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# Tandem MALDI/EI ionization for tandem Fourier transform ion cyclotron resonance mass spectrometry of polypeptides

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Dedicated to Professor F. Hillenkamp on the occasion of his 65th birthday.

**Abstract**

The tandem ionization (TI) technique in which protonated polypeptides $[M + H]^+$ produced by matrix-assisted laser desorption ionization (MALDI) are further ionized inside a Fourier transform (FT) mass spectrometer by >10 eV electrons has been extended to allow the formed radical dications $[M + H]^{\bullet 2+}$ to capture low energy electrons. The capture produces $[M + H]^{+*}$ ions which, being isoelectronic with the precursor ions, are electronically excited. The electron capture process is >10 eV exothermic and may cause fragmentation, mainly into even-electron **a** and **c**′ ions. This fragmentation bears similarity with UV photodissociation, but produces little secondary fragmentation. Up to date, 10 peptides from 1 to 3 kDa have been subjected to this electronic excitation dissociation (EED). The details and potential analytical applications of EED are discussed.


*Keywords:* Polypeptides; Tandem ionization; Electron capture dissociation; Post-ionization

## 1. Introduction

In matrix-assisted laser desorption/ionization (MALDI) [1], the average mass-to-charge ratio of protein molecular ions for large sample molecules is typically >10,000, compared to ≈1000 in electrospray ionization (ESI) and ≈5000 in plasma desorption [2]. Polypeptides in the 1–10 kDa range appear in MALDI spectra predominantly singly-charged, which simplifies mass spectra interpretation at low signal/noise and/or in the absence of isotopic resolution. However, this feature also limits the sequencing possibilities of tandem mass spectrometry, since the conventional fragmentation techniques collisionally activated dissociation (CAD) [3] and infrared multiphoton photodissociation (IRMPD) [4] are more efficient for multiply-charged ions. The novel technique electron capture dissociation (ECD) [5] cleaves more bonds than CAD and IRMPD [6], but it only works with ESI-generated multiply-protonated precursor ions.

Recently, we have reported on the possibility to increase the charge state of singly-protonated polypeptides by tandem ionization (TI) [7]. In TI, MALDI-produced $[M + H]^+$ ions between 1 and 3.5 kDa are

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further ionized by electron impact (EI) in collisions with >10 eV electrons inside a Fourier transform (FT) mass spectrometer [7]:

$$[M + H]^+ + e^-_{fast} \rightarrow [M + H]^{\bullet 2+} + 2e^- . \quad (1)$$

This tandem MALDI/EI ionization (TI) has been used for determination of the ionization energies of protonated polypeptides, which were in the range of 10–12 eV [7]. Even ESI-generated multiply-charged (up to 16+) ≤13 kDa radical polypeptide ions have been produced [8]. In our previous communication [9], we reported that the radical dications formed in (1) can undergo intramolecular hydrogen atom transfer, which has been revealed via fragmentation following electron capture:

$$[M + H]^{\bullet 2+} + e^-_{slow} \rightarrow [M + H]^{+*} \rightarrow \text{fragments}. \quad (2)$$

Here, we report details of this novel fragmentation technique called electronic excitation dissociation (EED). The EED fragmentation pattern is compared with those in CAD, ECD and UV photodissociation techniques, including data reported earlier in the literature.

## 2. Experimental

### 2.1. Mass spectrometry

A commercial 4.7 Tesla Ultima (IonSpec, Irvine, CA) FT mass spectrometer equipped with both MALDI and ESI ion sources was used in this study. The experimental details are given elsewhere [8]. Briefly, singly-protonated polypeptides were externally produced using a 337 nm nitrogen laser for smaller peptides (<2.5 kDa), and a Q-switched 2.94 μm Er:YAG laser (Bioptic, Berlin, Germany) for larger molecules. The ions were transferred from the external ion source to a rectangular FTMS analyzer cell through a 1.2 m rf-only quadrupole ion guide, and were gated-trapped inside the cell by increasing the trapping potential from 0 to +20 V. To assist the trapping, a 4 ms argon gas pulse was applied (argon pressure in the gas cylinder was 20 Torr). The precursor ions were then selected by stored waveform ion excitation, and irradiated for 10–100 s by 17–21 eV electrons emitted from a tungsten filament placed behind the cell. During the irradiation, the quadrupole ion guide could be set to a negative potential (−40 V) that repelled the electrons back to the cell (the rf voltage was off). Since the electron capture cross-section strongly depends upon the electron energy and the ionic charge [10], the dominant capture reaction was that between repelled low-energy electrons and the radical dications. The excess of the kinetic and internal energies of the trapped ions was removed by a 4 ms argon pulse after irradiation. Typically, 5–10 accumulations ("scans") were integrated to improve the signal/noise ratio.

