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Multiple ion/ion reactions in the 3D ion trap: Selective reagent anion production for ETD and PTR from a single compound

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ABSTRACT

Electron transfer dissociation (ETD) is dedicated for the sequence analysis of larger peptides and proteins. It is particularly suited for the identification of post-translational modifications (PTMs), as weakly bonded PTMs like phosphorylation or glycosilation survive the electron-induced fragmentation of the backbone of the amino acid chain. A drawback however is that ETD MS/MS-data of proteins are typically highly complex because a large number of multiply charged fragment ions are obtained. This complexity of the ETD MS/MS-data is significantly reduced when the initial electron-induced fragmentation is followed by a subsequent proton transfer reaction (PTR) to reduce the charge states of the multiple charge fragments.

Traditionally ETD and PTR are accomplished using different reagent ions generated from different neutral compounds. Here we show that the formation of reagent anions dedicated for either ETD or PTR can be accomplished from only one neutral compound. By altering the voltage of the negative chemical ionization source (nCI-source) either the odd electron anion (appropriate for ETD) or the even electron anion (appropriate for PTR) is extracted. The PTR-reagent as well as the ETD-reagent are exclusively generated at an appropriate ionization chamber voltage and no further mass selection of the reagent anion is needed. The voltage of the ionization chamber can be switched within milliseconds allowing applications for rapid sequential ion/ion reactions. One of the more interesting sequential ion/ion reactions is the combination of ETD followed by PTR, which make top–down sequencing of intact proteins possible for ion trap instruments.

1. Introduction

During the last decade, radiofrequency (RF) ion traps have become a workhorse in the analytical laboratory. Particularly for "bottom-up" proteomic experiments ion traps are widely used as these instruments are sensitive, fast, reliable and robust. The analysis of protein and peptide analysis is commonly based on use of tandem mass spectrometry, combining electrospray ionization and collision-induced dissociation (CID) [1–3]. The CID-fragmentation of peptides results in sufficient sequence information particularly for doubly charged precursors whose fragmentation mechanism has been extensively studied [4–6]. However, with increasing precursor charge state, the collisional activation leads to less sequence information because at higher charge states the loss of small neutral molecules like water or ammonia are getting preferred cleavage channels [7]. A major disadvantage of collisional activation is the internal heating of the parent ion, which predominantly yields cleavage of the weakest bond. Electron capture dissociation (ECD) [8], a complementary fragmentation technique to CID, has demonstrated its capabilities for the identification of weakly bonded post-translational modifications (PTMs), i.e., phosphorylation, sulfurylation or glycosilation, as it cleaves the peptide backbone even in the presence of the labile bonded PTM [9–11].

Electron transfer dissociation (ETD) [12–15] is a new peptide fragmentation technique that is equivalent to ECD. Both electron-induced fragmentation techniques, ECD and ETD, are particularly beneficial for identification of post-translational modifications [16–18]. Due to the difficulties for the trapping of free electrons in the RF trap [19,20], ETD is the favored electron-induced fragmentation process for the conventional less expensive ion trap instrument. The key process of the new fragmentation technique is the electron transfer from a reactant anion towards a multiply charged peptide cation, whereby the even-electron peptide cation is transformed into an odd-electron intermediate species [21,22]. Dissociations of the intermediate peptide radical cations result in

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a randomly distributed N–C (alpha) bond cleavages at each amino acid position of the peptide backbone to result in complementary c- and z-type fragment ions. In most cases, the c-fragment is observed as an even-electron species and z-type fragments is observed as an odd-electron species, which is due to its radical nature denoted as z* [23].

In prior approaches of ion/ion reactions in three-dimensional (3D) ion traps as well as in linear ion traps, reagent anions have been introduced from the same side as used for the analyte introduction [24], from the rear side of linear ion trap [12] or even through or in between the rods of the ion guide [25]. The introduction from the rear side requires several modifications: an additional ion transmission device for the reagent anions, the elongation of the vacuum manifold as well as the implementation of an additional turbo pump [15]. The design of the approach presented in this article realizes the orthogonal introduction of the reagent anion between the two octapoles of the main transmission device.

