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'Top Down' Analysis of Proteins for the Masses

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Abstract: During the last decade, 'top-down analysis' of proteins has been available to those scientists privileged by access to Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). Recent advances in ion trap and orbitrap technology coupled with developments like electron transfer dissociation (ETD) promise to make the 'top-down' strategy of protein analysis more widely available. Here, the general strategy of 'top-down analysis' is reviewed, an example of 'top-down analysis' of a small protein by ion trap mass spectrometry is described, and the importance of ETD is presented in the context of instrumentation based on the linear ion trap or as hybridized with transmission quadrupole mass spectrometers.

Keywords: mass spectrometry, amino acid sequencing, electron transfer dissociation, ion/ion reactions, MS/MS, protein identification.

1. Introduction

During the last four decades, protein analysis based on mass spectrometry has been conducted on a 'bottom up' strategy of degrading the protein in a controlled manner, usually by proteolysis, followed by identification of the resulting proteolytic fragments and their 'reassembly' to cover the sequence of amino acid residues in the intact protein. In the earliest applications of mass spectrometry to protein analysis, the proteolytic fragments were usually 'identified' by molecular mass, but during the last two decades the identification process often has involved some structural information from analyses of fragments by collisionally activated dissociation (CAD)-mass spectrometry/mass spectrometry (MS/MS) for a more reliable result. The seminal works by Biemann [1-3] and others [4, 5] provide the basis for deducing the sequence of amino acids from CAD-MS/MS data obtained from a protonated peptide produced by electrospray ionization, for example [6]. The 'bottom-up' approach can be quite labor intensive because of the need to purify the original protein by 1D- or 2D-gel electrophoresis [7], to isolate and process electrophretic 'spots', and subsequently to analyze the complex mixtures [8-10] resulting from its digestion.

In recent years, the strategy of 'top-down' analysis has been developing [11-13]. In principle, the difference between the measured mass (molecular weight) of a protein and its calculated mono-isotopic mass (based on its DNA-predicted sequence) indicates sequence errors and/or posttranslational modifications to the isolated analyte. This 'top-down' approach focuses on direct analysis of the intact protein, but the need for sample purity is less stringent than that for 'bottom-up' analysis

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because ion current corresponding to the protein of interest can be selected for further analysis. In this way, the 'top-down' approach brings the sensitivity of the analytical procedure more in line with detection limits achieved by mass spectrometry than does the 'bottom-up' approach, which can suffer from sample losses associated with various chromatographic, electrophoretic, and corresponding isolation procedures; yet, the two approaches can be complementary [14].

The 'bottom-up' approach has had the advantage of requiring analyses of relatively small molecules, which usually are amenable to the capabilities of ordinary mass spectrometers. However, with the 'top-down' approach, the straight forward analysis of an intact protein molecule having a mass of 10 kDa or more requires extraordinary resolving power and mass range. For this reason, the 'top-down' approach has been limited to a relatively small number of laboratories that are equipped with Fourier transform (FT) ion cyclotron resonance (ICR) mass spectrometers, which have a resolving power approaching 10^6 and a *m/z* range of tens of thousands. This elitism is being threatened by new developments in instrumentation that are commercially available in the form of the linear ion trap [15] and the orbitrap [16-21].

2. An illustrative example of top-down anal -ysis with a linear ion trap mass spectrometer

The capacity of ion traps to conduct analyses of relatively large proteins is illustrated in the following example of a 14.6-kDa protein [22]. In this case, multi-protonated molecules of the protein were prepared during electrospray ionization; in this way, the *m/z* values for the most abundant species was less than 1000 as presented in the mass spectrum in Figure 1.

As stated in the introduction, the 'top-down' approach to protein analysis requires sufficiently good resolving power that the isotope peaks representing the protonated molecule can be resolved. This level of performance will allow the charge state to be computed from the spacing of the isotope peaks; recall that spacing of the isotope peaks at 0.1 *m/z* units indicates a charge state of 10 in the corresponding ion. In this way, a measurement of the *m/z* value of an ion together with an assessment of the charge state allows the analyst to compute the mass of the ion.

The first requirement in 'top-down' analysis is to determine the mass of the intact protein. This can be accomplished by ESI-MS as illustrated by the mass spectrum shown in Figure 1, which was acquired at low resolving power at an acquisition rate of 4,400 *m/z* units s^{-1}.

A subsequent zoom scan (results not shown) at a resonant ejection scan rate of 1100 *m/z* units s^{-1} allowed isotope peaks to be resolved sufficiently that the charge state of the protein could be established. As shown in Figure 1, the clusters of isotope peaks centered at *m/z* 735.5 and at *m/z* 817.0 represent the protein containing 20 and 18 protons, respectively.

All other discernible clusters of peaks represent protein molecules differing by one in the number of protons attached; e.g., the cluster centered at *m/z* 774.1 indicates 19 protons attached to the molecule, while that at *m/z* 1469.6 indicates 10 protons attached to the molecule. These mass spectral results allow the mass of the protein to be experimentally determined as 14,688.3 Da, which is in good agreement with the calculated value of 14, 689.8 Da for the known sequence.

