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Methods for analyzing peptides and proteins on a chromatographic timescale by electron-transfer dissociation mass spectrometry

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Abstract

Advancement in proteomics research relies on the development of new, innovative tools for identifying and characterizing proteins. Here, we describe a protocol for analyzing peptides and proteins on a chromatographic timescale by coupling nanoflow reverse-phase (RP) liquid chromatography (LC) to electron-transfer dissociation (ETD) mass spectrometry. For this protocol, proteins can be proteolytically digested before ETD analysis, although digestion is not necessary for all applications. Proteins ≤30 kDa can be analyzed intact, particularly if the objective is protein identification. Peptides or proteins are loaded onto a RP column and are gradient-eluted into an ETD-enabled mass spectrometer. ETD tandem mass spectrometry (MS/MS) provides extensive sequence information required for the unambiguous identification of peptides and proteins and for characterization of posttranslational modifications. ETD is a powerful MS/MS technique and does not compromise the sensitivity and speed necessary for online chromatographic separations. The described procedure for sample preparation, column packing, sample loading and ETD analysis can be implemented in 5–15 h.

INTRODUCTION

A comprehensive and sensitive analysis of peptides using liquid chromatography (LC) online with tandem mass spectrometry (MS/ MS) has, until recently, mainly utilized collision-activated dissociation (CAD) for peptide fragmentation. In a traditional LC-MS/MS analysis of peptides, CAD is employed to dissociate small peptides derived from proteins by proteases, such as trypsin[1,2]. The peptide mixture is commonly separated by reverse-phase (RP) LC and introduced online into a mass spectrometer via electrospray ionization (ESI)[3]. Following mass analysis of the fragment ions resulting from peptide dissociation, the experimental data are usually searched *in silico* against theoretical peptides created from predicted protein sequences using database-searching algorithms[4,5]. Peptide identifications are then correlated to protein sequences in the database to identify the source proteins.

Although CAD has been widely adopted for peptide characterization, it has several shortcomings. These include the following: (i) CAD often promotes the loss of labile posttranslational modifications (PTMs) (e.g., phosphorylation), making PTM site-mapping difficult; (ii) CAD fails to generate random cleavage along the backbone of peptides that

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contain multiple basic residues and (iii) CAD provides limited sequence information for large (>30 amino acids), highly charged peptides and intact proteins[6].

Electron-transfer dissociation (ETD) is a powerful fragmentation technique that overcomes these limitations. ETD was first introduced by Syka *et al*. in 2004 (ref. [6]) and is the ion/ion analogue of electron-capture dissociation (ECD)[7]. ECD was first demonstrated on a Fourier transform ion cyclotron resonance mass spectrometer. ETD is performed on relatively inexpensive quadrupole ion trap mass spectrometers, requires short ion/ion reaction times (tens of milliseconds, short duty cycles) and promotes extensive fragmentation of the protein backbone within the time frame of a single spectrum acquisition[6,8]. Because ETD is highly efficient and takes place on a millisecond timescale, it is compatible with online chromatographic separations and can be used to analyze samples at the low femtomole level[6,9].

ETD results when multiply charged peptide cations in the gas phase are allowed to react with radical anions of a polyaromatic hydrocarbon such as fluoranthene[6,8] (Fig. 1). During the reaction, the radical anion transfers an electron to the multiply charged peptide cation.

$$[M+3H]^{+3}+C_{16}H_{10}^{-\bullet} \rightarrow [M+3H]^{+2\bullet}+C_{16}H_{10}$$

This process is exothermic and triggers highly selective fragmentation of N-C_α bonds along the peptide/protein amide backbone, and fragment ions of type c′ and z′• result[6] (Fig. 1). ETD is likely a nonergodic process, not a threshold energy dissociation process such as CAD. Peptides and proteins undergo highly efficient fragmentation, and labile PTMs remain intact upon peptide/protein dissociation[6,7,10]. Shown in Figure 2 is the ETD MS/MS spectrum of phosphorylated peptide, SGDpSDEELIRTVR. The observed c′-and z′•-type fragment ions are labeled above and below the peptide sequence, respectively. These ions facilitate complete sequence analysis of the phosphorylated peptide, including the unambiguous assignment of the modified serine residue.

Recent studies indicate that ETD can also be employed to characterize PTMs on large peptides, providing a means for identifying long-range, combinatorial modifications[11,12]. When the primary sequence of the protein being studied is known, digestion of the protein can be tailored to generate large peptides. These peptides are generally more amenable to fragmentation via ETD and are often produced by proteases other than trypsin such as endoproteinases Lys-C, Asp-N and Glu-C[12-14]. If the primary sequence of the protein is not known, then Lys-C is often an appropriate choice for protein digestion as it both generates large peptides and ensures the presence of a C-terminal lysine which facilitates interpretation of the resulting ETD spectra[6,13,15]. If the peptide or protein exists in multiple charge states, the best sequence coverage (optimal formation of c′- and z′•-type fragment ions) is likely to be obtained by acquiring an ETD spectrum on one of the higher rather than lower charge states. Competitive with the production of c′-and z′•-type ions is the process of charge reduction. In this latter pathway, the multiply charged peptide or protein accepts an electron from the fluroanthene radical anion and undergoes backbone cleavage, but the resulting c′ and z′• fragments fail to dissociate because they are held together by multiple hydrogen bonds or salt bridge interactions. In Figure 2, charge reduction of the $[M + 3H]^{+3}$ ion generates the $[M + 3H]^{+2\bullet}$ and $[M + 3H]^{+1\bullet\bullet}$ ions at *m/z* 779 and *m/z* 1,558, respectively. Supplemental activation can be employed to facilitate selective dissociation of these ions, to improve the yield of c′- and z′•-type fragment ions[6,16] while preserving labile PTMs.

