiply charged ($\leq 16+$) polycations of proteins up to 17 kDa have been ionized (Zubarev et al., 2000). The ionization cross section, measured to be $(1.3 \pm 0.4) \times 10^{-15}$ cm^2 for 20 eV electrons and vasopressin $[M+H]^+$ cations, was found to increase with the molecular mass, in direct contradiction with previous beliefs. Approximately 30% of the oxidized bovine insulin B-chain could be converted to dications (Budnik & Zubarev, 2000), a much higher efficiency than is typical for EI of neutrals. Although some theories do predict a nearly unit quantum yield of ionization of large molecules by 20 eV electrons (Berkowitz, 2000), the question remains whether a stabilization mechanism for the leaving electron is needed for irreversible ionization, as has been implied by Schlag and

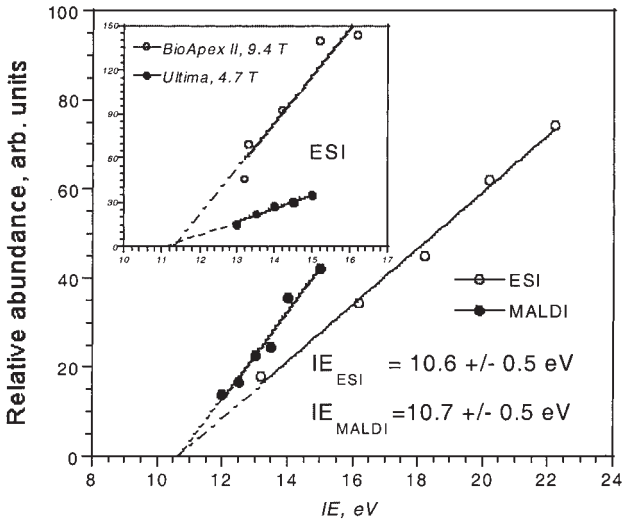

FIGURE 13. Ionization curves of monocations of substance P obtained by ESI (9.4 T Bruker FTMS instrument; closed circles) and MALDI (4.7 T IonSpec FTMS instrument; open circles). The deduced ionization energy values are 11.0 ± 0.4 eV and 10.7 ± 0.5 eV, respectively. (Reproduced from Budnik et al., 2002b with permission. Copyright 2002 John Wiley & Sons, Ltd.)

co-workers (Schlag, Grotemeyer, & Levine, 1992). Indeed, whenever $n+$ molecular ions were ionized by electrons, reduced $(n-1)+\cdot$ molecular species always accompanied the process (Zubarev et al., 2000). In a FT cell, where ion–ion collisions often occur during their storage, capture by neighboring polycations provides stabilization for the leaving electron (Zubarev et al., 2000):

$$([\mathrm{M}+n\mathrm{H}]^{n+}\cdots[\mathrm{M}+n\mathrm{H}]^{n+})+\mathrm{e}^- \rightarrow [\mathrm{M}+n\mathrm{H}]^{(n+1)+\cdot}+[\mathrm{M}+n\mathrm{H}]^{(n-1)+\cdot}+\mathrm{e}^-. \quad (15)$$

Because the importance of this stabilization mechanism for the ionization of large molecules is yet to be determined, the question whether large molecules ionize *in an isolated state* remains, in our opinion, open.

Another peculiarity associated with EI of polypeptide n-charged cations is the rapid loss of small groups by the formed radical $(n+1)$-cations. These losses were typically 44 Da for peptides with the acidic C-terminus and 74 Da for that in the amide form (Nielsen et al., 2002). The 44 Da loss corresponds to the CO_2 group leaving the C-terminal carboxylic acid as a result of rearrangement from it of the acidic hydrogen. The 74 Da loss turns out to be more difficult to account for. The current explanation is that it can either be a loss of the methionine side chain minus one hydrogen atom (i.e., C_3H_6S (74.019 Da)), or a loss of the resonance-stabilized radical C_2H_4NS (74.006 Da) from the N-terminal cysteine residue. Additional experimental data are obviously needed to draw a more specific conclusion.