The sequence of the protein can be confirmed only if adequate fragmentation of the protonated molecule can be achieved by subjecting it to CAD. The CAD fragmentation pattern is a function not only of energy, but of charge state of the ion. However, from previous work, it is known that the CAD fragmentation pattern is usually not a continuous function of the charge state, but rather there are likely to be abrupt changes in the CAD pattern with charge state such that there are usually no more than two or three distinct

patterns. Therefore, it is necessary to perform a survey scan of the product ion spectra of the precursor ions of each charge state. The survey scan at 1100 *m/z* units s^{-1} allows each of the multi-protonated molecules to be obtained in a few seconds; at this scan rate, the resolving power does not permit unit resolution, but it is adequate for obtaining the general appearance of the fragmentation pattern as described below.

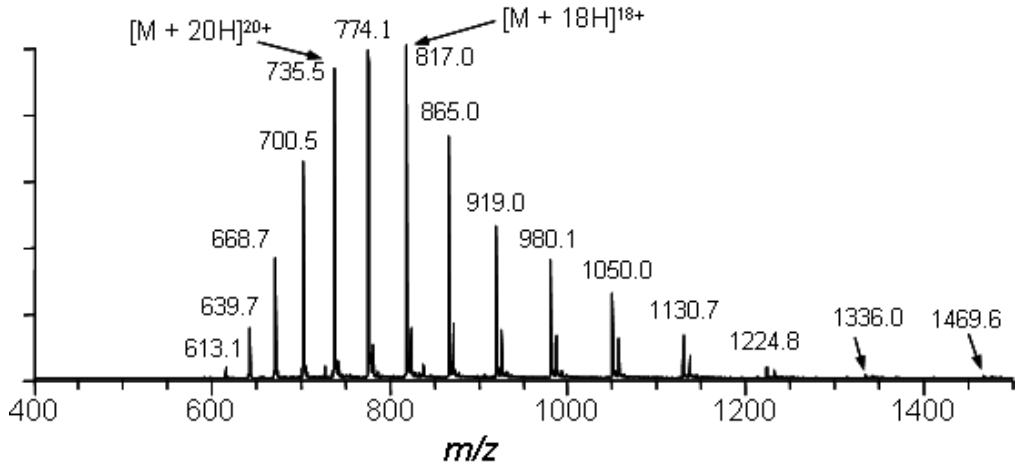

Figure 1. *ESI Mass Spectrum of a Protein Prior to Top-Down Analysis.* Data courtesy of Prof. Gavin Reid, Dept of Chemistry, Michigan State University.

In this example, the CAD behavior of the variously protonated forms of the protein fell into two distinct groups, one group consisted of those molecules containing 18 or fewer protons and the other group consisted of molecules containing 20 or more protons. The CAD fragmentation pattern of the protein molecule containing 19 protons (data not shown) seemed to be a blend of those of the two distinct groups. Because of the high abundance of protein molecules containing 18 and 20 protons (see peaks in Figure 1 at *m/z* 817.0 and at *m/z* 735.5, respectively), these species were subjected to CAD-MS/MS in separate experiments. The CAD product ion spectrum of *m/z* 817.0 representing $[M + 18H]^{+18}$ is shown in Figure 2, which shows a pattern that is obviously different from that for CAD of *m/z* 735.5 representing $[M + 20H]^{+20}$ in Figure 3.

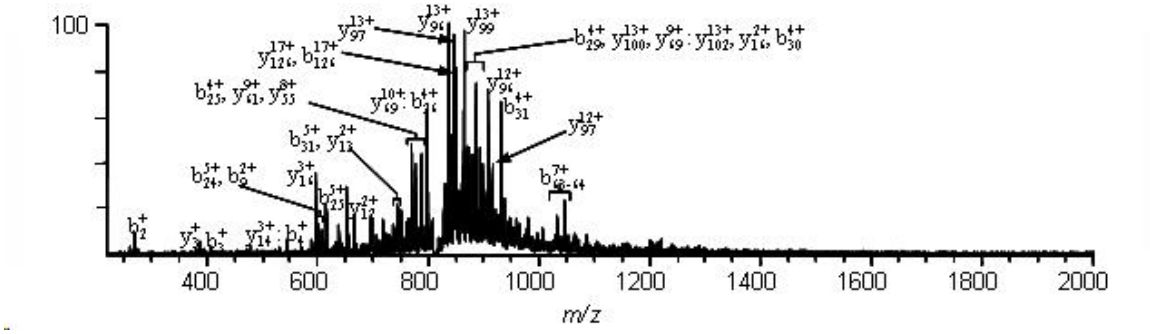

Figure 2. *CAD product-ion spectrum of m/z 817.0 (from spectrum in Figure 1) representing [M + 18H]+1.*

