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Advantages of External Accumulation for Electron Capture Dissociation in Fourier Transform Mass Spectrometry

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A combination of external accumulation (XA) with electron capture dissociation (ECD) improves the electron capture efficiency, shortens the analysis time, and allows for rapid integration of multiple scans in Fourier transform mass spectrometry. This improves the signal-to-noise ratio and increases the number of detected products, including structurally important MS^3 fragments. With XA-ECD, the range of the labile species amenable to ECD is significantly extended. Examples include the first-time determination of the positions of six GalNAc groups in a 60-residue peptide, five sialic acid and six O-linked GalNAc groups in a 25-residue peptide, and the sulfate group position in a 11-residue peptide. Even weakly bound supramolecular aggregates, including nonspecific peptide complexes, can be analyzed with XA-ECD. Preliminary results are reported on high-rate XA-ECD that uses an indirectly heated dispenser cathode as an electron source. This shortens the irradiation time to ≥1 ms and increases the acquisition rate to 3 scans/s, an improvement by a factor of 10−100.

Shortly after the introduction of electron capture dissociation (ECD) by McLafferty et al.,[1] it became clear that there are two major application areas for the new technique: de novo MS sequencing of proteins[2,3] and determination of labile posttranslational modifications in polypeptides.[4−6] These two applications set somewhat contradictory requirements on the electrospray interface. While the first goal is best achieved when the parent ions are activated collisionally prior to ECD so that the weak intramolecular bonds break and a stretched configuration is acquired,[7] the latter objective requires preservation of the labile groups, which often means avoiding any activation at all. The currently popular Fourier transform mass spectrometry (FTMS) external accumulation (XA) technique used in an electrospray interface seems to serve both purposes. It has been shown that prolonged ion storage in a linear multipole trap leads to fragmentation.[8] The phenomenon, termed multipole storage-assisted dissociation (MSAD), is believed to be a kind of collision-activated dissociation (CAD).[9,10] On the other hand, at certain experimental conditions, ion−neutral collisions can be deactivating (cooling).[11] Collisional ion−neutral cooling in a quadrupole is used, for example, in time-of-flight instruments with orthogonal injection.[12,13] By choosing appropriate experimental conditions (pressure, trapping potentials, the number of stored ions, etc.), the energizing conditions in the storage multipole can be avoided, and large populations of labile molecular ions can be accumulated and stored for many minutes.[14] This is potentially beneficial for ECD, where the ion signal is reduced due to partial charge neutralization and where the more homogeneous than in CAD bond-cleavage frequencies[2] often result in low-intensity fragments.

This study reveals that XA provides more than one benefit for ECD. Besides the higher abundances of the parent ions, XA produces a more centered ion beam. Since the ion cloud remains largely on-axis after trapping in the ICR cell, its overlap with the electron beam is maximized and so is the efficiency of electron capture. The formed fragments also remain close to the axis and, thus, are more efficiently detected. In effect, the overall efficiency of ECD improves significantly, and the need for the assisting gas

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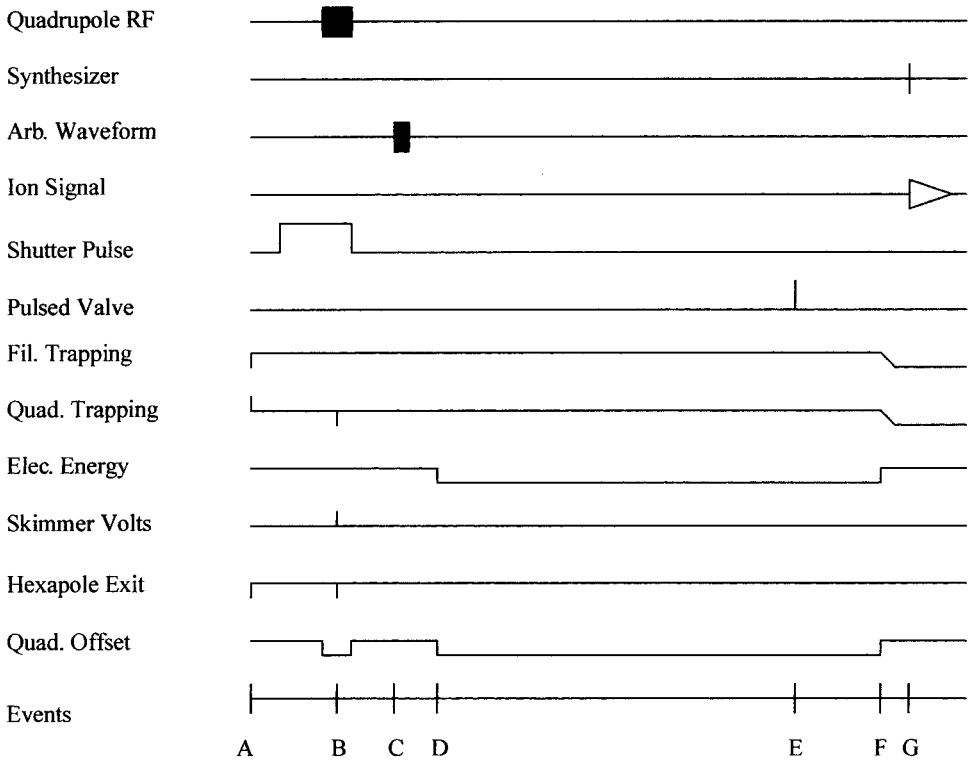