Can EI of large polypeptides possibly compete with MALDI and ESI? The answer to this question may depend upon whether it will be possible to convert the radical cations obtained in EI into more stable (and more familiar to mass spectrometrists) even-electron species. Because the EI-generated radical ions are hydrogen-deficient, this conversion requires the addition of a hydrogen atom. The reaction

$$[\mathrm{M}+\mathrm{H}]^{2+\cdot}+m \rightarrow [\mathrm{M}+2\mathrm{H}]^{2+}+(m-\mathrm{H})\cdot \quad (16)$$

has been attempted, unsuccessfully, with m being both molecules and free hydrogen atoms (Budnik & Zubarev, 2000; Zubarev et al., 2000). Now the hope of achieving this reaction rests with internal H· transfer within an ionized cluster. Irradiation by 20 eV electrons of electrospray droplets should increase their charge state and lead to increased protonation of the emerged polypeptide ions, a step possibly leading towards absolute sensitivity.

B. Electron-Detachment Dissociation (EDD)

Although most of the research literature, both experimental and fundamental, concerns polypeptide cations, negative polypeptide ions may be just as important. One of the typical reasons for less than 100% sequence coverage in detecting proteolytic fragments by mass spectrometry is the use of only positive polarity. Many acidic peptides that readily form anions produce no or little signal in the positive mode, especially in mixtures where competition for charge results in suppression of less basic species. Some important PTMs, such as phosphorylation and sulfation, are acidic and, therefore, are much more labile in cations than in anions. At the same time, vibrational activation of anionic polypeptides often yields little analytical information. All these factors warrant developing a fragmentation technique for anions analogous to ECD of polycations.

Straightforward application of this idea seems impossible because the ability of anions to capture electrons is much smaller due to Coulombic repulsion. Employing positron irradiation should improve the situation and lead to new fragmentation phenomena (Zubarev et al., 2000), but is expensive and inexpedient. The solution was found in replacing the positrons with $\geq$20 eV electrons that ionize the anions, creating in them a positive radical charge (hole). This hole recombines with one of the negative charges, which results in electronic excitation (recombination is $\approx$5 eV exothermic) and eventually in cleavage of backbone bonds (Budnik & Zubarev, 2000). This phenomenon was termed EDD. As an example, Figure 14a shows the EDD spectrum of dianions of the sulfated peptide caerulein, dications of which were too labile in the gas phase to produce any meaningful information on the sulfate group position. EDD cleaved all inter-residue bonds (Fig. 14b), including several $N–C_\alpha$ bonds. At the same time, vibrational excitation of the same species gave only losses of neutral molecules with little backbone cleavage. Useful analytical results were obtained with EDD on a range of other peptides, both sulfated and unmodified (Haselmann et al., 2002a).

Among EDD fragments, $a\cdot$ and a ions usually dominate. The presence of abundant radical species and the excess energy deposited by energetic electrons should lead to secondary fragmentation. However, although the losses of 1 and 44 Da are rather common in EDD, the presence of d ions has yet to be observed.

Being analogous to ECD for negative ions (''intramolecular ECD''), EDD is likely almost as fast as ECD, and should in the future become a powerful tool for analysis of acidic polypeptides, including peptides with labile modifications. This development is hindered by tamed general interest to negative peptide ions, which in turn stems from the poor understanding of their fragmentation. Except for a few recent accounts (Harrison, 2000; Brinkworth et al., 2001), there are very few systematic studies of peptide anion fragmentation—a situation that must change in the future.