Using the method detailed in this protocol, we often interrogate large peptides and intact proteins. ETD of these large, highly charged species commonly results in complex MS/MS spectra containing fragment ions in a variety of charge states. To simplify spectra, another ion/ion reaction termed proton-transfer reaction (PTR) is employed sequentially to deprotonate multiply charged fragment ions[8]. PTR is currently available from another instrument vendor (i.e., Bruker Daltonics), but is not yet available on the Thermo Scientific ETD-enabled linear ion trap (LTQ XL). Investigators are also utilizing extended ETD reaction times in order to simplify spectra and ease spectral interpretation[17].

ETD shows great promise in the field of proteomics, and here we present a protocol for implementing ETD for dissociation of both peptides and proteins on a chromatographic timescale. We demonstrate the utility of this method on a peptide containing a labile PTM, a large peptide (90 residues in length), and an intact protein (21 kDa) that were successfully interrogated via ETD.

MATERIALS

REAGENTS

- (NH_4HCO_3): 100 mM, pH 8 (Sigma Aldrich)
- DTT (Sigma Aldrich)
- Iodoacetamide (Sigma Aldrich) ▲ **CRITICAL** Prepare the solution immediately before use because of light sensitivity.
- Proteases: endoproteinases Lys-C, Asp-N and Glu-C (Roche Diagnostics), Trypsin (Promega)
- Glacial acetic acid, ≥99.9% purity (Sigma Aldrich)
- Peptide standards: angiotensin I human acetate hydrate, ≥90% purity; vasoactive intestinal peptide fragment 1–12 human, porcine, rat, ≥97% purity; adrenocorticotropic hormone (ACTH) fragment 18–39 (Sigma Aldrich)
- HPLC-grade acetonitrile, ≥99.8% purity (Mallinckrodt)
- RP-HPLC solvent A = 0.1 M acetic acid in deionized water
- RP-HPLC solvent B = 0.1 M acetic acid in 70% acetonitrile, 30% deionized water (vol/vol)
- Formic acid, >99% purity (Pierce)
- Fluoranthene, 99% purity (Thermo Scientific, manufactured by Supelco)
- Gas for chemical ionization: methane, min. 99.995% (Thermo Scientific, LTQ modified in-house for ETD); nitrogen, min. 99% (Thermo Scientific, LTQ XL)

EQUIPMENT

- Fused silica capillary tubing for precolumn: 360 μm o.d. × 75 μm i.d. (Polymicro Technologies)
- Fused silica capillary tubing for analytical column: 360 μm o.d. × 50 μm i.d. (Polymicro Technologies)
- Lichrosorb Si60 material for frit: 5-μm mean particle size (EMD Chemicals)
- C18 RP resin: 5-μm diameter, 120-Å pore size resin (YMC) for analytical columns; 5–20-μm diameter, irregular, 120 Å pore size resin (YMC) for precolumns

- C8 RP resin: 5-μm diameter, 300-Å pore size resin (YMC)
- C4 RP resin: 5-μm diameter, 300-Å pore size resin (Vydac) for analytical columns; 5–15-μm diameter, 300 Å pore size resin (YMC) for precolumns
- Laser-based micropipette puller (Sutter Instrument, model P-2000)
- Teflon heat shrink tubing for connecting analytical column to precolumn: 0.06 in o.d. × 0.012 in i.d. PTFE tubing (Zeus Industrial Products)
- High-pressure column packer and sample loader (pressure bomb) *Note*: We use a pressure bomb manufactured in-house. Proxeon offers a high-pressure column packer and sample loader.
- HPLC delivery system (HP 1100; Agilent Technologies) with post-HPLC split for nanoflow[18]
- Nanoflow microelectrospray ionization source (Thermo Scientific)
- Quadrupole linear ion trap mass spectrometer enabled for ETD *Note*: We use either a Thermo Scientific LTQ XL or Thermo Scientific LTQ modified in-house for ETD[6].

EQUIPMENT SETUP

RP chromatography—RP chromatography gradient; Step 9:

Time interval (min)	Gradient (% B, vol/vol)
0–60	0–60
60–70	60–100
70–75	100
75–80	100–0
80–100	0

A sensitive analysis is achieved using small i.d. columns (50–75-μm diameters) and 50–100 nl min^{-1} flow rates. We prefer using an Agilent 1100 binary pump delivering solvent at 0.2 ml min^{-1} and splitting the flow prior to the column as previously described[18]. We apply a spray voltage of 2 kV for all online nanoflow experiments. We prefer acetic acid as the ion-pairing agent when separating peptides for online LC-MS/MS. Acetic acid or formic acid can be utilized for analyzing intact proteins via LC-MS/ MS. You can also use higher organic content (>70% acetonitrile (vol/vol)) for solvent B when analyzing intact proteins. Under the ESI operating conditions outlined above, we observe that acetic and formic acids afford ion currents that are more than five times higher than those obtained with stronger ion-pairing agents.

PROCEDURE

Sample preparation

1. Obtain protein sample and use pH 8 buffer to achieve 5–10 pmol μl^{-1} of protein. We use 100 mM NH_4HCO_3. If protein is dried down, reconstitute sample in 100 mM NH_4HCO_3. If sample is concentrated, sample can be diluted with NH_4HCO_3. If protein is on-beads[19], exchange the suspension buffer with 100 mM NH_4HCO_3.
2. Reduce and carbamidomethylate the cysteines within the proteins. Examples for final concentrations of DTT and iodoacetamide have been detailed previously[13,20].

3. Decide whether proteins need to be digested (see option A) or if the proteins can be mass analyzed intact (see option B).

   A. Peptide analysis

   i. If the sample is complex, contains proteins of varying abundances or if the main objective includes site-mapping of PTMs, the sample should be digested to optimize dynamic range. Immobilized metal affinity chromatography can be utilized for enrichment of phosphopeptides from the peptide mixture if the sample contains proteins with low-level (<1%) phosphorylation sites[13,21,22]. If protein sequences are not known, then endoproteinase Lys-C, which cleaves C-terminal to lysine residues, is a good choice for protein digestion. If protein sequences are known, then select a protease that would generate larger peptides that will hold three or more charges.