In order to distinguish EED fragments from products of other reactions, control experiments were performed, in which the transfer quadrupole potential was switched to a positive value (+10 V), thus preventing the slow electrons from being repelled back to the cell. EED fragmentation pattern was compared to CAD fragmentation of 1+ and 2+ of the same molecules, as well as to ECD of 2+ (doubly-charged species were ESI-produced).

### 2.2. Sample preparation

Peptides were obtained from Sigma (St. Louis, MO). Sample molecules were deposited on a stainless steel probe covered with an appropriate matrix (2,5-dihydroxybenzoic acid, DHB, for UV-MALDI and glycerol or succinic acid for IR-MALDI).

Typically, the samples were allowed to dry for several minutes in air prior to insertion into the mass spectrometer. ESI samples were prepared as $10^{-5}$ M solutions in water–methanol–acetic acid mixture (49:49:2, v/v) and electrosprayed from metallized glass nano-capillaries (MDS Proteomics, Odense, Denmark).

## 3. Results and discussion

EED spectrum of substance P (Fig. 1a) shows an extended series of **a** ions and three $\mathbf{c}'$ ions, $\mathbf{c_2}'^{+}$,

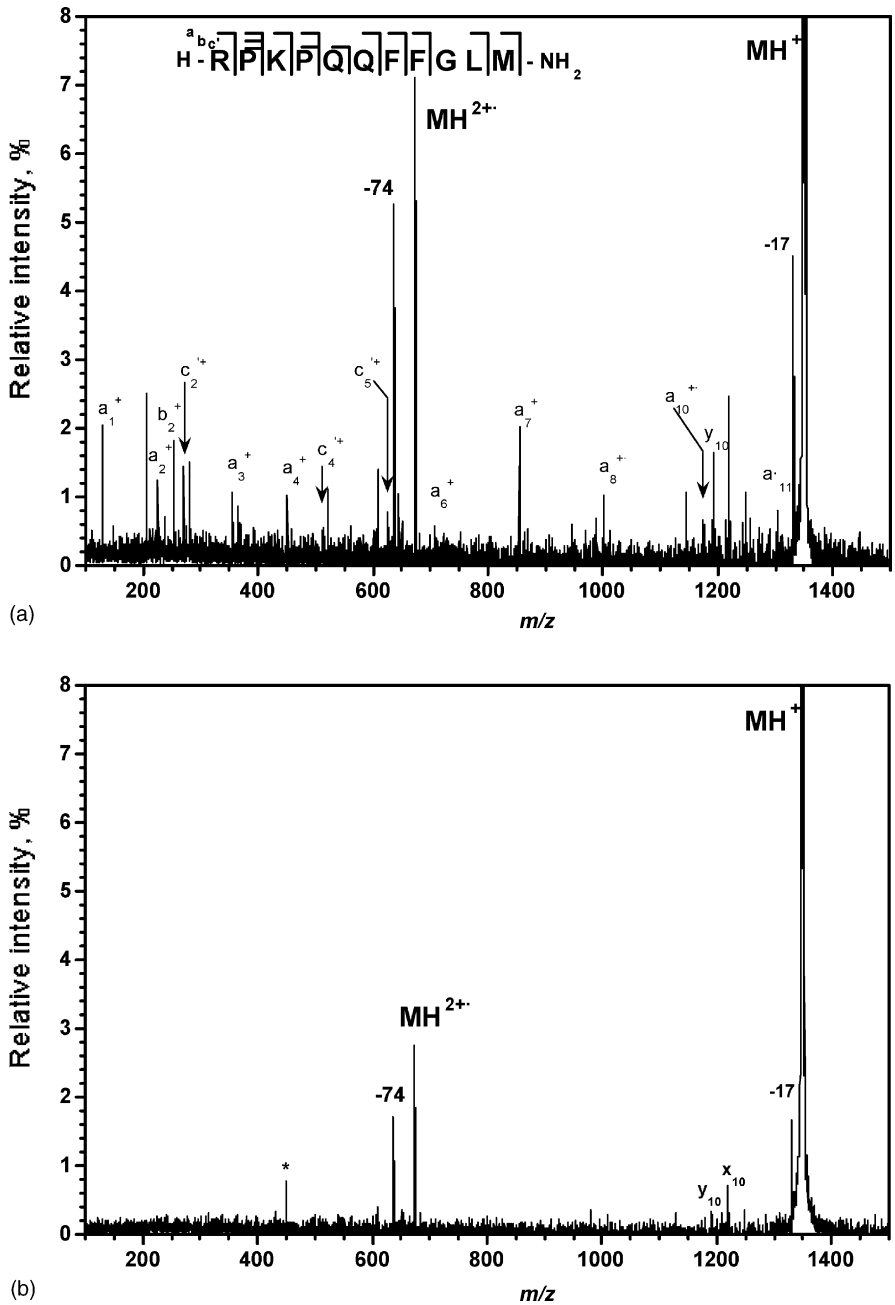


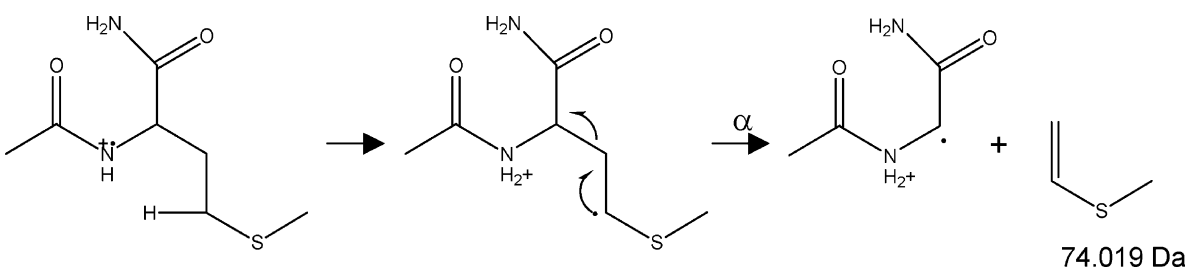

Fig. 1. Electronic excitation dissociation of MALDI-produced $[M + H]^+$ ions of substance P at different quadrupole bias voltages: (a) −30 V; (b) +10 V. Note the 74 Da loss from the radical dications. The artifact peak due to harmonics is marked by a star.