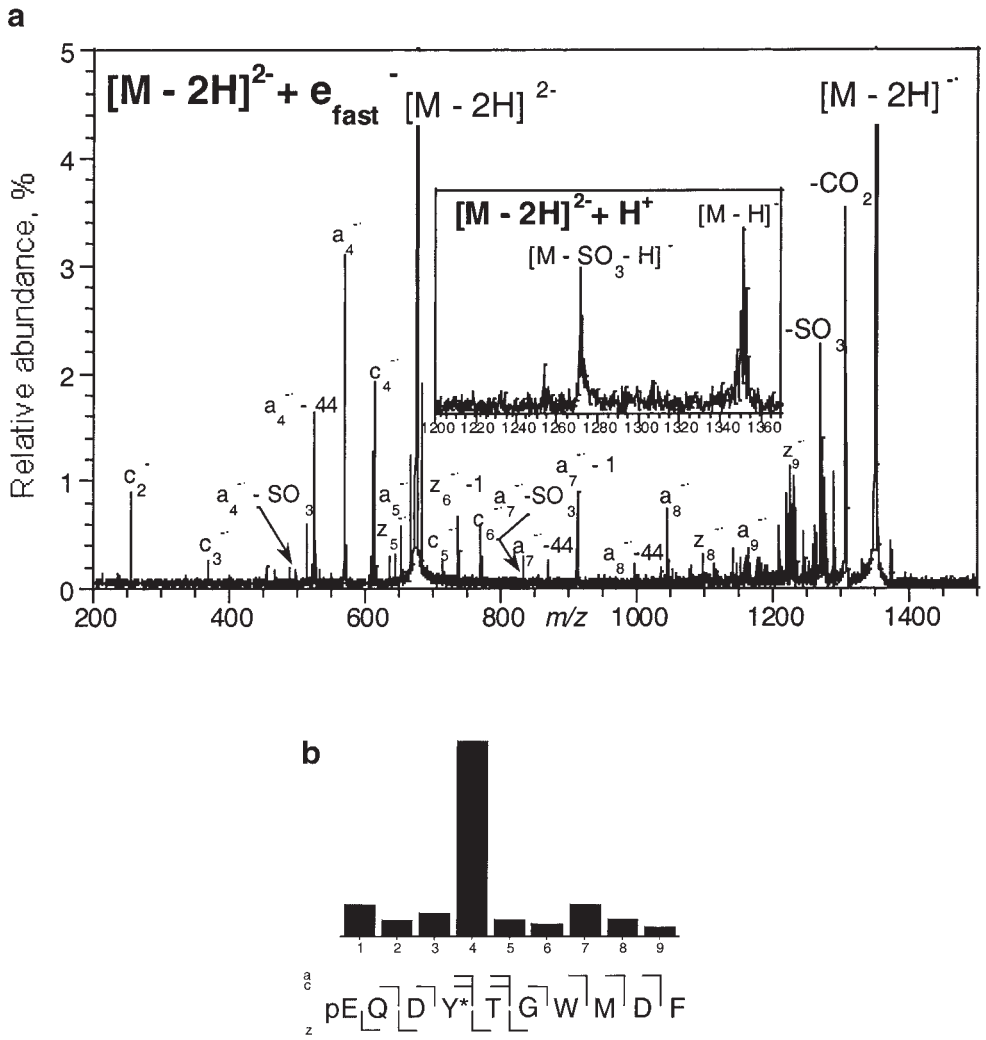

FIGURE 14. **a**: Electron-detachment dissociation (EDD) spectrum of dianions of the sulfated peptide caerulein; (**b**) obtained backbone cleavages. (Reproduced from Budnik, Haselmann, & Zubarev (2001) with permission. Copyright 2001 Elsevier Science.)

VII. CONCLUSIONS

In this review, we attempted to give a summary of recent research that involves novel ion–electron reactions. Some of these reactions have just been discovered, which means that more surprises are probably underway. The importance of ion–electron reactions for mass spectrometry of polypeptides is in the potential of these reactions for addressing the existing challenges, such as protein *de novo* sequencing, characterization of PTMs and gas-phase structures, as well as achieving absolute sensitivity. This goal makes the area an exciting research field that requires ''only'' devotion and concerted efforts of many research groups. To encourage such efforts was one of the main purposes of this review.

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