   B. Protein analysis

   i. Proteins can be mass analyzed intact especially if the sample is of reasonable complexity. Because LC separation of proteins can be challenging, LC-MS/MS analysis is best suited for simple protein mixtures with proteins ≤30 kDa in molecular weight.

4. Quench the peptide or protein mixtures with glacial acetic acid, bringing the sample to pH 3.5.

   ■ **PAUSE POINT** Acidified peptides and proteins can be stored for several weeks at −80 °C.

RP capillary column assembly

5 For online nanoflow experiments construct a capillary precolumn (see option A) and analytical column (see option B).

   A. Precolumn

   i. Create a LiChrosorb Si60 frit in the end of a 360 μm o.d. × 75 μm i.d. fused silica capillary (Fig. 3B). To make the LiChrosorb frit, expose 1–2 mm of glass at one end of the fused silica capillary by removing the polyimide coating. Dip exposed glass into powdered LiChrosorb 10–50 times. Heat the end of the capillary with a nitrous oxide flame for 1–3 s to form the LiChrosorb frit. To pack the precolumn, make a slurry of RP resin in 70:30 vol/vol acetonitrile:isopropanol. Use a pressure bomb[23] to pack 4–7 cm of resin into the fritted fused silica capillary (Fig. 3A). Rinse the capillary with several column volumes of 0.1% (vol/vol) acetic acid. For peptide analysis, the precolumn should be packed with C18 RP resin. For protein analysis, the precolumn should be packed with C8 or C4 RP resin.

   B. Analytical column integrated with an electrospray emitter tip

   i. To optimize sensitivity, we suggest constructing an analytical column with an integrated electrospray emitter

tip. It is necessary to first create a restriction in a short section of 360 μm o.d. × 50 μm i.d. fused silica capillary to retain packing material. To construct a bottleneck restriction, remove 2–3 cm of polyimide coating ~4 cm from one end of the fused silica capillary. Generate a 15 μm i.d. bottleneck using a laser-based micropipette puller (Fig. 3F). Before packing the analytical column, it is necessary to pack the bottleneck with <2 mm of irregular RP resin to create a restriction that is resilient at >500 psi (Fig. 3E). After washing the column with several column volumes of 0.1% (vol/vol) acetic acid at >500 psi, pack the analytical column with 5–10 cm of 5-μm diameter resin (Fig. 3D). The analytical column should be packed with a similar RP material to that decided in Step 5A. Wash the column with several column volumes of 0.1% (vol/vol) acetic acid. To create an integrated emitter tip, use a micropipette puller to generate a 2 μm i.d. tip ~5 mm from the bottleneck (Fig. 3G).

? TROUBLESHOOTING

Sample loading

6 Pressure-load sample at 1 μl min^{-1} onto a RP capillary precolumn as previously described[20]. We suggest loading 500 $fmol^{-1}$ pmol of each protein to optimize dynamic range.

7 Rinse the precolumn with several column volumes of solvent A.

8 Connect the precolumn to an analytical column using heat shrinkable teflon tubing (Fig. 3C)[20].

Setting up an instrument method

9 Set up the instrument method with the following parameters:

Method parameter	Value
Activation type	ETD
Reagent AGC target (radical anions)	2×10^5 ion counts
Full AGC target (MS1 precursor cations)	1×10^4 ion counts
MSn AGC target (MS2 isolated precursor cations)	2×10^4 ion counts
Isolation window	3 *m/z*
MS1 scan range	300–2,000 *m/z*
Reaction time	100 ms*
Repeat count	2
Repeat duration	20 s
Exclusion duration	30 s

The table above lists the parameters that are appropriate for a data-dependent MS/MS analysis. We recommend using a method where a full-scan mass spectrum is acquired followed by six full-scan MS/MS spectra acquired sequentially on the six most abundant ions detected in the initial full-scan. The time to complete a duty cycle is lengthened as the number of data-dependent scans increases, and a significant increase in duty cycle time will negatively affect the dynamic range. We prefer acquiring six data-dependent scans as the average duty cycle is ~2 s. Sequence information from peptides and proteins can typically be

obtained routinely at the level of 10 and 100 fmol, respectively. To evaluate instrument performance, two peptides, angiotensin I and vasoactive intestinal peptide, are employed as internal standards at the 100 fmol level.

The reaction time, denoted with an asterisk in the table above, is an important parameter that needs to be customized for each experiment. For options, see options A–C for peptide analysis with ETD, peptide analysis with ETD and supplemental activation, and peptide/protein analysis using ETD/PTR or extended ETD, respectively.

A. Peptide analysis with ETD

i. Ion/ion reaction rates are dependent on the charge state of the precursor ion[8,24]. Reaction times need to be increased as precursor charge states decrease.

B. Peptide analysis with ETD and supplemental activation

i. For doubly or triply protonated precursors, consider applying supplemental activation[16] to facilitate dissociation of charge-reduced ions to c′- and z′•-type fragment ions.

C. Peptide/protein analysis using ETD/PTR or extended ETD

i. Highly charged peptides and proteins require less reaction time for proficient ETD. As described previously, ETD of highly charged species often yields complex MS/MS spectra[8]. We utilize a second ion/ion reaction (PTR)[8] to simplify spectra; however, although PTR is currently not available on the Thermo Scientific LTQ XL, employing a longer ETD reaction time has been shown to achieve similar results[17].

? TROUBLESHOOTING

Nanoflow LC-MS/MS analysis

10 Perform online RP separation of peptides or proteins using a flow rate of 50–100 nl min^{-1}. See suggested gradient in equipment setup. Gradient-elute peptides or proteins into an ETD-enabled mass spectrometer equipped with a nanoflow microelectrospray ionization source. We use either a LTQ (Thermo Scientific) modified in-house with a chemical ionization source for generation of fluoranthene radical anions for ETD, which is described in Syka *et al.*[6], or a LTQ modified with a Thermo Scientific upgrade to enable ETD (LTQ XL).