Figure 1. Script sequence of XA-ECD. The events: (A) purging the ICR cell and the hexapole from remaining ions; (B) extraction of the accumulated ions from the hexapole to the ICR cell; (C) isolation of ions of interest; (D) electron irradiation; (E) gas pulse (optional); (F) termination of irradiation; (G) excitation and detection of ions in the ICR cell.

pulse[15] vanishes. Without the gas, the MS/MS experiment (including ion trapping, isolation, ECD, and detection) can be much shorter than the typical[1−7] 10−30 s. Moreover, the absence of the gas eliminates conditions for collisional activation of ions, which is particularly important in analysis of labile molecules. Here we demonstrate this on a range of samples of increased lability, from the standard peptide melittin to O-glycosylated peptides ≤60 residues long to sulfated peptide to a nonspecific peptide complex. We also explore the possibilities offered by the collisional activation XA regime, where abundant b- and y-fragments can be precursors for ECD and yield structurally important MS^3 information. And finally, the first results on the new efficient electron source are reported that eliminate the bottleneck in the ECD performance, the long time of electron irradiation.

EXPERIMENTAL SECTION

Materials. A 60-residue human-derived peptide (triple repeat of 20 amino acids of the MUC1 mucin) and a 25-residue TAP25 (TAPPAHGVTSAPDTRPAPGSTAPPA) were O-glycosylated in vitro. Melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), bombesin (pEQRLGNQWAVGHLM-NH_2), and adrenocorticotropine hormone (ACTH) peptide fragment 1−10 (SYSMEHFRWG) were obtained from Sigma (St. Louis, MO) and used without further purification. Met oxidation in Y_6-sulfated drosulfakinin (DQFD-DYGHMRF-NH_2) happened during storage.

Sample Preparation. The sample molecules were dissolved in a water−methanol−acetic acid mixture (49:49:2 v/v) to a concentration of $\sim 10^{-5}$ M. Aliquots of 4 μL were loaded into a capillary needle (MDS Proteomics, Odense, Denmark) for nanoelectrospray (nanoESI).

Mass Spectrometry. A 4.7-T Ultima (IonSpec, Irvine, CA) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer was used to perform electron capture dissociation. External accumulation in the hexapole of the ESI source (Analytica of Branford, MA, modified with a heated metal capillary), followed by gated trapping was used before the desired charge states where selected by applying a preprogrammed waveform. In some cases, several charge states were selected simultaneously. Isolated cations were irradiated by electrons from a heated tungsten filament for 3−9 s. Due to the electron thermal energy and the nonzero potential in the center of the cell, +(0.75−1.15) V bias of the filament center was necessary to ensure <0.2 eV electron energy in the region occupied by the ions. In one experiment, the tungsten filament was replaced by an indirectly heated dispenser cathode with the emitting surface area of 1.6 mm^2. The cathode was factory-produced in Russia but is not available commercially. With the voltage drop on the cathode heater of 5 V, an electron current of up to 2 mA was produced.

In Figure 1, a schematic representation of the script sequence is shown. For performing external accumulation, the following in-house-written script was used: the capillary exit and skimmer and hexapole exit potentials were set at 75, 30, and 20 V, respectively. For external accumulation of labile ions, the potentials were further reduced to 25, 5, and 5 V, respectively. The offset of the hexapole acting as a linear ion trap was set at +1 V. After a preset time of accumulation (500−3500 ms), the skimmer and the hexapole exit potentials were shifted to +250 and −100