Particularly for larger and highly charged peptides, ETD has demonstrated its capabilities for sequence analysis [26–28]. ETD allows the sequence analysis of mixtures of highly modified large peptides where, e.g., the detection of individual post-translational modification of the N-terminal tail of the investigated histone in context of one another results in invaluable information in deciphering the histone code [27].

But ETD-data of multiply charged proteins ($z > 10$) show a highly complex mixture of multiply charged fragments ($z = 1$–9). The analysis of the resulting fragmentation spectrum can be rather complicated, because a plethora of multiply charged and overlaid fragment ions is expected. It has been demonstrated that the complexity of the ETD spectrum of smaller proteins can be significantly reduced if electron transfer dissociation is followed by a consecutive proton transfer reaction [27,29]. In the pioneering works, PTR-reagent anions from PDCH [30] have been used. ETD-anions from fluoranthene and PTR-anions from benzoic acid has been selected for the sequential application of multiple ion/ion reactions [27]. The PTR-reagent anions have a significantly higher proton affinity than the multiply charged ETD-fragments. Each basic PTR-reagent takes one proton from the multiply charged cation and becomes neutralized, thus reducing the charge state of the resulting cation by one. As a consequence, via several proton transfer reactions, the charge state of a multiply charged cation is decreased.

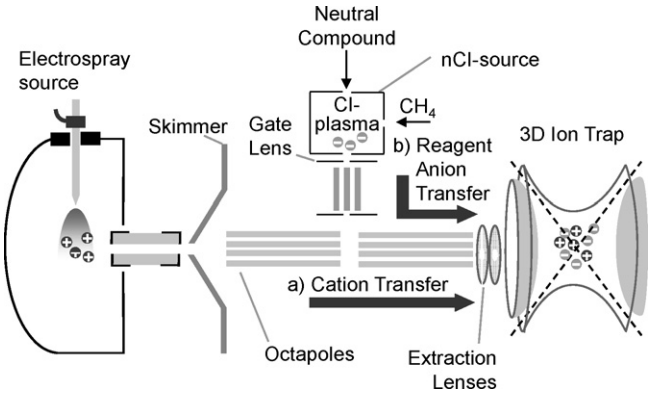


Fig. 1. General scheme of the HCTultra PTM Discovery System including electrospray source, skimmer, octapoles, extraction lenses, 3D ion trap and nCI-source. Methane and neutral molecule are supplied to the nCI-source. Electron impact of methane produces CI-plasma which leads to the reagent anions. Peptide cations and reagent anions are transferred by applying different voltages to the ion transfer system: (a) peptide cations transfer from the electrospray source. Reagent anions are blocked in the chemical ionization source by applying a repulsive voltage at the gate lens (b) reagent anion transfer from the nCI-source. Peptide cations are blocked by applying a repulsive voltage at the skimmer.

In the method described in this article, anions needed for both types of ion/ion reaction are extracted from the negative chemical ionization source (nCI-source) starting from one volatile neutral compound. The formation of the resulting reagent anion, either the odd electron anion (appropriate for ETD) or the even electron anion (appropriate for PTR), is controlled by changing the voltages applied to the chemical ionization source.

2. Experimental

2.1. Materials

Standard samples of ubiquitin (from bovine red blood cells), cytochrome *C* (from horse heart), Substance-P and the polyaromatic hydrocarbons anthracene and fluoranthene were purchased from Sigma (St. Louis, MO) and used without further purification. Electrospray solutions from proteins and peptides were prepared in 50:49:1 acetonitrile/water/acetic acid to a final concentration of 0.5–1.0 pmol/μl.

2.2. Mass spectrometry

The experiments described in this paper were all performed on a HCTultra PTM Discovery System (Bruker Daltonik, Bremen, Germany), where transmission and accumulation of target cations as well as reagent anions is under the full control of the instrument software.