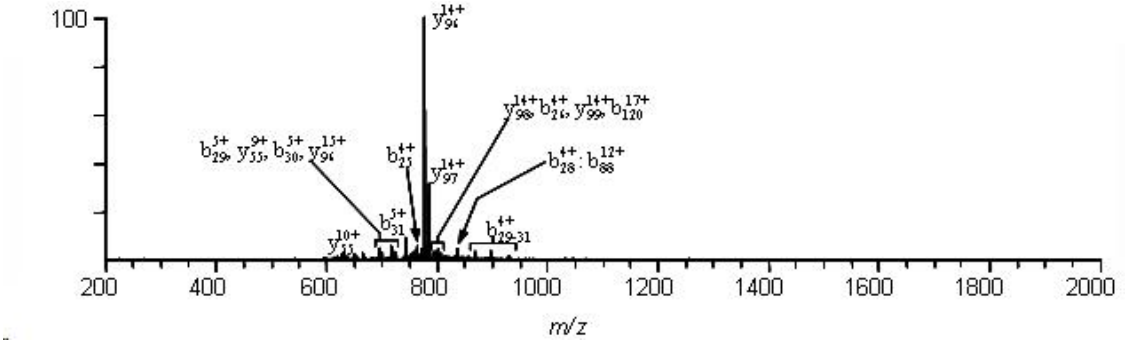

Figure 3. *CAD product-ion spectrum of m/z 735.5 (from spectrum in Figure 1) representing [M + 20H]$^{+20}$.* Data courtesy of Prof. Gavin Reid, Dept of Chemistry, Michigan State University.

Because of the trade-offs in quality of data versus the operating parameters that must be imposed on an ion trap of limited *m/z* range, e.g., *m/z* 2000, the product-ion mass spectra shown above in Figures 2 and 3 were re-acquired in the 'zoom' mode 'at a rate of 1100 *m/z* units s^{-1} over a period of 15 min [22]. Although the mass spectral data re-acquired in the 'zoom' mode have the same general compact appearance as those shown in Figures 2 and 3, the high-resolution quality is apparent in the expanded format of selected segments of the data as shown in Figure 4.

The data in Figure 4A are from an expanded segment of the re-acquired spectrum (similar to that displayed in Figure 2) of product ions of *m/z* 817.0 representing $[M + 18H]^{+18}$. In the narrow *m/z* range displayed in Figure 4A, one peak was sufficiently resolved for accurate measurement, and its *m/z* value was sufficiently close to the calculated value for b_{26}^{4+} to qualify as an 'identification'.

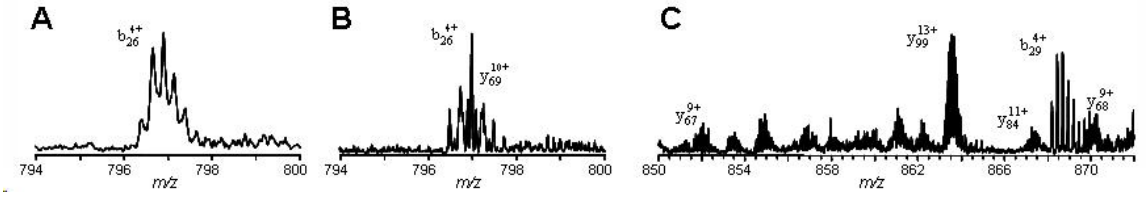

Figure 4. *Panels A, B, and C show expanded segments of the product-ion spectrum of m/z 817.0 representing [M + 18H]$^{+18}$ at high resolving power allowing for identification of specific ions as described in the text.* Data courtesy of Prof. Gavin Reid, Dept of Chemistry, Michigan State University.

The data in Figure 4B are from the same narrow *m/z* range as displayed in Figure 4A, but were acquired in the 'ultra-zoom' mode at a rate of 28 *m/z* units sec^{-1} during an acquisition period of 30 minutes. The resolution is now sufficient to resolve additional mass spectral peaks, one of which has an experimentally measured value of *m/z* that is sufficiently close to the calculated value for y_{69}^{10+} to constitute an 'identification'.

The data in Figure 4C are from another expanded region of the spectrum similar to that shown in Figure 2, but were acquired in the 'ultra-zoom' mode; sufficient mass accuracy was achieved to identify additional ions. Other peaks (data not shown) in the complete scan at this low data-acquisition rate (high resolving power) allowed for identification of 36 amide bond cleavages or 28% sequence coverage. In all the MS/MS data available

from CAD of representing $[M + 18H]^{+18}$, approximately half of all the amide bonds in the protein were identified [22].