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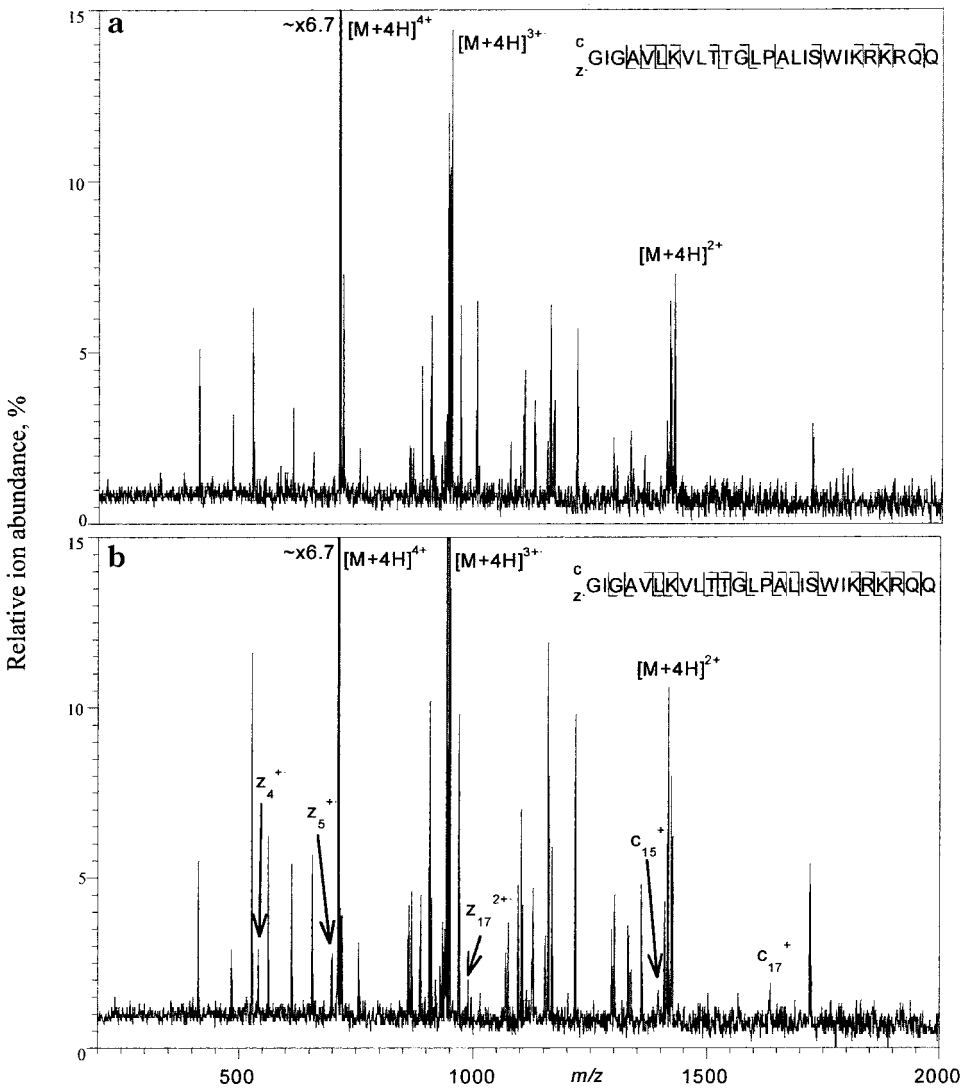

Figure 2. ECD one-scan mass spectra of melittin 4+ (712.4 Th) using (a) gas pulse-assisted trapping, total script length 10 s; and (b) external accumulation (500 ms) with gated trapping, total script length 5 s.

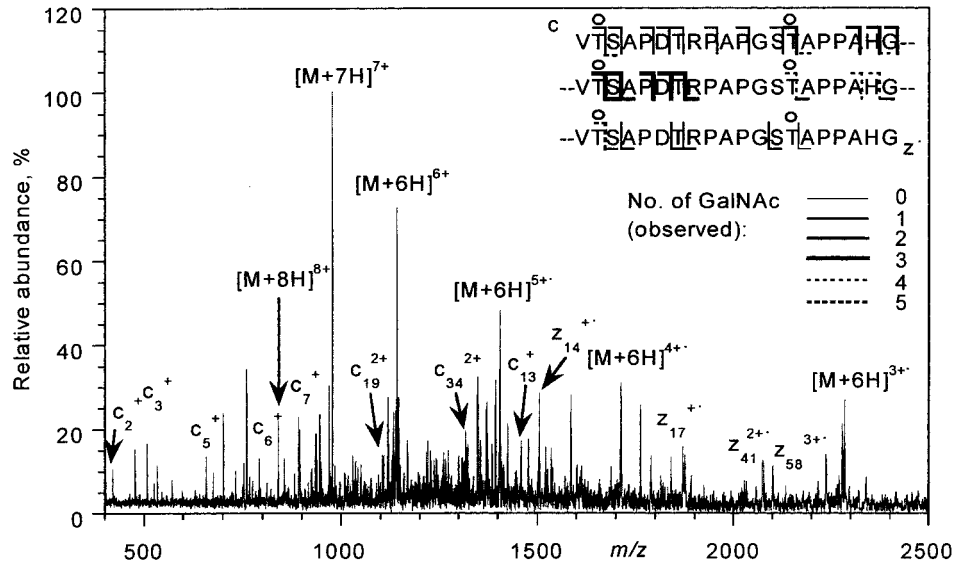

Figure 3. ECD mass spectrum (200 scans) of the 60-residue human MUC1 peptide O-glycosylated with *N*-acetylgalactoseamine (GalNAc) by the enzyme GalNAc-transferase T11 (MW 6841). Cleavage sites and positioning of the GalNAc groups are shown.