Data analysis

11 Use a database-searching algorithm to search c′- and z′•-type fragment ions present in ETD MS/MS spectra against an appropriate protein database. We recommend using the Open Mass Spectrometry Search Algorithm (OMSSA)[5,9,13] and/or validating MS/MS spectra via manual interpretation. OMSSA can be downloaded from http://pubchem.ncbi.nlm.nih.gov/omssa. We use precursor and product ion mass tolerances of ±1.5 and 0.35 Da, respectively.

● **TIMING**

Steps 1 and 2, reduction/carbamidomethylation: 2 h

Steps 3A–4, protein digestion: 6–8 h

Step 5, column packing: 1–2 h

Steps 6–8, sample loading: depends on sample concentration and volume (typically we perform this step in <1 h)

Steps 9 and 10, mass spectrometric analysis: <2 h per sample

Step 11, data analysis: searching depends on the size of the database, number of MS/MS scans and number of PTMs to be searched. A search can take from 30 min to 8 h depending on these parameters. The time needed for manual validation is variable and can require several days. Although 1–2 h is sufficient to validate the most abundant peptides in the sample, much more time (hours to days) may be required for complete characterization of proteins (including PTM assignment).

? TROUBLESHOOTING

Step 5

If a C8 column was used for LC-MS/MS analysis and poor chromatography resulted from the intact proteins having excessive retention times, then a C4 column may be more appropriate for separation.

Step 9

Upon data analysis in Step 11, you may observe ETD MS/MS spectra wherein charge-reduced ions constitute the majority of the ion current. Supplemental activation can be employed in Step 9 to disrupt intramolecular interactions (e.g., hydrogen bonds) present within these charge-reduced species. Supplemental activation increases the relative abundances of c′-and z′•-type ions and results in more extensive sequence information. These features of supplemental activation are shown in Figure 4.

ANTICIPATED RESULTS

Phosphorylation site assignment on a triply protonated peptide

Figure 2 shows the ETD MS/MS spectrum recorded on $[M + 3H]^{+3}$ ions derived from the phosphorylated peptide, SGDpSDEELIRTVR. This peptide was derived from HIV type 1 protein, Rev (*r*egulator of *e*xpression of *v*irion products)[25,26]. Product ions of type c′ and z′• allow for extensive sequence coverage of the peptide and enable assignment of the modified serine residue. Identification of the phosphorylated residue was achieved following gradient elution of the Rev peptide from a C18 RP column. The ETD reaction time employed was 60 ms, and the total time required to obtain the MS/MS spectrum was <350 ms.

Figure 4 displays MS/MS spectra recorded from ETD, with and without supplemental activation, on $[M + 3H]^{+3}$ ions of the 18–39 peptide of ACTH (18–39). An extended ETD reaction time of 400 ms was used to ensure the $[M + 3H]^{+2\bullet}$ reduced-charge species was the most abundant peak in the MS/MS spectra before implementing supplemental activation. The MS/MS spectrum acquired after ETD followed by supplemental activation is shown in Figure 4b,c. The $[M + 3H]^{+2\bullet}$ and $[M + 3H]^{+1\bullet\bullet}$ charge-reduced products were activated. Note that although the $[M + 3H]^{+1\bullet\bullet}$ species is outside the scanned mass range, it is retained in the LTQ, and, because the *m/z* of the $[M + 3H]^{+1\bullet\bullet}$ is <2,500, it will be activated by the supplemental activation procedure. Following supplemental activation, ETD product ions are increased in relative abundance, and, furthermore, product ions are present that were previously not detected without supplemental activation. As seen in ECD[27], activation of charge-reduced product ions resulting from electron transfer can yield ions of type c′ and z′• that have undergone radical transfer reactions; a z′•-type radical ion abstracts a hydrogen radical from the c′-type ion with which it is in complex. Therefore, observed masses for z′•-type ions are increased by 1 Da, and c′-type ions are decreased by 1 Da.

Assignment of methylation sites on a large, 90-residue peptide

Figure 5 displays the ETD/PTR MS/MS spectrum of an Asp-N-generated, 90-amino acid peptide derived from the RNA-binding protein Sam68 (*S*rc-*a*ssociated in *m*itosis of *68* kDa)[28,29]. Because the sequence of the protein was known, Asp-N was selected for digestion to generate the 90mer peptide harboring a C-terminal lysine, making the peptide amenable to dissociation via ETD. To obtain this spectrum, the Sam68 peptide was gradient-eluted from a C18 RP column into the mass spectrometer. The total scan time (including both ETD and PTR times) was <3 s. Two arginine methylation motifs are present within this large peptide, and the coverage obtained with doubly charged c′-type ions facilitated identification of the dimethylated arginine residue indicated in the spectrum.

Identification of intact 21-kDa protein

Figure 6 displays the ETD/PTR MS/MS spectrum of the 21-kDa protein subunit, p21, of the Arp2/3 (*a*ctin *r*elated *p*rotein 2/3) complex[30]. Identification of p21 was achieved following gradient elution of the intact protein from a C4 RP column. Less than 2 s was required to dissociate p21, resulting in extensive sequence information of the N- and C-termini of the intact protein. Nanoflow RP chromatography coupled to ETD MS/MS enabled the identification of p21 from a mixture of all the intact Arp2/3 protein subunits.