The general scheme of the instrument including electrospray source, skimmer, octapoles, extraction lenses, 3D ion trap and nCI-source is depicted in Fig. 1. The nCI-source fits directly into the standard vacuum housing of the commercial ion trap. Neither an extension to the vacuum manifold nor an additional turbo pump is required.

Multiply charged peptide cations are generated in the electrospray source. The ion transfer device including capillary exit, skimmer, octapole and extraction lenses is switched to passing voltages and cations are accumulated in the ion trap. During the time for the cation introduction and precursor isolation, the reagent anions are blocked by applying a repulsive voltage at the gate lens of the nCI-source.

After the cation injection, a negative set of voltages is applied to the transmission device that the anions from the nCI-source can be transferred in the ion trap.

Solids compounds of either anthracene or fluoranthene are placed in a crucible connected to the nCI-source. The neutral molecules sublimes into the nCI-source by heating the crucible to 60 °C. The pressure of methane inside the ionization chamber is held at about 0.1 mbar and electron impact of methane is therefore the primary ionization channel. Due to the high methane pressure, the resulting methane molecular ions as well as the released electrons collide with neutral methane gas molecules thus creating a chemical ionization plasma via a cascade of reactions. The plasma contains low-energy electrons and electron attachment of these thermal electrons to the neutral molecules of anthracene or fluoranthene leads to the production of the radical anion.

By switching the gate lens potential of the nCI-source towards positive voltages, the reagent anions are orthogonally injected into the main transmission device and introduced into the ion trap. Under an initial set of anion extraction conditions, the odd-electron radical anion is ejected out of the nCI-source and further transferred into the ion trap. The radical anions are then used for electron transfer dissociation.

Even-electron anions which are needed for the proton transfer reaction are produced by lowering the ionization chamber volt-

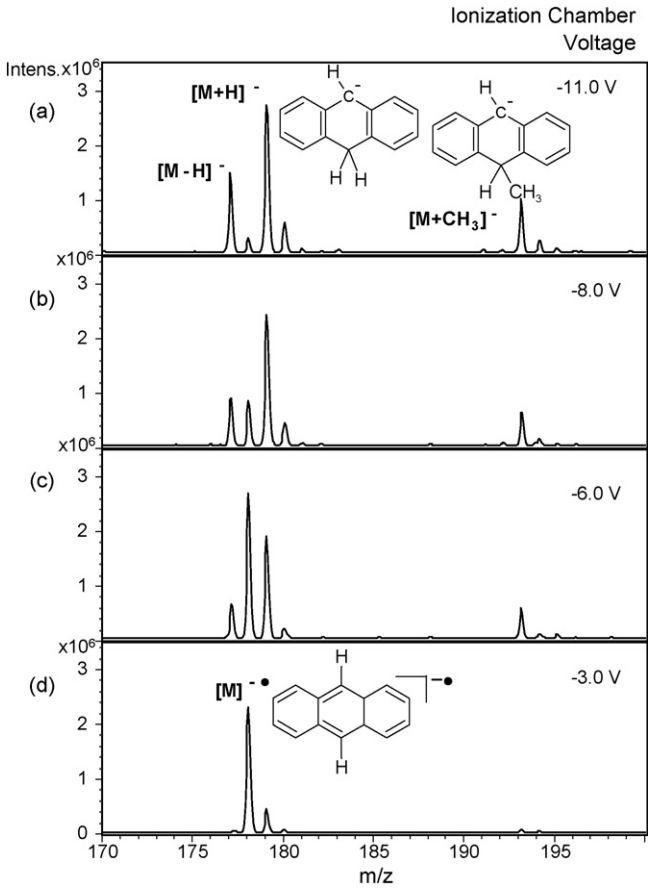


Fig. 2. Selective reagent anion production of anthracene. At ionization chamber voltages of −11.0 V (a), PTR-reagents at *m*/*z* 177 and at 179 are generated in the nCI-source. Increasing the voltage towards −8.0 V (b) results in the partial production of anthracene radical anion at *m*/*z* 178 and at −6.0 V (c) both types of reagent anion are obtained in equal abundance. The ETD-reagent anion at *m*/*z* 178 is exclusively produced at −3.0 V (d).

age within a few milliseconds. The radical anions in the nCI-source are then entirely converted into even electron species, which have a strong proton affinity. These reagent ions are then introduced into the ion trap for the proton transfer reaction step. After the PTR and the ejection of the PTR-reagent anions out of the ion trap, the mass spectrum is acquired. The multiple ion/ion reactions presented in this method are under full control of the instrument software.