$\mathbf{c_4}'^{+}$ and $\mathbf{c_5}'^{+}$ ("′"denotes the presence of an extra hydrogen atom compared to homolytic bond cleavage, in accordance with the notation introduced in [11]). Control experiments did not yield this fragmentation, thus proving its origin from electron capture (Fig. 1b). The first six **a** ions were even-electron species, as expected for the fragmentation of an even-electron precursor. This is in contrast to the radical **a** ions formed in ECD [5], where the immediate precursors are hydrogen-abundant radical cations $[M + nH]^{\bullet(n-1)+}$. The even-electron **a** ions in EED were 1 Da lighter than the ECD **a** ions, indicating hydrogen-atom abstraction. The $\mathbf{a_8}$, $\mathbf{a_{10}}$ and $\mathbf{a_{11}}$ ions were radical species, as in ECD. All $\mathbf{c}'$ ions in EED were of the same types as in ECD, i.e., exhibited +1 Da mass shift. In ECD, this shift is due to the incorporation of the neutralized proton [5]; the same shift in EED indicates intramolecular hydrogen atom transfer prior to bond cleavage. In a separate study, we postulated that such a transfer could happen in radical dications prior to electron capture, driven by the competition for hydrogen atoms in hydrogen-deficient cations [9]. This transfer involves the most labile of the hydrogen atoms, typically the phenolic hydrogen in the tyrosine residue or the acidic hydrogen of the C-terminal carboxylic group. In the latter case, a facile loss of 44 Da ($CO_2$ group) is observed [8,9]. The substance P was in the amide form, and the loss of 74 Da was detected instead.