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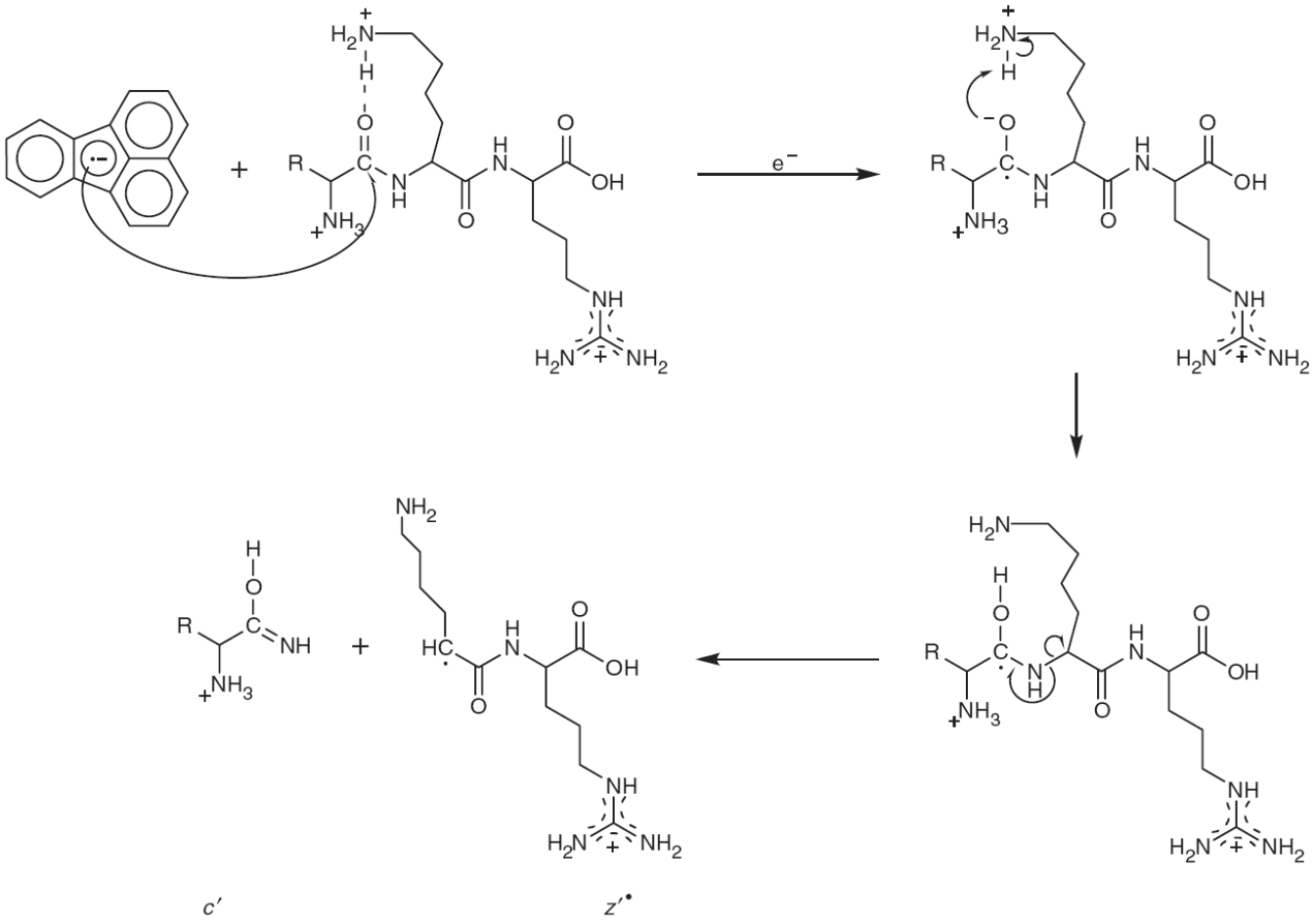

Figure 1.

Fragmentation scheme for production of ions of type c′ and z′• resulting from the reaction of a fluoranthene radical anion and a multiply protonated peptide[6,8]. c′- and z′•-type product ions are generated following transfer of a low-energy electron from fluoranthene to a carbonyl group solvated to the side chain of a basic residue, such as lysine.