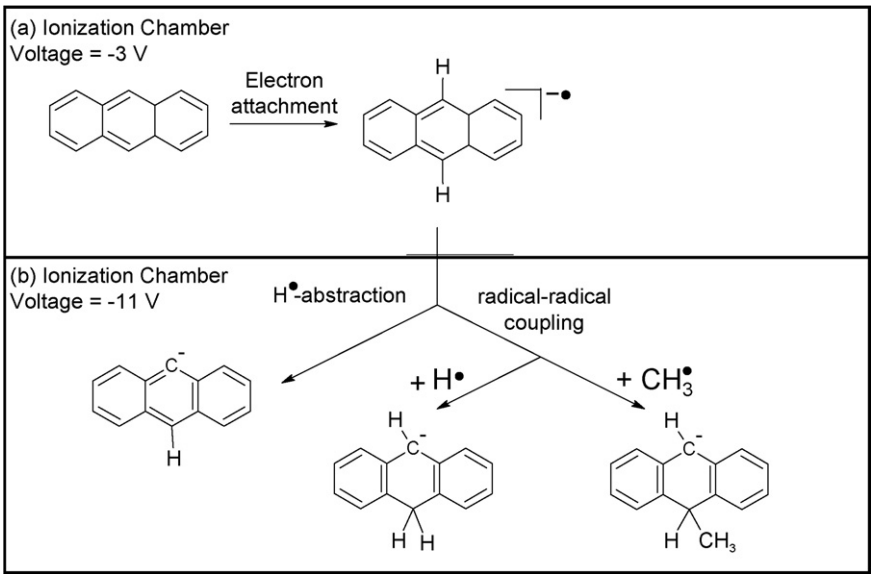
3. Results and discussion

3.1. Selective reagent anion production for ETD and PTR—starting from anthracene

The reagent anion spectra of anthracene for different ionization chamber voltages are shown in Fig. 2. Although the radical anion at *m*/*z* 178, resulting from the electron attachment to anthracene, was expected, the reagent anion spectrum at an ionization chamber voltage of −11.0 V (Fig. 2a) shows two even-electron species at *m*/*z* 177 and 179. The presence of these even electron species of anthracene has been previously shown in the literature [31]. The formation of the anion at *m*/*z* 177 can be explained by the loss of one hydrogen atom from the anthracene radical anion. The anion at *m*/*z* 179 is believed to be formed by radical–radical coupling of the anthracene radical anion with free hydrogen atoms (Scheme 1), which are expected to be formed in the ionization plasma inside the nCI-source (Fig. 1).

It is worthwhile to notice that in Fig. 2a another anion at *m*/*z* 193 occurs in lower abundance. This anion does have an even electron number as well and matches to the molecular formula $C_{15}H_{13}$. We believe that this anion is also formed out of a radical–radical coupling where the anthracene radical anion couples with the methyl radical which is produced in the plasma of the ionization chamber. This reaction is a homologue reaction to the hydrogen-attachment yielding anion at *m*/*z* 179 as described above (Scheme 1).

As the voltage on the ionization chamber is increased (Fig. 2b) it can be seen that the ionization chamber voltage is influencing secondary and tertiary reactions of the anthracene radical anion with the radical compounds (hydrogen atoms or methyl radical) from



Scheme 1. Interpretation of the selective formation of anthracene reagent anions. Electron attachment results in anthracene radical anion when −3.0 V (a) is applied to the ionization chamber voltage suitable for ETD. Lowering the voltage to −11.0 V (b) controls the formation of the reagent anions towards anions with even number of electron. The PTR-reagents are produced by hydrogen-abstraction or radical–radical coupling.