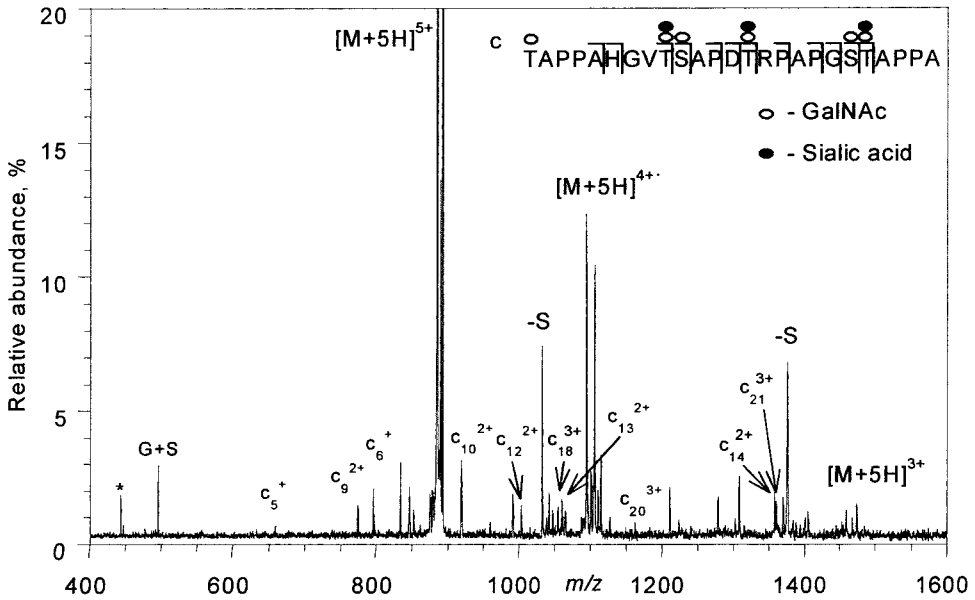

Figure 4. ECD mass spectrum (50 scans) of the O-glycosylated peptide TAP25 containing six GalNAc and three sialic acid groups (MW 4416). Asterisk marks an artifact peak due to the second harmonic.

V, respectively, to eject the accumulated ions. The negatively biased quadrupole ion guide transmitted the ions to the cell where the trapping plate potential was lowered from 3 to 0 V and back within a given trapping interval (600−1000 μs), providing the so-called gated trapping.[16] In addition to ion transmission, the −40 V potential on the quadrupole ion guide served to repel the electrons during the irradiation event, thus increasing the electron capture efficiency.

RESULTS AND DISCUSSION

Gas-Assisted versus Gated Trapping. One-scan electron capture dissociation spectra of melittin 4+ (m/z = 712.4 Th) ions obtained in both regimes with the same electron irradiation conditions (filament bias +1.25 V; 3-s electron irradiation) are shown in Figure 2. In the gas pulse-assisted trapping mode (Figure 2a), 11 c-ion and eight z˙-ion fragments were observed, which corresponds to 46 and 33% of all theoretically possible fragments. In the gated-trapping spectrum (Figure 2b), the electron capture is more efficient, the parent ions are more depleted, and the reduced peak $[M + 4H]^{3+\bullet}$ is 11% larger. A total of 15 c-ions and 11 z˙-ions were recorded, which corresponds to 63 and 50% of all theoretically possible fragments, respectively. The higher electron capture efficiency achieved in the gated trapping mode is explained by trapping the ion cloud closer to the center of the cell than in gas-assisted trapping. Since no gas is typically used in the external accumulation mode,[16] no extra time for pumping down is necessary. Many scans can therefore be accumulated in a rapid sequence without pressure buildup inside the cell. As an example demonstrating the utility of multiple-scan accumulation in ECD, a 50-scan spectrum accumulated within 4.5 min (not shown) gave not only a 7-fold increase in the signal-to-noise ratio (S/N) but also many more fragments. In total, 19 c-ions and 22 z˙-ions were detected, which corresponds to 80 and 92% of all theoretically possible fragments, respectively (all amenable N−C_α bonds were cleaved). Importantly, the number of the complementary fragment pairs that are invaluable for sequence determination[3] increased from 10 in the one-scan spectrum to 18 in the 50-scan spectrum.

Sample number	Sequence, cleavage site and glycan position	Charge state used for ECD	MW
1	TAPPAHGVTSAPDTRPAPGSTAPPA	3+	2,323
2	TAPPAHGVTSAPDTRPAPGSTAPPA	4+	2,730
3	TAPPAHGVTSAPDTRPAPGSTAPPA	4+	3,718
4	TAPPAHGVTSAPDTRPAPGSTAPPA	4+	4,010
5	TAPPAHGVTSAPDTRPAPGSTAPPA	5+	4,416
6	TAPPAHGVTSAPDTRPAPGSTAPPA	5+	4,707
7	TAPPAHGVTSAPDTRPAPGSTAPPA	5+	4,999

○ = GalNAc ● = Sialic acid

Figure 5. Analyzed O-glycosylated TAP25 peptides together with the observed c-ion fragments and the deduced glycan positions.

O-Glycosylated Polypeptides. In Figure 3, the XA-ECD spectrum is presented of a GalNAc glycosylated 60-peptide (MW 6841). This peptide is ∼2 times larger than those previously reported,[5] the improvement being mainly due to external accumulation. The peptide was glycosylated with the GalNAc-T7 enzyme (*N*-acetylgalactoseamine transferase 7); six GalNAc residues were incorporated. Due to the high lability of the molecular ions, mild source conditions of ion accumulation had to be selected by reducing the skimmer voltage from 75 to 50 V and by lowering the hexapole exit potential to 10 V. Three different