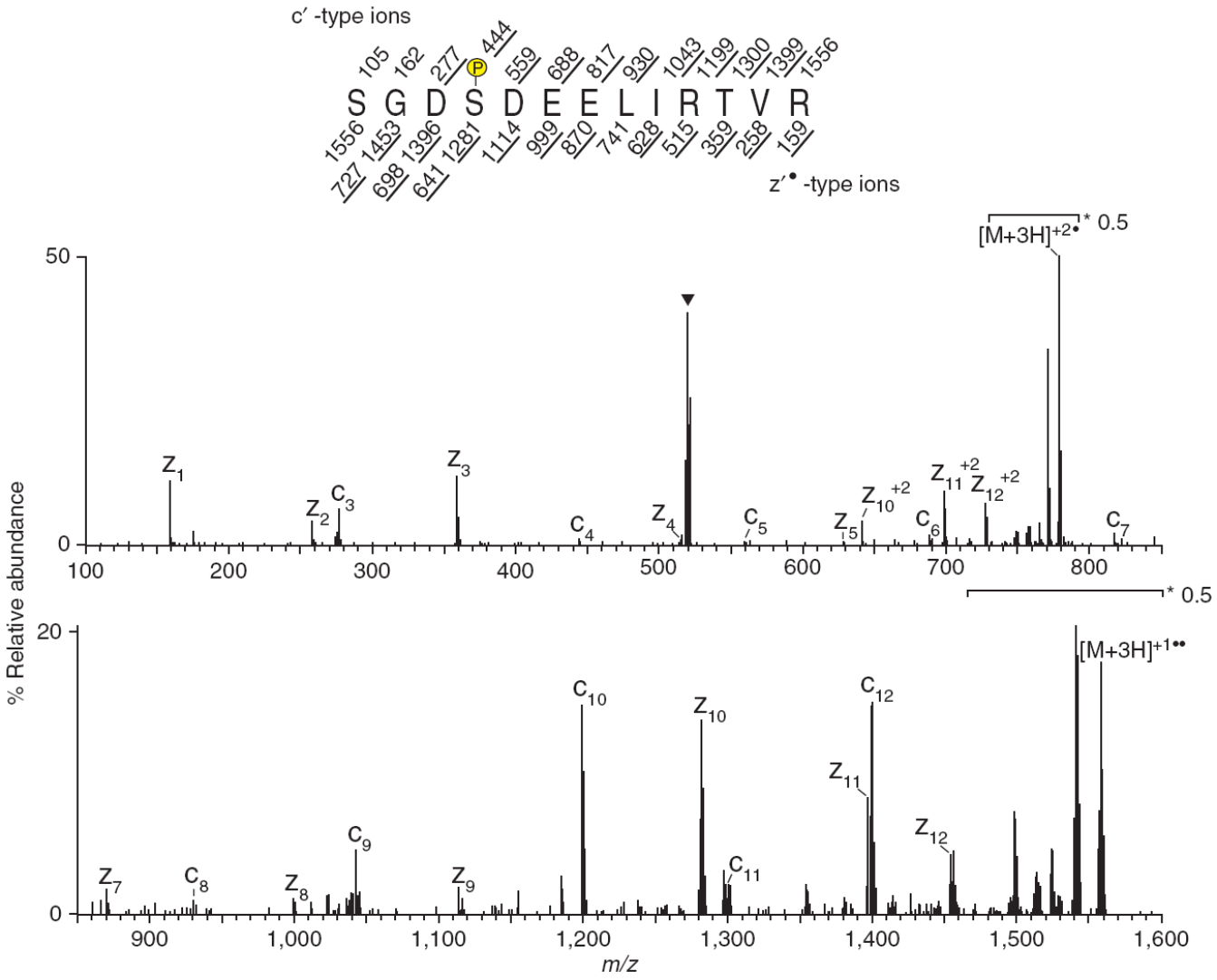

Figure 2.

ETD mass spectrum of the $[M + 3H]^{+3}$ ion (*m/z* 519.8) of a phosphorylated peptide derived from the HIV-1 Rev protein. Singly and doubly charged product ions of type c′ and z′• are listed above and below the peptide sequence, and the observed ions are underlined and labeled in the MS/MS spectrum. Singly and doubly charged product ion masses were calculated as monoisotopic and average masses, respectively, and are listed with no decimal places. Ⓟ represents a phosphate group and is positioned above the phosphorylated serine residue. A triangle (▼) is positioned above *m/z* peaks that are within the precursor isolation window. Charge-reduced species and species resulting from neutral losses are bracketed.

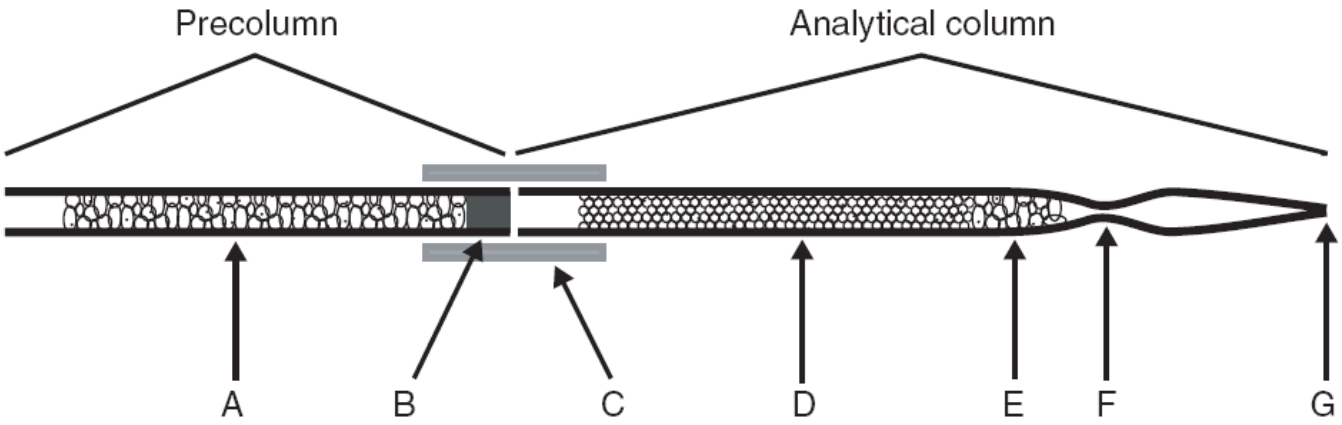

Figure 3.
Diagram of a capillary column utilized for online nanoflow LC analyses. Irregular sized RP resin (A) is contained in a length of fused silica capillary tubing by a LiChrosorb frit (B). An analytical column is attached to the precolumn using teflon tubing (C). The analytical column is packed with regular-sized RP resin (D). The regular-sized resin is retained in the column with a small amount of irregular RP resin (E) and a bottleneck restriction (F). Capillary columns are equipped with an integrated electrospray emitter tip (G).