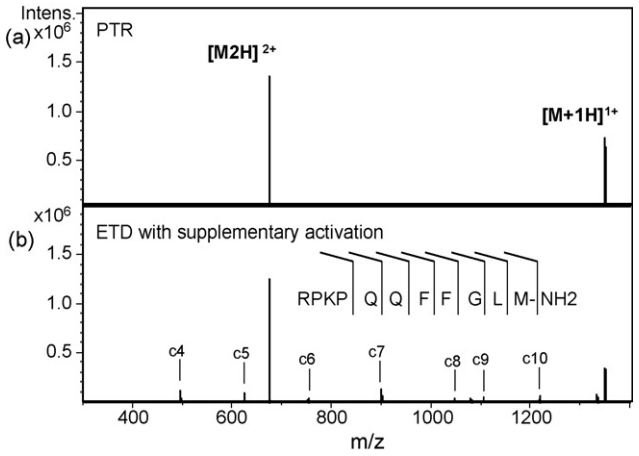


Fig. 3. Ion/ion reaction of Substance-P $[M+2H]^{2+}$ at m/z 450 with different reagent anions of anthracene by altering ionization chamber voltage between (a) −11 V and (b) and −3 V. (a) Selective deprotonation of Substance-P by the anion at m/z 177 and at m/z 179 at −11 V. (b) ETD of Substance-P by the radical anion at m/z 178 at −3 V.

the ionization plasma. At −6.0 V for example (Fig. 2c), both types of reagent anions – even electron anions as well as radical anions – are obtained in equal abundance. At −3.0 V (Fig. 2d), the formation of the even electron reagent anions are entirely suppressed and the anthracene radical anion is obtained. Further increasing of the voltage above −2 V leads to a dramatic drop of the anion intensity (data not shown). This may be attributed to decreasing transmission efficiency into the ion trap. The total ion current of the different reagent anion spectra in Fig. 2a–d is nearly constant indicating the quantitative conversion of ETD-reagent anions into PTR-reagent anions (depending on the operation condition of the nCI-source within an appropriate range). From the data set shown in Fig. 2 it is clear that the voltage applied to the ionization chamber is truly controlling the formation of the different reagent anions.

3.2. Ion/ion selectivity of the different anthracene reagent anions

In the following ion/ion reaction of Substance-P only the voltage applied to the ionization chamber has been changed in order to demonstrate the ion/ion selectivity of the different anthracene reagent anions. As shown in Fig. 3, the anthracene reagent anions with either odd or even number of electrons generated with the ionization chamber voltage of −11 V or −3 V lead to significantly different ion/ion reactions. The doubly charged Substance-P cation is selectively deprotonated by the anion at m/z 177 and 179, indicating the selective proton transfer reaction when anions with closed shell configuration are used for ion/ion reaction (Fig. 3a). Because the even electron species are resulting in proton transfer this result is consistent with other reports which suggest that anthracene is not preferred for ETD [31]. The anthracene radical anion at m/z 178 with the odd electron number results in the electron transfer reaction and here the c-ion series of Substance-P is observed (Fig. 3b). This result demonstrates that with the appropriate form of the reagent ion anthracene can be used as reagent for ETD.

For ECD it is known that fragmentation of the non-dissociated intermediate species can be assisted by additional ion activation [32–35]. For ETD the supplementary activation is needed as well, and particularly doubly charged cations result in better dissociation efficiencies, when the singly charged radical cation intermediate is resonantly activated [36,37]. Therefore, the electron transfer reaction with the anthracene radical anion is followed by a supplemental ion activation that is under fully control of the acquisition software. In these experiments the acquisition software determines the charge state of the parent ion and then determines if supplemental activation is necessary [36]. In the case of Substance-P the supplemental activation results in the appearance of the c4 and c5 ions that are only observed when the intermediate is activated. These experiments do clearly show that by simply changing the ionization chamber voltage the ion/ion reaction can be changed from PTR to ETD.