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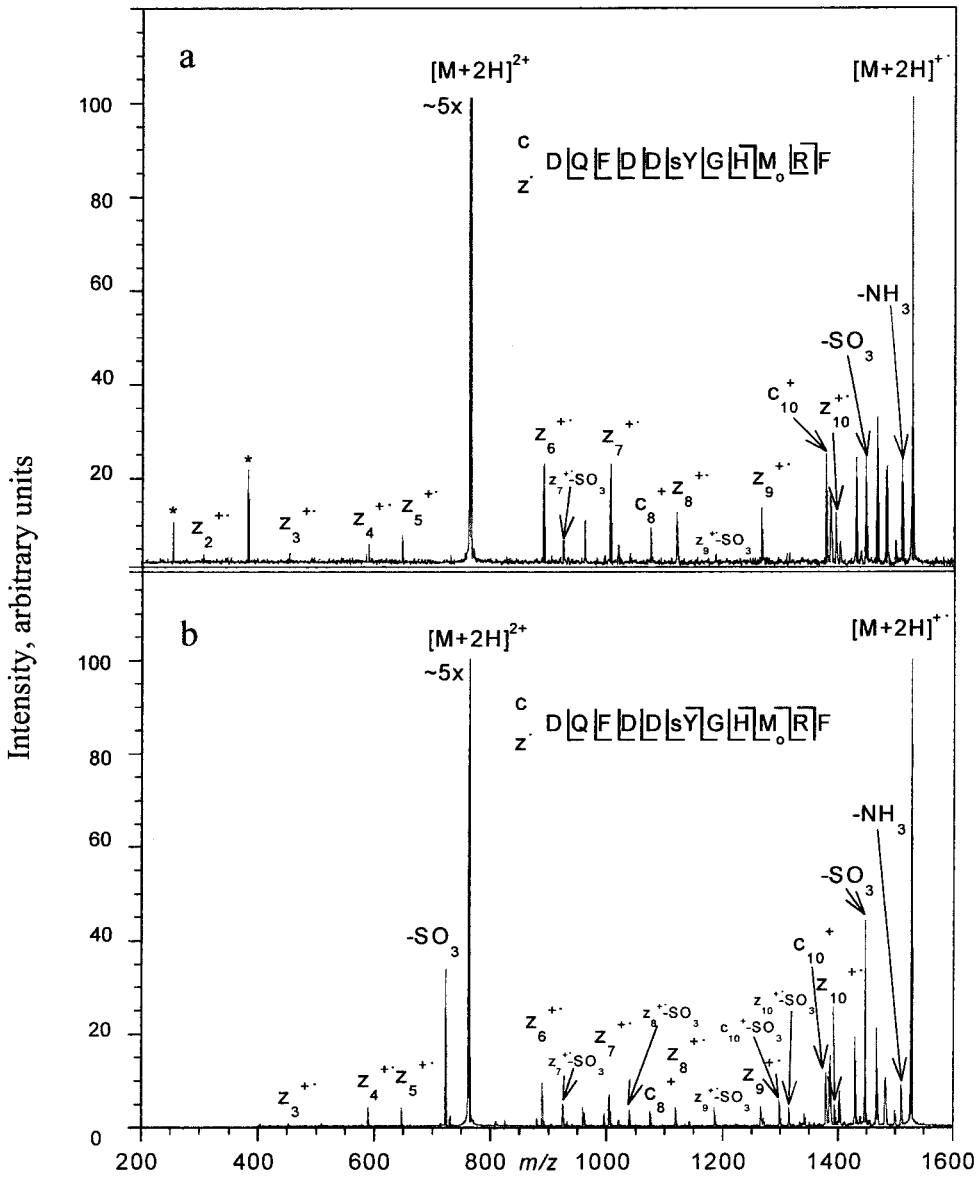

Figure 6. XA-ECD mass spectrum (50 scans) of drosulfakinin: (a) without any gas pulse; (b) with a gas pulse (2 ms, N_2 at 20 Torr) during electron irradiation. Asterisk marks an artifact peak due to the second and third harmonics.

charge states (6+, 7+, 8+) were selected for irradiation with <0.2 eV electrons; 200 scans each 10 s long were accumulated. The spectrum is rather complicated, due to the presence of several charge states and imperfect ejection of sodium adducts. In the spectrum, 21 c-ion fragments (48% of the theoretically possible) were observed without losses of GalNAc residues and 14 z•-ion fragments (32% of the theoretically possible). The smaller amount of z•-ions was expected[5] as these radical cations are less stable and can undergo further fragmentation. Despite the incomplete sequence coverage (not all bonds were cleaved) it was possible to locate four of the GalNAc groups at T_2, T_{14}, T_{22}, and T_{42}. Due to the absence of cleavages between $S_{33}-T_{34}$ and $S_{53}-T_{54}$, two glycosylation sites could not be unambiguously determined. In Figure 4, GalNAc residues were assigned at T_{34} and T_{54} based on the knowledge of the glycosylating enzyme action.