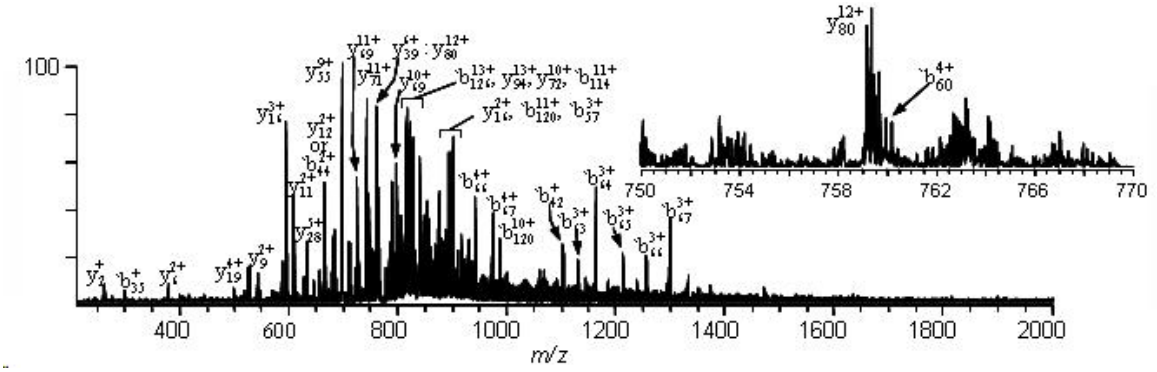

Figure 5. *CAD-MS/MS/MS Product-Ion Scan of m/z 735.5 (from the primary mass spectrum in Figure 1).* Data courtesy of Prof. Gavin Reid, Dept of Chemistry, Michigan State University.

Because the vast majority of the ion current in the CAD-MS/MS spectrum shown in Figure 3 of *m/z* 735.5 (representing $[M + 20H]^{+20}$ in the primary ESI mass spectrum in Figure 1) is due to y_{96}^{14+} (as represented by the predominant peak in Figure 3), this primary fragment ion was selected for further CAD and a third stage of analysis by MS, i.e., MS^3. Figure 5 is the CAD-MS/MS product-ion spectrum of y_{96}^{14+} (CAD-MS/MS/MS of *m/z* 735.5 from the primary mass spectrum in Figure 1) acquired in the zoom mode; these data provided the identification of 29 amide cleavages in the original protein. The inset in Figure 5 shows a segment of this CAD-MS/MS/MS product-ion spectrum acquired in the 'ultra-zoom' mode; the increased resolution in the data allowed 47 amide bonds to be identified.

Table 1 shows the number of amide bonds identified as a function of the 'zoom' and 'ultra-zoom' data acquisition modes. Some amide bonds were identified in all data acquisition modes, but other amide bonds were identified in only one mode. Overall, the $CAD\text{-}MS^2$ and $CAD\text{-}MS^3$ data provided evidence for 85% sequence coverage in the protein [22]. In some cases, additional amide bonds could be identified from data acquired in the 'ultra-zoom' mode because the lower resonance ejection rate produced better resolution in the mass spectral data. In other cases, amide bonds that were identified in the 'zoom' mode because of adequate resolution and peak intensity could not be identified in the 'ultra-zoom' mode because further reduction in the peak intensity associated with this high-resolution scan mode made it impossible to distinguish the analyte signal from noise.

Table 1. *Number of Amide Bonds Identified and Percent Sequence Coverage from Indicated CAD Scan Modes*

Mode	MS/MS of $[M + 18H]^{+18}$	MS/MS of $[M + 20H]^{+20}$	MS/MS/MS of $[M + 20H]^{+20}$
'Zoom' mode	30 amide bonds (24%)	30 amide bonds (24%)	29 amide bonds (23%)
'Ultra-Zoom' mode	36 amide bonds (28%)	15 amide bonds (12%)	47 amide bonds (37%)

3. Specialized fragmentation processes for 'Top-Down' analyses

3.1. Electron capture dissociation

The "top-down" approach to structural analysis requires some means of causing the high-mass ions to fragment in a way that reveals structural features. In the top-down mass spectrometry approach, the protonated analyte is dissociated, and the resulting fragment ion masses are compared with corresponding calculated masses expected from the cDNA-predicted protein sequence in an effort to recognize the locations of any translation errors or posttranslational modifications [23, 24].

The 'top-down' approach became viable after the discovery of electron capture dissociation (ECD) [25-27]. The conventional process of CAD is ineffective for analyzing ions with an *m/z* value exceeding 1500. However, ECD promotes efficient fragmentation of massive ions containing two or more protons, thereby allowing sequence information to be obtained directly from analysis of the intact protein using FTICR MS [27-32]. In ECD, highly positive ions (usually multi-protonated species from ESI) readily interact with low-energy electrons to diminish the high charge state, and effectively convert it from an even-electron ion to an odd-electron species as illustrated in Scheme 1.

$$MH_2^{2+} + e^-_{5\,eV} \rightarrow MH^{+\bullet} + H^{\bullet}$$

Scheme 1

As long realized from classical mass spectrometry of odd-electron molecular ions formed during electron ionization (EI), radical sites often promote homolytic cleavage of bonds adjacent to the atom possessing the odd electron. Because the site of electron capture is highly distributed in a protonated protein, extensive cleavage is observed as illustrated in Figure 6. Fragment ions associated with side-chain losses are reportedly comparable in abundance to those resulting from backbone cleavage of peptides (up to 14-mers) following ECD and, thus, should be taken into account in data interpretation [33]. In advanced studies of protein fragmentation following ECD, considerable loss of CO has been observed from double charged **b** ions, suggesting the linear open-chain acylium structure as opposed to the protonated oxazolone structure predicted by *ab initio* calculations [34].