3.3. Selective reagent anion production for ETD and PTR—starting from fluoranthene

It is of interest to prove if the selective anion production from anthracene can be applied to other compounds. Because of its high electron transfer dissociation efficiency [13], fluoranthene is one of the most frequently chosen ETD-reagent anion. In comparison towards anthracene, the radical anion of fluoranthene is known to yield a higher number of electron transfer dissociation fragments. Because of these two reasons, fluoranthene was chosen as the second compound for testing the method of ETD and PTR-reagent ions production starting from one neutral compound.

The common ionization chamber voltage for generating fluoranthene radical anion at m/z 202 ($C_{16}H_{10}$) is −6 V where the radical anion is exclusively generated (Fig. 4a). For lower ionization chamber voltages, the intensity of radical anion decreases and reagent anions at m/z 201 and 203 are observed. Both anions at m/z 201 and 203 are even electron anions and match to the molecular formulas $C_{16}H_9$ and $C_{16}H_{11}$, respectively. As depicted in Fig. 4b, the formation of the radical anion is suppressed when −15 V is applied at the ionization chamber. We believe that the fluoranthene radical anion is accelerated at the outlet of the ionization chamber at a decreased ionization chamber voltage of −15 V. As the methane pressure inside the ionization source is held at 0.1 mbar, the accelerated radical anions are forced to collide with neutral as well as radical species present in the ionization plasma. The activation of the radical anions results partially in hydrogen abstraction yielding anion at m/z 201. The dominated reaction channel of the activated radical anion is the radical–radical coupling with a hydrogen atom resulting in anion at m/z 203. The radical–radical coupling is believed to be followed by a ring opening reaction yielding an aryl-substituted naphthyl anion (Fig. 4b).

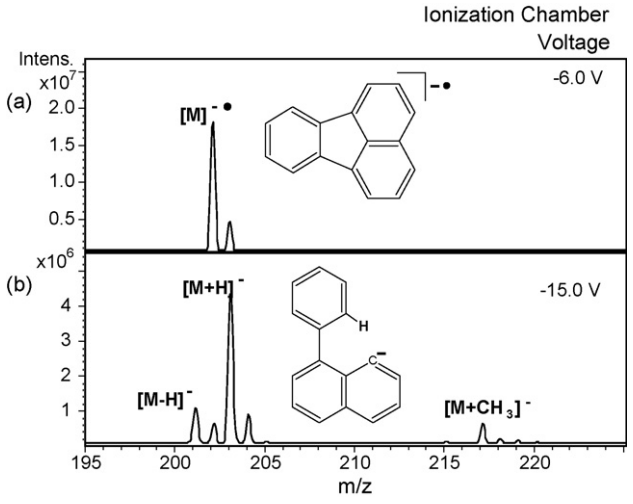


Fig. 4. Selective anion production of fluoranthene. At ionization chamber voltages of (a) −6.0 V, ETD-reagent at m/z 202 is exclusively obtained. Voltage of (b) −15.0 V results in the production of the PTR-reagent anions at m/z 201 and 203.