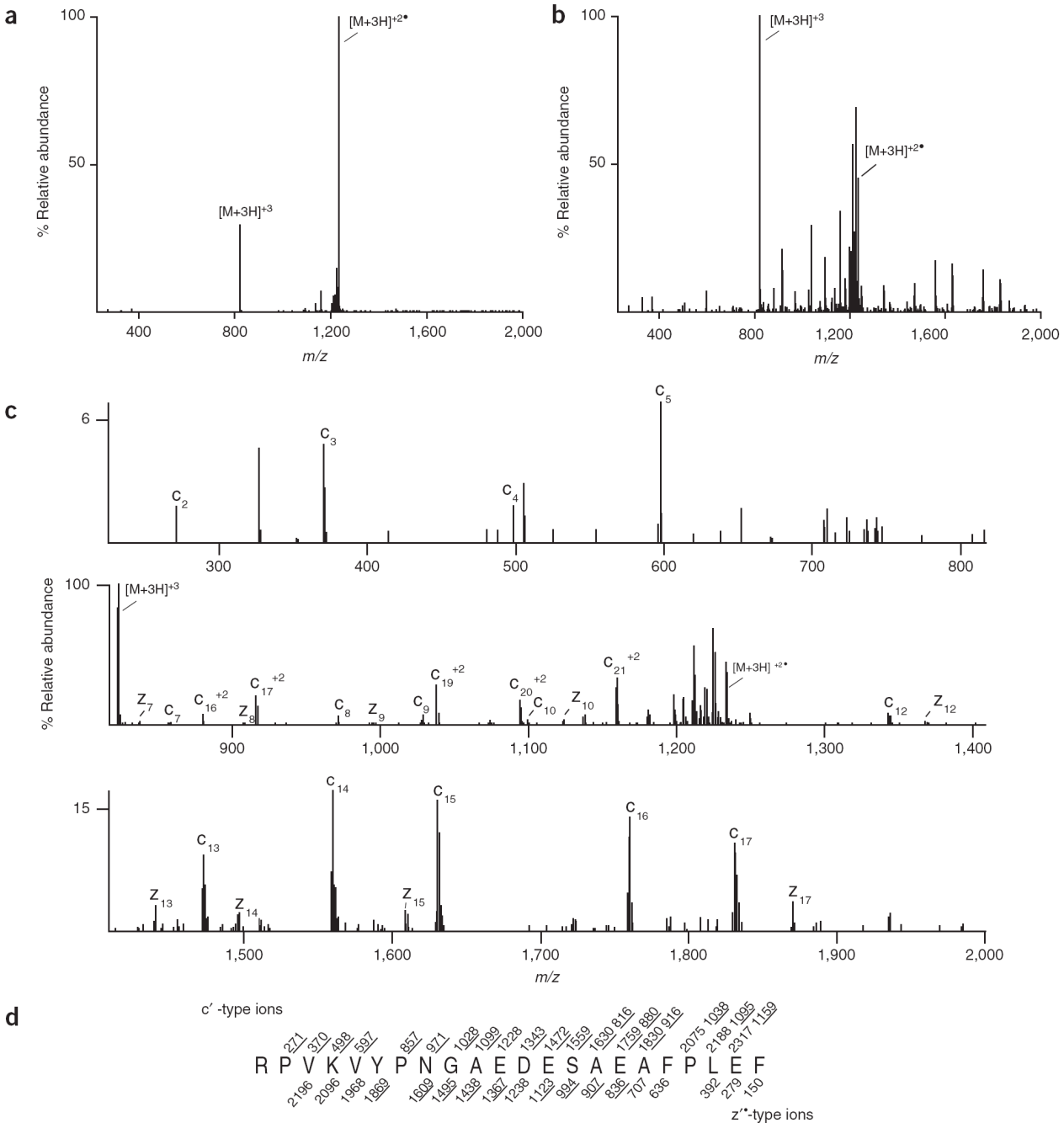

Figure 4.

ETD mass spectra recorded, with and without supplemental activation, on $[M + 3H]^{+3}$ ions (*m/z* 823) from residues 18–39 of Adrenocorticotropic Hormone (ACTH 18–39). (**a**) ETD spectrum (reaction time, 400 ms) dominated by the charge-reduced species $[M + 3H]^{+2\bullet}$ at *m/z* 1,233. (**b**) and (**c**) Same ETD spectrum, displayed in 1 and 3-panel views, respectively, acquired after supplemental activation was applied to the charge-reduced $[M + 3H]^{+2\bullet}$ and $[M + 3H]^{+1\bullet\bullet}$ ions at *m/z* 1,233 and 2,466. (**d**) Predicted *m/z* values for singly and doubly charged fragment ions of type c′ and z′• displayed above and below the amino acid sequence of ACTH (18–39). Ions observed in the spectrum are underlined. Note that hydrogen radical

transfer from c′- to z′•-type ions in the long-lived, charge-reduced species can shift the observed signals by minus and plus 1 Da, respectively.