Analysis of peptides with sialic acids attached to O-linked GalNAc groups presents a great challenge to mass spectrometrists due to the high lability of the corresponding molecular cations in the gas phase.[17] This challenge can be successful met by XA-ECD, as is demonstrated in Figure 4, where the ECD spectrum of a 4.4 kDa 25-peptide TAP25 is shown. This peptide was glycosylated with both GalNAc-T7 and a sialyl-transferase; six GalNAc and three sialic acids were incorporated. The charge state 5+ was selected within a ±5 Th m/z window, and 50 scans were accumulated. The spectrum contains 12 c-ion fragments (71% of theoretically possible) without any loss of GalNAc residues. Although the sialic acid losses account for some of the major peaks, these losses are only observed from the reduced species, except for the c_{24}-ion that lost one sialic acid group out of the total three. The ECD information allowed for unequivocal determination of the glycosylation pattern: T_1, T_9, S_{10}, T_{14}, S_{20}, and T_{21} are the sites of the GalNAc attachment, with T_9, T_{14}, and T_{21}

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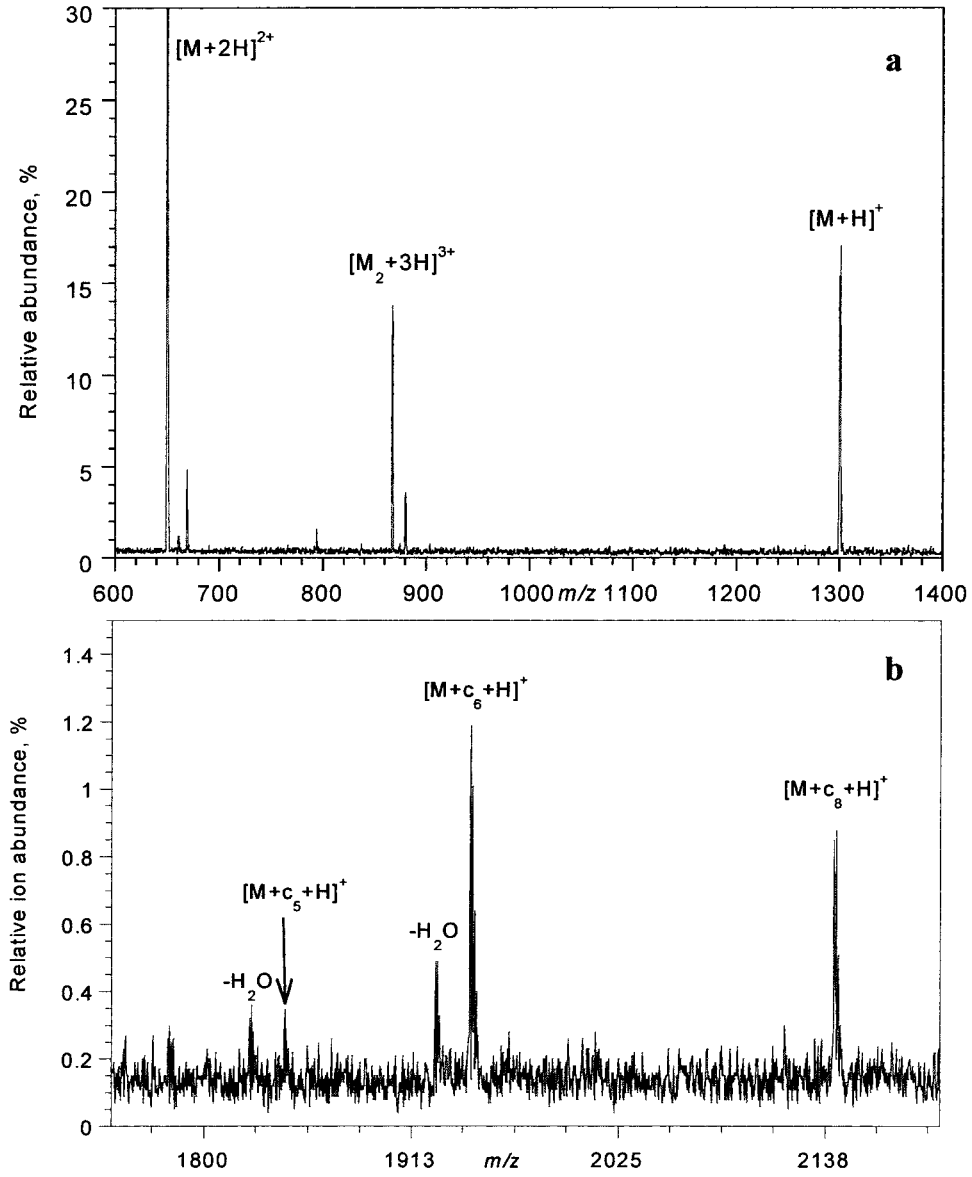

Figure 7. (a) Parent ion mass spectrum of 10^{-4} M solution of ACTH 1−10; (b) part of a ECD mass spectrum of a peptide homodimer trication $[2M + 3H]^{3+}$ (M = TTTDSTTPAPTTK) obtained from 10^{-4} M solution. Electron capture by the trications was followed by the backbone N−C_α cleavage, giving supramolecular $[M + c_x + H]^+$ (x = 5, 6, 8) fragments and the complementary $z^{+\bullet}$-fragments.

carrying also sialic acid. This successful glycan localization led to the analysis of a series of O-glycosylated TAP25 polypeptides modified at different positions and to a different extent. All TAP25 polypeptide samples analyzed are shown in Figure 5 together with ECD cleavages, the corresponding positions of the glycans, and the charge states used in the ECD experiments. All glycan residue positions were unambiguously identified except for one position in sample 4 (no cleavage between T_{14} and R_{15}). On the basis of the acquired knowledge of the glycosylating enzyme action, the glycan position was assigned to S_{20}. The biological implications of these findings are discussed in a separate publication.[18] Here we just note that the order of glycan incorporation by the enzyme can be directly determined from the ECD spectra, as samples 3−4 and 5−7 represent consecutive glycosylation.