ECD has the advantages of cleaving between a high proportion of amino acids, without loss of such posttranslational modifications as glycosylation, carboxylation, and phosphorylation [35]; when coupled with the formation of charged derivatization, there is a dramatic improvement in sequence coverage realized by ECD during the analysis of O-glycosylated and O-phosphorylated peptides [36]. An exception has been reported in the ECD of double-protonated O-sulfated peptides which cleanly expelled SO_3 upon ECD; however, ECD of divalent complexes of these O-sulfated peptides allowed recognition of the sites of sulfation [37]. The top-down analytical approach has matured to the point that it can be conducted on the chromatographic time scale as demonstrated in the analysis of a whole cell lysate of yeast [38]. The mechanism and energetics of bond cleavage following ECD has been well described by Turecek [39, 40]; experiments with labeled peptides show evidence for at least two mechanisms, one slow, the other fast [41].

Although use of ECD with a quadrupole has been reported, the adverse influence of RF voltage on electron trajectories needs to be addressed before ECD can be used with ion traps [42].

3.2. Electron transfer dissociation

Electron transfer dissociation (ETD) appears to be on track to replace electron ECD, in part because a superconducting magnet is not necessary for the instrumentation which can

be simply an ion trap mass spectrometer [43-48]. (The electron capture approach is not practical in quadrupole ion traps because the thermal electrons are quickly excited and ejected by the radio frequency (RF) fields.)

ETD adds a low-energy electron to a multi-protonated molecule via an ion/ion reaction, thereby converting it from an even-electron species to a radical cation, which readily dissociates via a variety of pathways in a manner analogous to those observed in ECD [44, 45, 49]. The efficiency of dissociation is improved substantially by combining ETD with CAD in a procedure called ETcaD, which reportedly increases dissociation of the protein to permit nearly 90% sequence coverage [50].

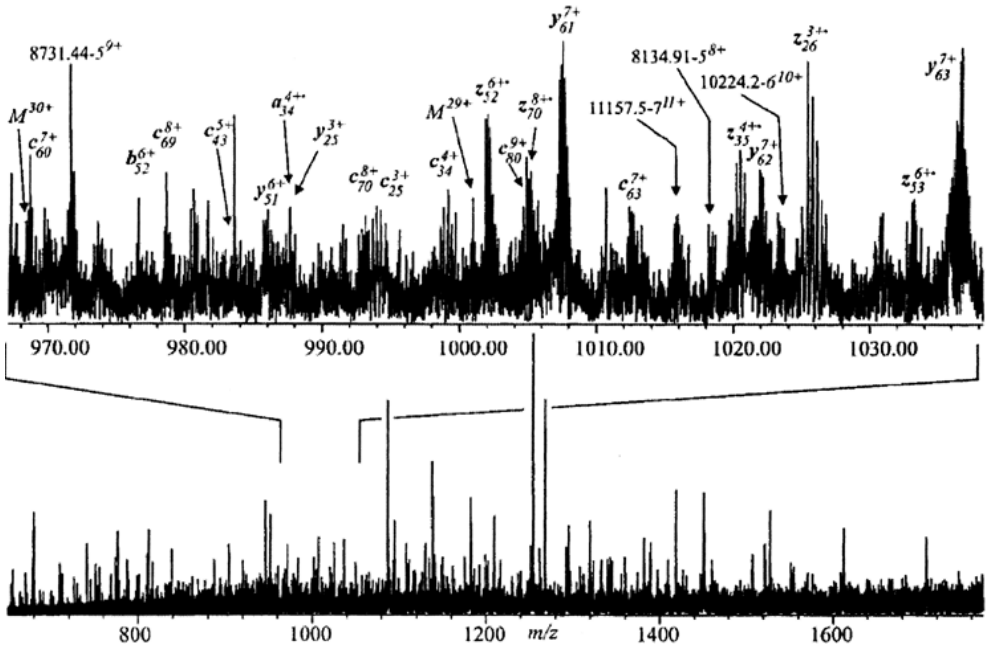

Figure 6. *A selected segment of the activated ion ECD spectrum of carbonic anhydrase B (29 kDa). Reprinted from Horn, Ge, & McLafferty, PNAS 97: 10313 (2000) with permission from the National Academy of Science.*

The thermal electron of interest in ETD is transferred to the protonated analyte by interaction with an externally formed anion ($\mathbf{A^{-\cdot}}$) as represented conceptually in Scheme 2.