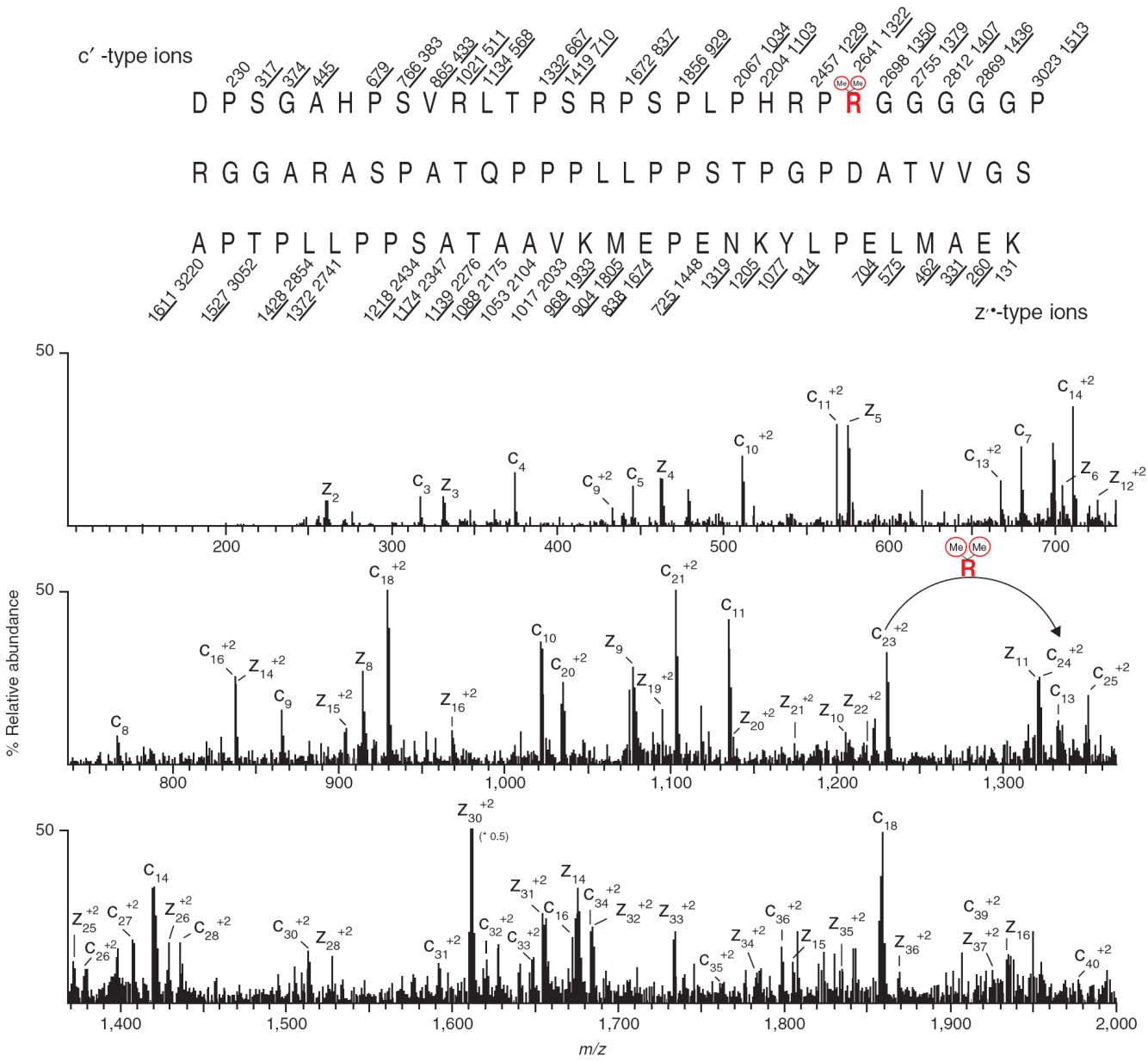

Figure 5.

ETD/PTR mass spectrum of a 90-residue peptide derived from the Sam68 protein. The ion selected for dissociation was the $[M + 13H]^{+13}$ ion (*m/z* 699.4). ETD and PTR times were 40 ms and 135 ms, respectively. The c′- and z′•-type product ions (singly and doubly charged) that occur from cleavage at the N- and C-terminal 30 residues of the peptide are listed above and below the sequence, respectively. Note that cleavage N-terminal to proline residues does not occur under ETD conditions. Observed singly and doubly charged product ions are underlined and labeled in the MS/MS spectrum. Me Me is positioned above the arginine residue found to be dimethylated. A triangle (▼) is positioned above *m/z* peaks that are within the precursor isolation window.

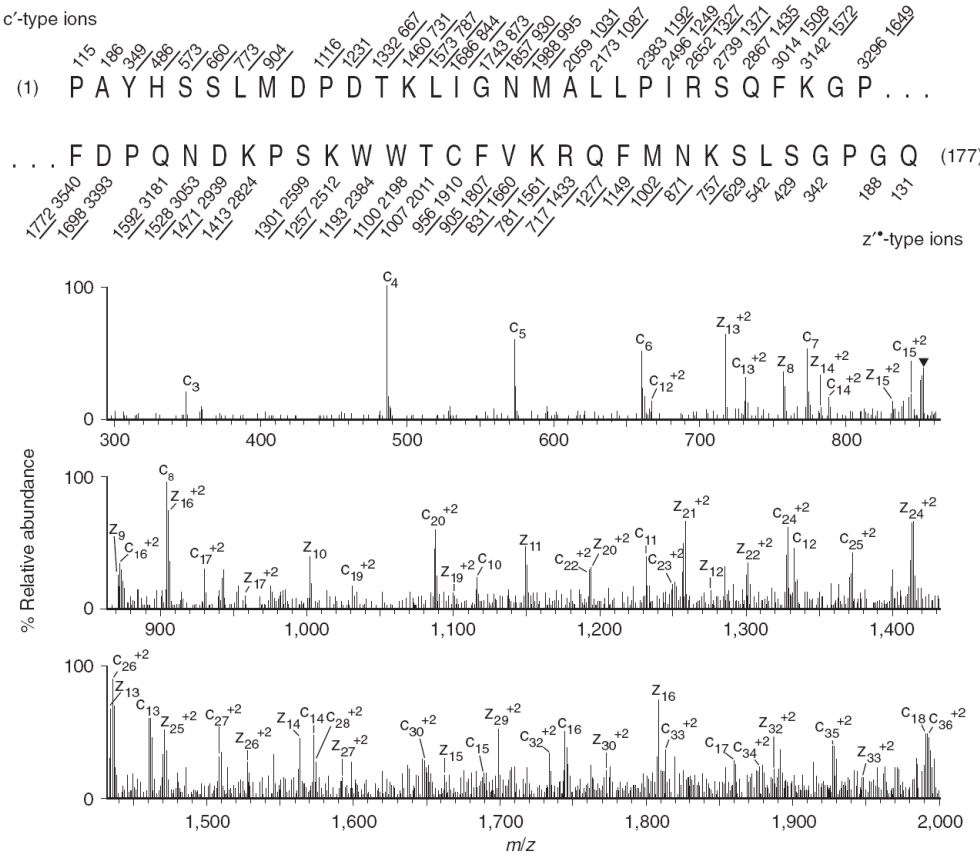

Figure 6.

ETD/PTR mass spectrum of intact 21-kDa protein, p21. The ion selected for dissociation was the $[M + 24H]^{+24}$ ion (*m/z* 851.6). ETD and PTR times were 40 and 135 ms, respectively. The first (N-terminal) and last (C-terminal) 30 residues are shown, whereas the remaining 117 residues of the protein are not shown. The c′- and z′•-type product ions that occur from cleavage at the N- and C-terminal 30 residues of the peptide are listed above and below the sequence, respectively. Note than cleavage N-terminal to proline residues does not occur under ETD conditions. Observed singly and doubly charged product ions are underlined and labeled in the MS/MS spectrum.