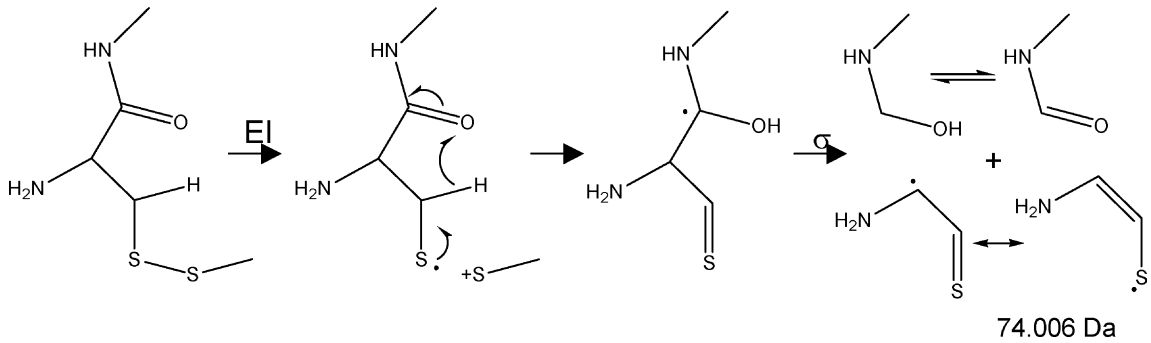
The origin of this loss has been a mystery, since out of the totally 10 peptides between 1 and 3 kDa studied up to date with EED, only melittin did not follow the empirical rule that the 44 Da loss occurred from an acidic C-terminus while the 74 Da loss appeared with an amidated C-terminus. We finally abandoned the attempts to give a unified explanation for this loss, although such an explanation would support the possibility of rapid differentiating of peptide C-termini. Our current interpretation of the 74 Da loss from radical dications is the following.

Substance P and bombesin both have an amidated C-terminal methionine. Upon ionization of, e.g., the backbone amide nitrogen, a hydrogen atom rearrangement occurs from the methionine side chain (Scheme 1). This rearrangement leaves a radical that induces α-cleavage to produce the $C_3H_6S$ loss of 74.019 Da, consistent with the measured value of 74.013 ± 0.007 Da. This loss from peptide radical cations has also been reported by Cooper et al. [12] who however did not suggest any mechanism. When substance P is in the free acid form at the C-terminus, this loss occurs at much lower rate than the competing decarboxylation (loss of 44 Da). The 74 Da loss is also seen in EED of vasopressin, which is also amidated but does not contain any methionine. Instead, it possesses a disulfide bond between two cysteins that can open upon ionization (Scheme 2) yielding one radical and one positively charged sulfur atoms. At the radical site, hydrogen rearrangement to the carbonyl oxygen leads to direct C–C bond dissociation with a loss of the resonance-stabilized radical $C_2H_4NS$ (74.006 Da) from the N-terminal cysteine residue.

CAD of 1+ and 2+ ions of substance P gave mass spectra (not shown) dominated by **b**, **y** fragmentation and losses of water and ammonia molecules from both precursor and fragment ions. At higher excitation, internal fragments appeared resulting from




Scheme 1. Suggested mechanism of 74 Da loss from the C-terminal methionine.




Scheme 2. Suggested mechanism of 74 Da loss from the N-terminal cysteine involved in a disulfide bond.

multiple cleavages of peptide bonds, in contrast to EED where no small molecule losses were observed from the fragments. The closest resemblance of the EED pattern was to the UV photodissociation (UV PD) spectrum obtained by Barbacci and Russell [13] with 193 nm photons (6.43 eV per photon) in a MALDI time-of-flight mass spectrometer. Like in EED, the UV PD spectrum was dominated by a series of **a** ions, also mostly even-electron species.