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Sulfated Peptide. Although an ECD of a peptide containing sulfated cysteine residue has been reported,[4] our previous attempts to unequivocally locate the sulfated tyrosine residue in drosulfakinin using gas-assisted trapping have not been successful due to insufficient abundances of the key ECD fragments. Figure 6 demonstrates that this problem is now solved with XA-ECD. Additionally, the influence of the gas pulse during electron irradiation was investigated. Without the gas pulse, 12% of the total fragment ion intensity was due to SO_3 loss (Figure 6a), compared to 60% with the gas pulse (Figure 6b). This is because the increased pressure in the gas-assisted ECD converts part of the ionic kinetic energy into internal energy. Note also the SO_3 losses from the 2+ parent ions in Figure 6b. These losses are due to the background pressure increase in the gas-assisted ECD. As a result of this increase, the parent ions are excited collisionally during their isolation in subsequent scans.

On the other hand, two more c-ions were observed with the gas pulse, also due to collisional activation.[7] Therefore, in deciding whether to use the gas pulse during ECD, one must choose between the desire to obtain more backbone cleavages[7] and the necessity to avoid losses of labile groups.

Supramolecular Complexes. Mild conditions (nozzle−skimmer voltage difference, 20 V; hexapole exit voltage, 5 V) allowed for observation of specific and nonspecific peptide complexes, provided the sample concentration was increased to 10^{-4} M and the solution was stored for some time at room temperature. Peptide *n*-mers survive external accumulation and transmission to the ICR cell and can account for a significant fraction of the total ion current (>10%, see Figure 7a). Even more strikingly, ECD of triply charged dimers gave supramolecular fragments in which breakage of the backbone N−C_α bond did not lead to separation of the fragment from the partner molecule (Figure 7b).[19] The supramolecular fragments $[M + c_x + H]^+$ (x = 5, 6, 8) were accompanied by the corresponding complementary $z^{+\bullet}$ fragments (not shown). Investigation of other supramolecular systems is underway.

MS³. Increasing the accumulation time in external accumulation induces fragmentation,[8] which can be used for a multistage MS experiment. In Figure 8, an MS^3 experiment of bombesin (MW 1619) ions is presented. Electrosprayed molecular ions subjected to the usual 500-ms external accumulation produced the mass spectrum of 2+ to 4+ molecular ions (inset in Figure 8a). When the accumulation time was increased to 3.5 s, abundant series of b-ions appeared (Figure 8a). The b_{13}^{2+} ions (m/z = 736.8 Th) were then isolated in the ICR cell and irradiated with electrons for 10 s. The resulting ECD mass spectrum is presented in Figure 8b. The contribution to the fragmentation pattern from the incompletely ejected 2+ ions present at 5% level was minimal. CO loss from the reduced b-ion confirms its identity (we discuss these losses in a separate work).[20] The full c-ion series from c_3 to c_{12} were detected, with only c_1 and c_2 not present due to the detection window cutoff at m/z = 400. Compared to the ECD of $[M + 2H]^{2+}$ ions (Figure 8c), the bonds between R_3 and N_6 have now been broken. The observed fragments confirm the c-ion identities in the ECD spectrum of $[M + 2H]^{2+}$ and reveal the identities of the two $z^{\bullet}$-ions, allowing for determination of the whole sequence.

High-Rate ECD. The bottleneck of the ECD technique has been in the long time of irradiation by electrons, resulting from inefficient electron capture.[6] Although XA reduced the irradiation time from 30 to 3 s, it was still too long for such applications as LC/MS, where a peptide chromatographic peak typically elutes within a few seconds. It was known that a simple increase in the current through the filament does not provide a higher electron capture rate.[15] Recently, we replaced the tungsten filament with an indirectly heated dispenser cathode[22] with a much larger emitting area and a lower working temperature (~1000 °C). The preliminary results in our laboratory and in the Uppsala group that followed us in this approach[23] are very encouraging. The