$$\mathbf{MH_2^{2+} + A^{-\cdot} \rightarrow MH^{+} + AH^{+\cdot}}$$

Scheme 2

An analyte is typically ionized by ESI to form a multi-protonated molecule, which is guided into a reaction chamber for interaction with a beam of electron-rich anions formed in a separate ion source; ETD is readily accomplished due to electrostatic attraction between oppositely charged ions in the interaction chamber. Because the movement of both the reagent ions and the analyte ions can be controlled by electric fields, there are a variety of ways by which the ion/ion interaction can be conducted. Some of these are represented in Figure 7 as conceived by the McLuckey group. One means of performing these methods of ion/ion interaction is by use of an axial-ejection linear quadrupole ion trap in the position of a *Tandem-In-Space* mass spectrometer. In such an implementation of ETD, a transmission quadrupole can be used as MS 1 to select either analyte ions (multi-

ple-protonated ions) or anions (which can be referred to as reagent ions). After the ETD, in some cases in combination with CAD, is accomplished, the resulting product ions are analyzed according to their *m/z* values using a second transmission quadrupole (MS 2).

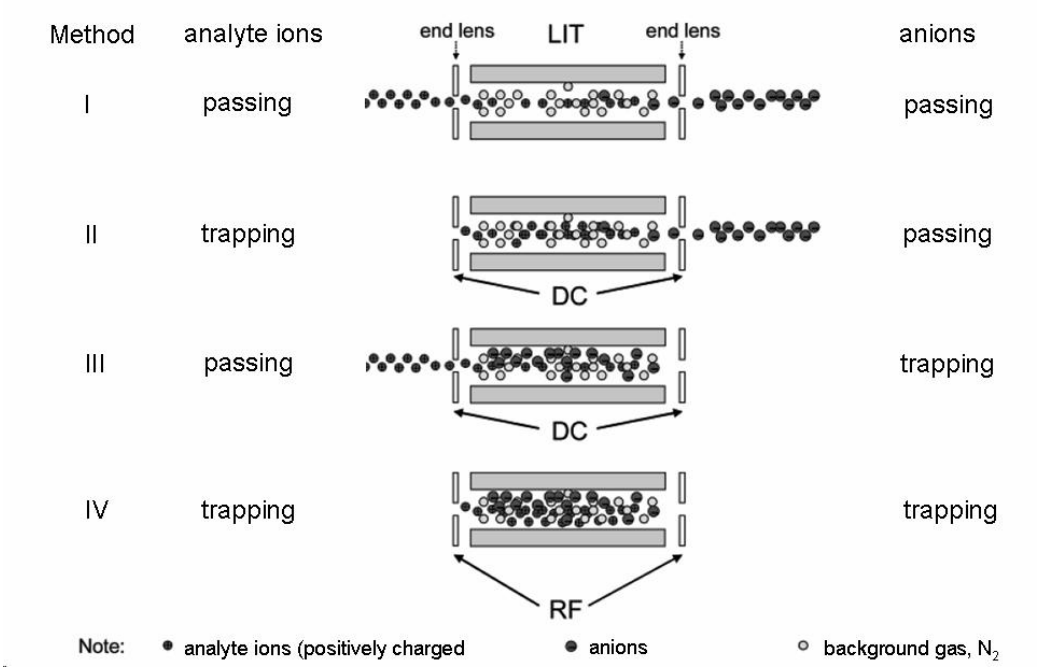

Figure 7. *Four methods of effecting ion/ion electron-transfer dissociation experiments in an LIT: (I) passage of both polarity ions; (II) positive ion storage/negative ion transmission; (III) positive ion transmission/negative ion storage; (IV) mutual storage of both polarity ions. Reprinted* from Liang, X; Hager, JW; McLuckey, SA "Transmission Mode Ion/Ion Electron-Transfer Dissociation in a Linear Ion Trap" *Anal Chem* ASAP article published on Web 3/28/2007, with permission from the Amer. Chem. Soc.

One implementation in which quadrupole mass filters are used to control the input (MS 1) and output (MS 2) of ions for interaction in a linear ion trap is shown in Figure 8 [51]. Another implementation uses a pulsed triple ionization source, using a common atmospheric/vacuum interface and ion path, to generate different types of ions for sequential ion/ion reaction experiments in a LIT-based tandem mass spectrometer [52, 53]. Unlike the low-mass electrons, the heavier anions (~100 Da) are not adversely affected by the RF fields in quadrupole ion traps, thereby allowing such relatively inexpensive instrumentation to be used for 'top-down' analysis of proteins that was once exclusively reserved for those with FTMS instrumentation [46, 54, 55].

There have been two commercial implementations of ETD on quadrupole ion traps (QITs). One is the product introduced by Agilent Technologies/Bruker Daltonics at PittCon 2006 based on their high-capacity 3D QITs [56, 57]. This implementation involves the isolation of multiple-protonated molecules of a single *m/z* value followed by the introduction of the negative-charge reagent ions. There is no isolation of reagent ions in this process, which means that if multiple reagent-ion species are formed, they compete with one another in their reactions with the analyte ions.