This similarity is not surprising, since both techniques involve electronic excitation. However, unlike the EED fragments, the photofragments were accompanied by abundant losses of 17 Da ($NH_3$) and by side-chain-specific and internal-cleavage products; no **c** ions were reported [13]. The losses from the UV PD fragments could be due to the absorption of more than one UV photon from the nanosecond-long laser pulse, with a rapid internal conversion of the electronic excitation into vibrational energy. The long irradiation time in EED ensured that at the moment of electron capture, the dications were mostly relaxed to the ground vibrational state (the relaxation time $<1$ s [14]). The absence of internal cleavages simplified the data interpretation in EED compared to UV PD.

Not surprisingly, EED spectrum of 1+ of substance P was very different from the ECD spectrum of 2+ shown in [15]. Despite the fact that both processes involve electron capture, there are significant differences between the two techniques in terms of exothermicity and electronic parity of the fragmenting species [9]. Noticeably, in the related field of dissociative ion–electron recombination (DR) dealing with small ($<100$ Da) singly charged ions [16], no distinction is made between recombination of odd- and even-electron ions, e.g., $H_2O^{\bullet+}$ and $H_3O^+$. In the context of the current study, the ($H_2O^{\bullet+} + e^-$) reaction is an EED-like process, while ($H_3O^+ + e^-$) is similar to ECD. As it is clear now, these two processes may lead to quite different fragmentations.

As another example of the EED performance, the spectrum of angiotensin II is presented in Fig. 2. EED produced a series of four even-electron **a** ions ($\mathbf{a_3}^+$, $\mathbf{a_4}^+$, $\mathbf{a_6}^+$ and $\mathbf{a_8}^+$), a smaller sequence coverage compared to substance P. This may be due to the free acid form of the C-terminus, which facilitates the intramolecular hydrogen transfer [9], and may inhibit fragmentation. Possibly, the radical site at the C-terminus acts as a trap for the hot hydrogen atom formed upon the electron capture [17], thus preventing the hydrogen capture by the backbone carbonyl and suppressing backbone cleavage.

Another possible site of the radical trap is the tyrosine residue. The transfer of the phenolic hydrogen from tyrosine is substantiated by the presence in Fig. 2 of the loss of 53 Th from dications, corresponding to the loss of the $C_7H_6O$ (106.042 Da) group. The mechanism of the tyrosine side chain loss is shown in Scheme 3.

The **y** and **b** fragments observed in the same spectrum were present at similar abundances without repelled electrons and without the argon pulse, which points to their origin from vibrational excitation of