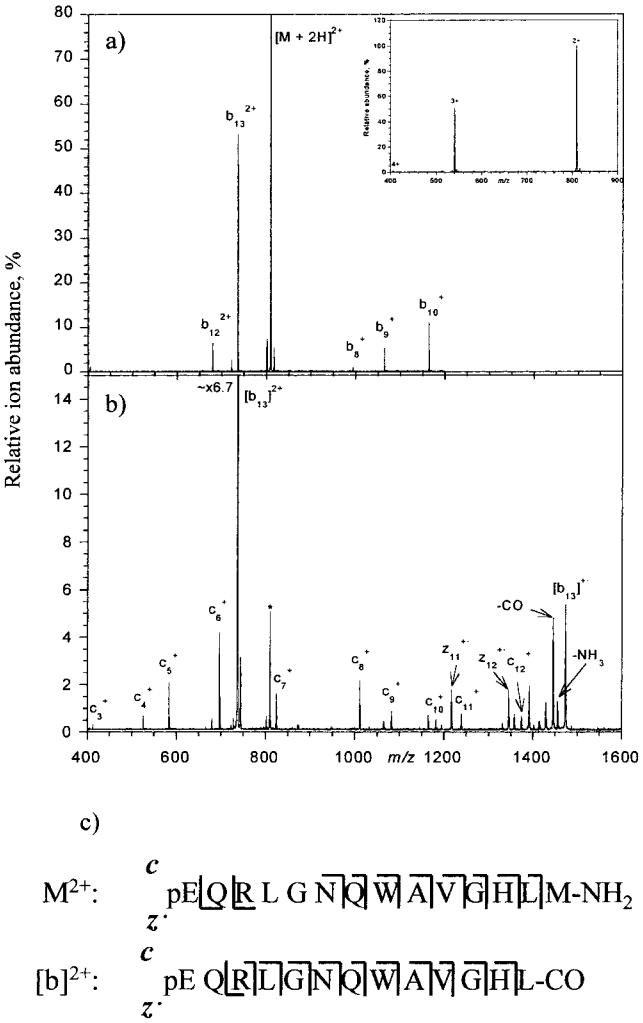

Figure 8. Mass spectra of bombesin: (a) MSAD with 3500 ms of external accumulation (inset: intact parent ions obtained with 500 ms of external accumulation); (b) 20-scan ECD mass spectrum of b_{13}^{2+} (m/z = 736.8 Th); asterisk denotes incompletely ejected 2+ ions; (c) ECD cleavages from 2+ and b_{13}^{2+} ions.

shortest ECD event obtained so far was just 1 ms long without parent ion isolation (Figure 9) and 10 ms with parent ion isolation. With ECD no longer being the rate-limiting event, the highest acquisition rate was 3 scans/s using constant ion accumulation (no hexapole purging in the beginning of the script). Each scan included pulsed ion transfer, gated ion trapping, parent ion selection, electron irradiation for 50 ms, product ion excitation, and detection with 64K data points. At such a high acquisition rate, the shutter performance was inadequate, and we removed it altogether; this did not result into noticeable spectra deterioration.

After this paper has been submitted, we observed a greatly improved stability of ECD operation with the new electron source. The electron capture efficiency was less dependent upon the gas pressure in the cell, trapping conditions, and even electron energy. This was attributed to the trapping properties[24] that an intense and broad electron beam exhibits for positive species, the parents ions, and the ECD fragments.

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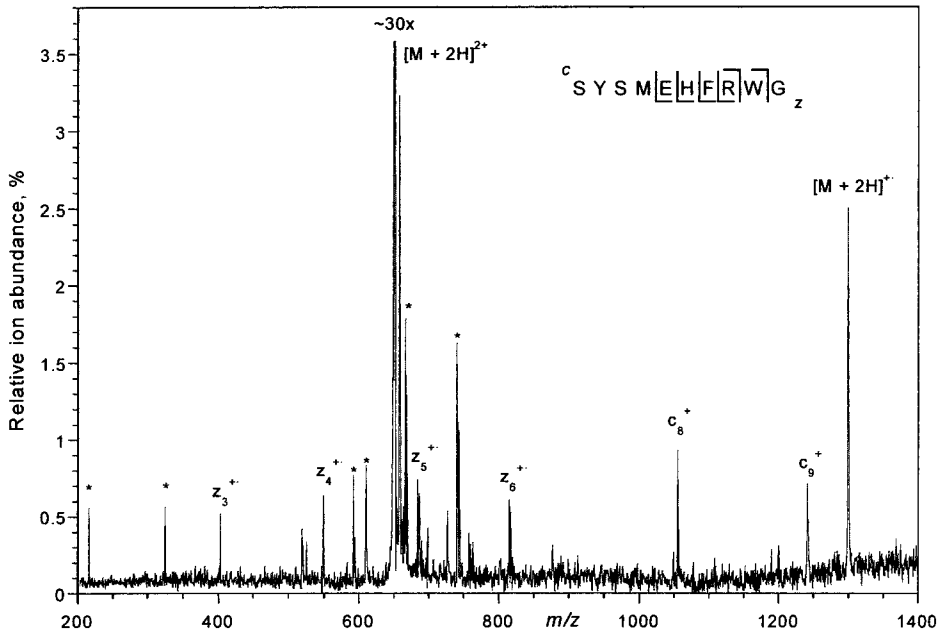

Figure 9. ECD of ACTH 1−10 2+ ions without parent ion isolation with 1-ms electron irradiation (200 scans). Asterisks denote artifact peaks and contamination ions.

CONCLUDING REMARKS AND PERSPECTIVES

We predict that the XA-ECD combination will become a standard technique in FTMS for peptide sequencing, determination of the position of labile modifications, and structural studies on supramolecular complexes. The absence of the gas pulse in XA-ECD is a major advantage, which shortens the data acquisition time and facilitates the analysis of labile molecules, where preservation of the labile group upon the backbone cleavage is of a prime importance. The high-rate XA-ECD opens the way for direct coupling of liquid chromatography and capillary electrophoresis with XA-ECD, which is important for high-throughput, high-sensitivity studies such as proteomics research.

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