The other implementation was introduced by Thermo Fisher Scientific based on their LTQ

XL linear quadruple ion trap mass spectrometer at PittCon 2007 [57]. Because the LTQ XL is a segmented ion trap (see Figure 9), the analyte ions are isolated in the main body of the trap in the usual manner. The ions of a single *m/z* value can then be moved to the first segment (segment on the trap entry side). The reagent ions are then allowed to enter the trap from a conventional EI/CI ionization source. Reagent ions of a specific *m/z* value are isolated in the main body of the trap. The analyte ions then are allowed to return to trap's main body where the ion/ion reaction and dissociation takes place with the storage of product ions. The flexibility offered by three segments of the LQT XL translates into the capability for carrying out the combination of electron-transfer dissociation and collisionally activated dissociation (ETcaD) discussed above.

In one application of ETD [43, 45, 46], multi-protonated protein molecules are allowed to react with fluoranthene radical anions; the electrophilic anions are so much more massive than free electrons that their trajectories are not adversely affected by RF. Electron transfer to the multi-protonated protein promotes random dissociation of the N-C_α bonds of the protein backbone. The resultant multi-protonated fragment ions are then deprotonated during a second ion/ion reaction with the carboxylate anion of benzoic acid. The *m/z* values for the resulting single- and double-charged ions are used to read a sequence of up to 15-40 amino acid residues at the N- and C-termini of the protein. This information and the measured mass of the intact protein are used to search protein or nucleotide databases for possible matches, detect post-translational modifications, and determine possible splice variants [58]. In another application, ETD allows differentiation of isoaspartic acid and aspartic acid residues using the same **c** + 57 and **z** - 57 peaks to define both the presence and position of isoaspartic acid in the same way previously reported using ECD [59].

The use of ETD avoids the problem of the low-mass cutoff when using the ion trap for CAD. That is, when the ion trap is used for CAD, an RF amplitude is imposed to excite only the precursor ions (not any other ions that may be in the trap). The conditions that make this selective excitation possible also limits the *m/z* range of product ions that can be trapped during the CAD process down to an *m/z* value that is one third that of the precursor mass, the so-called low-mass cutoff problem in transmission quadrupole mass spectrometers. By using ETD, rather than CAD, to dissociate the precursor ion, the ion trap can be adjusted to trap product ions down to a very low value of *m/z*; this additional advantage of ETD allows the ion trap to capture low-mass immonium ions that are important for identifying amino acid residues that constitute the protein under investigation. A recent report describes the implementation of ETD on a hybrid linear ion trap-orbitrap to produce accurate mass measurements (2 ppm) at high resolving power (60,000), an advancement that promises to accelerate adoption of top-down protein analyses by research laboratories that are not blessed with super-conducting FT-ICR-MS instrumentation [60].

While highly charged cations are good candidates for ETD, doubly protonated molecules of certain compounds like phosphopeptides may be difficult to form. In such cases, the McLuckey group has found that phosphopeptides readily form negative ions, which can then be converted to doubly protonated molecules via an ion/ion reaction multiply with protonated amino-terminated dendrimers; the indirectly formed doubly protonated phosphopeptides can then be subjected to ETD in the usual way to yield characteristic **c**- and **z**-type fragment ions by dissociation of the N-C_α bond along the peptide backbone while preserving the labile posttranslational modifications [47, 54, 61, 62]. In a developing approach to ETD that circumvents difficulties associated with vaporizing a precursor

molecule, ESI is used to produce ions of arenecarboxylic acids that can be dissociated subsequently into ETD reagent ions [49, 53]. The efficiency of dissociation is improved substantially by combining ETD with CAD in a procedure called ETcaD, which reportedly increases dissociation of the protein to permit nearly 90% sequence coverage [50].

In a demonstration of ETD for analyses on the chromatographic time scale, a modified linear ion trap using sequential ion/ion reactions to achieve electron transfer then proton transfer was used to dissociate intact proteins from the Escherchia coli 70S ribosomal protein complex then convert the resulting peptide fragments to a mixture of singly and doubly protonated species [46]. In this way, multi-protonated molecules of the proteins formed during electrospray ionization are allowed to react with fluoranthene radical anions. Electron transfer to the multi-protonated molecules promotes random fragmentation of amide bonds along the protein backbone. Multi-protonated fragment ions are then deprotonated in a second ion/ion reaction with even-electron benzoate anions to diminish the charge state down to singly and doubly protonated molecules. The ETD spectra of these singly and doubly protonated radical cations provide fragment ion peaks that can be related to the sequence of 15-40 amino acids at both the N-terminus and the C-terminus of the protein. These data, along with the measured mass of the intact protein, are employed to identify known proteins and to detect the presence of post-translational modifications. In this application, 46 of the 55 known unique components were identified during analysis of the intact proteins from the Escherchia coli 70S ribosomal protein complex during a single, 90-min, on-line, chromatographic experiment [46].