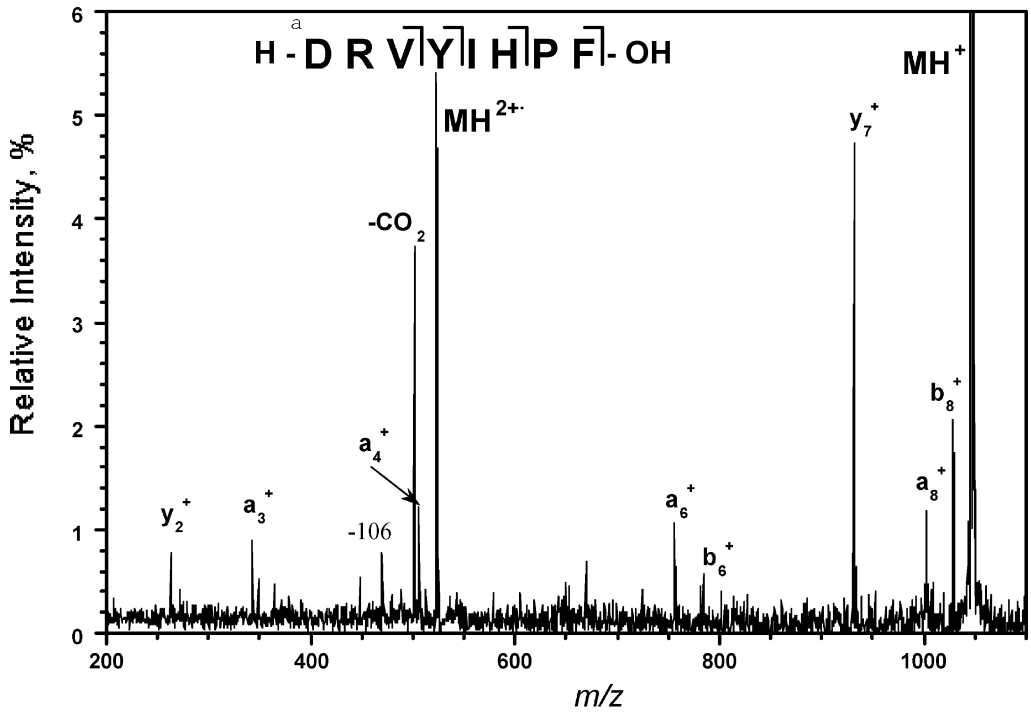
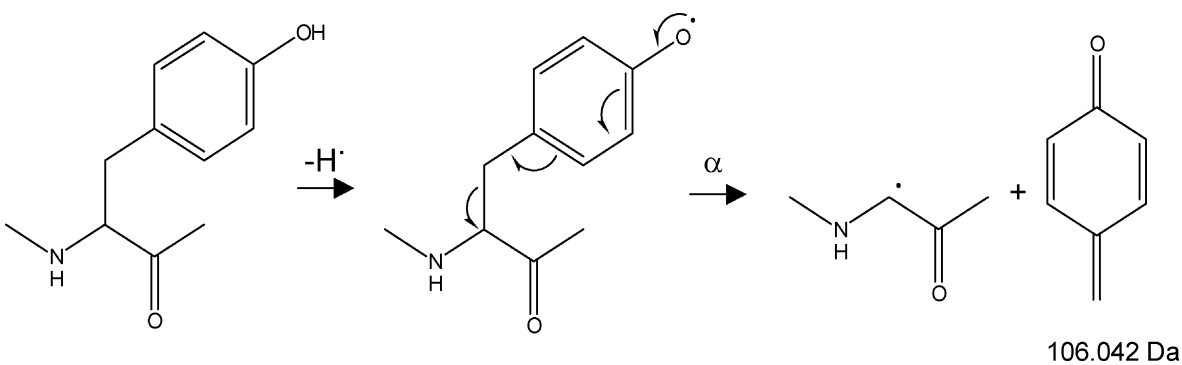



Fig. 2. Electronic excitation dissociation of MALDI-produced $[M+H]^+$ ions of angiotensin II. The **b** and **y** ions resulted from vibrational excitation of the precursor ions by electrons (EIEIO) [18]. Note the 44 Da ($CO_2$) loss from the radical dications.

the precursor cations by electrons (so-called EIEIO process [18]). Currently, EED is a rather inefficient process that requires a high abundance of the precursor $[M + H]^+$ ions; that is why ionization and electron capture are combined in one process. The major bottleneck in EED is in the production of radical dications. We hoped that employment of a more effective electron source would improve the EED performance. Recently, such a source has been installed on the ESI part of the instrument [19], which can also generate singly-protonated peptide species. Preliminarily, it was found that tandem ESI/EI




Scheme 3. Suggested mechanism of 106 Da loss from tyrosine.

ionization is most effective at rather low electron currents that result in long (>3 s) irradiation times. This can be explained by higher survival probability of radical di-cations when they are produced from the ground vibrational state. In the absence of ion–neutral collisions, the most abundant ground-state population is obtained when the average interval between electron collisions with the same ion is longer than the ionic vibrational relaxation time.

## 4. Conclusions and outlook

The potential analytical utility of EED is in the different fragmentation pattern compared to other fragmentation techniques, noticeably ECD and low-energy CAD. For example, EED of both peptides shown here gave **a** ions from the bond N-terminal to proline, which is immune to the ECD $\mathbf{c}'$, $\mathbf{z}^{\bullet}$ cleavage. More work is needed to make the technique practically applicable for high-sensitivity tasks, e.g., in proteomics studies. The bottleneck seems to be in the effective production of radical dications.

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