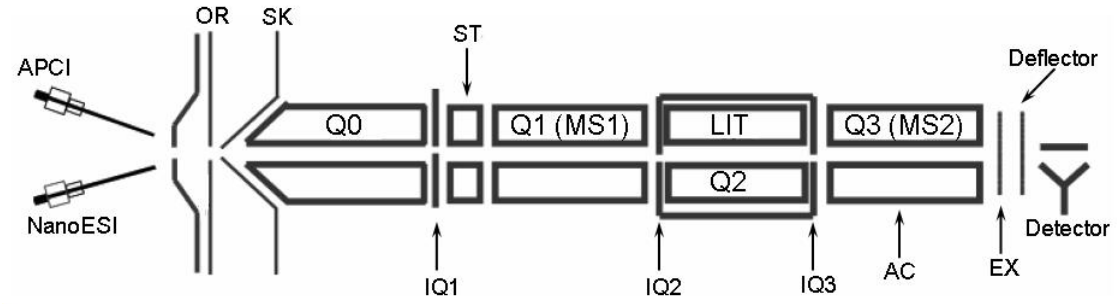

Figure 8. Illustration of Applied BioSystems/MDS Sciex Qtrap Tandem-In-Space mass spectrometer arranged so that the LIT is in the position of the collision cell rather than being positioned as Q3 (MS2) as is the case with currently commercially available instruments. This illustration shows a homemade dual ion source; nanospray (for formation of multiple-protonated analyte molecules) and APCI (for formation of reagent anions). Q1 can be set to alternately pass the two different polarity ions with storage of either in the LIT. Q3 is used for m/z analysis of the resulting product ions. Reprinted from Liang, X; Hager, JW; McLuckey, SA "Transmission Mode Ion/Ion Electron-Transfer Dissociation in a Linear Ion Trap" Anal Chem ASAP article published on Web 3/28/2007, with permission from the Amer. Chem. Soc.

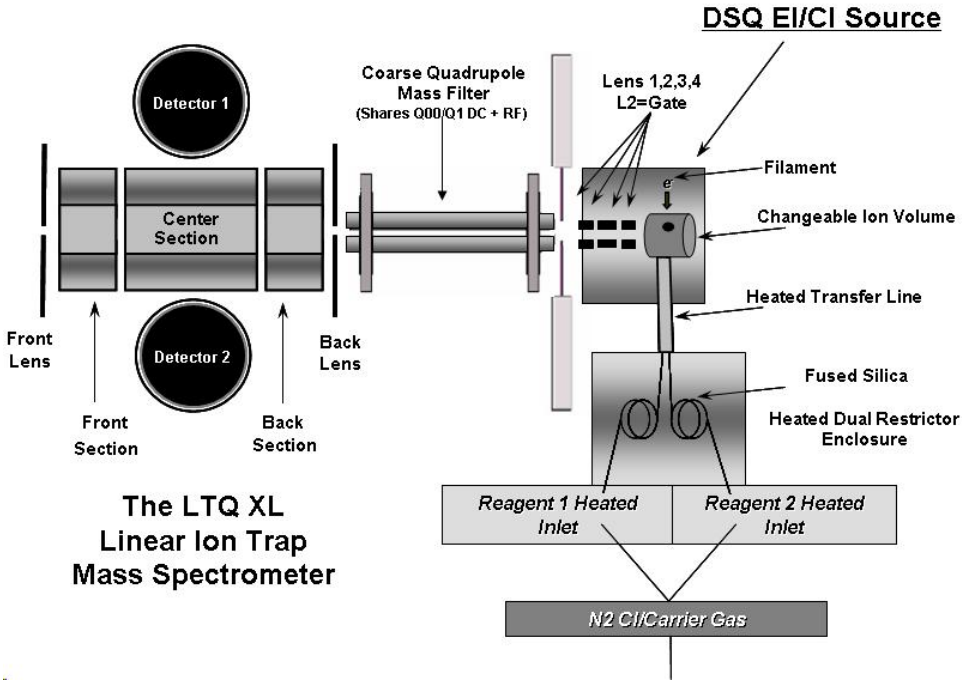

Figure 9. Illustration of the Thermo Fisher Scientific segmented-linear quadrupole configured for electron-transfer dissociation (ETD). Courtesy of Thermo Fisher Scientific.

4. Conclusion

The technology of electron-transfer dissociation (ETD) allows instrumentation based on linear ion traps and transmission quadrupoles to be used for 'top-down' analysis of proteins providing analytical results comparable to those achieved by electron capture dissociation (ECD) with ion cyclotron resonance (ICR) FTMS instrumentation. In this way, the 'top-down' approach to protein analysis should become more widely available as the ion trap and quadrupole instrumentation is less expensive to purchase and to maintain than the FT-ICR-MS instrumentation.

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