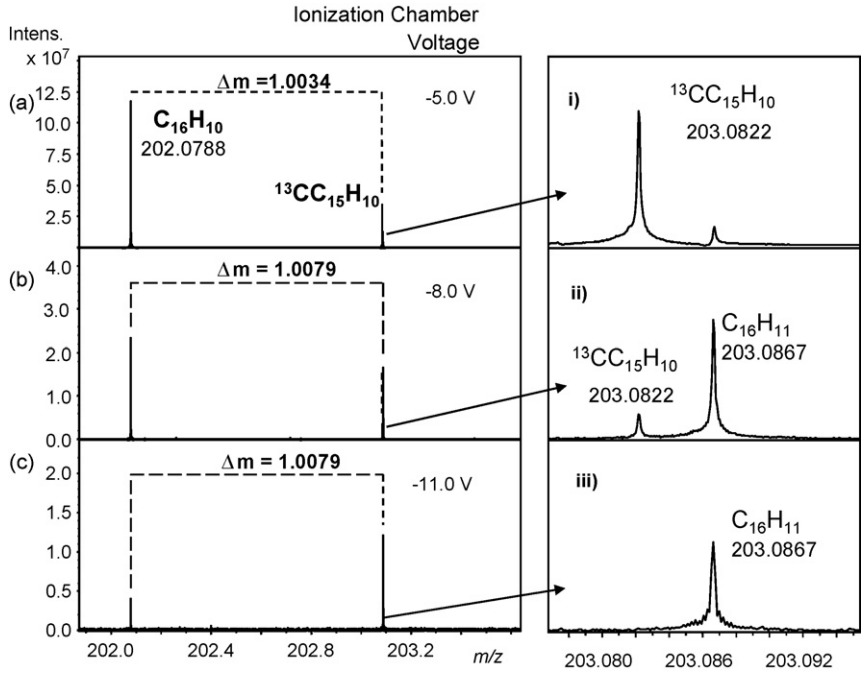


Fig. 5. High accuracy mass measurements of the different fluoranthene reagent anions on a 9.4 T FT ICRMS with a resolving power of about 1,000,0000. The molecular formula of the fluoranthene radical anion at *m*/*z* 202.0788 (a) and the naphthyl anion at *m*/*z* 203.0867 (c) are confirmed. As depicted in the subset (ii), the FT ICR instrument resolves the ^{13}C isotope of the radical anion at *m*/*z* 203.0822 from the naphthyl anion at *m*/*z* 203.0867.

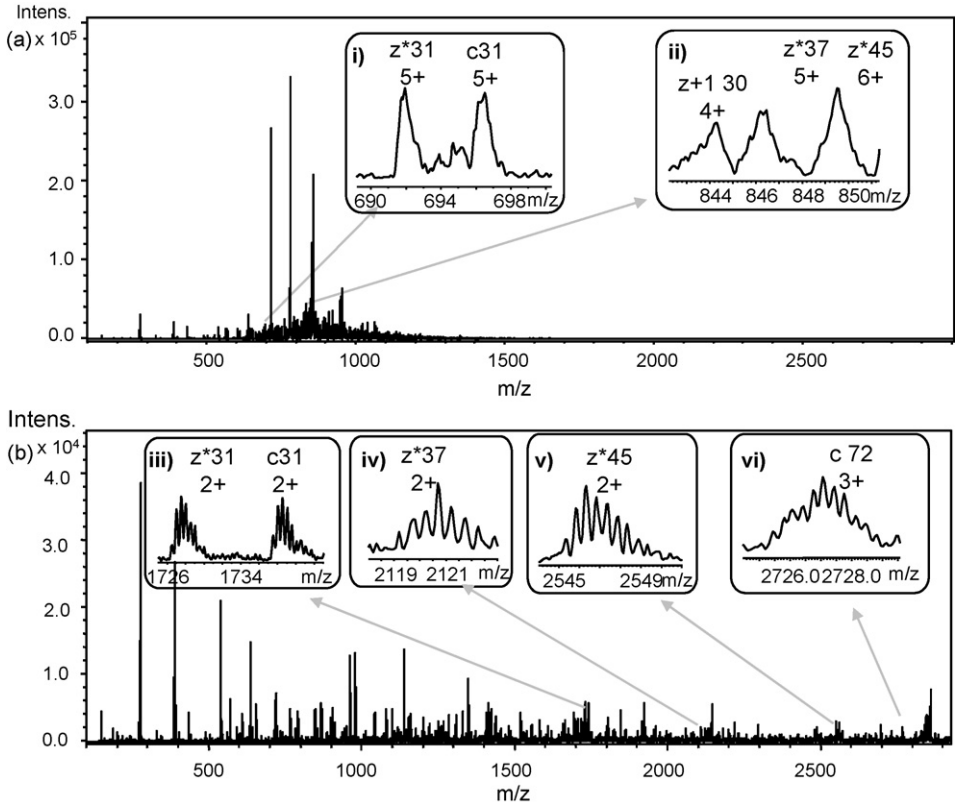


Fig. 6. Ion/ion reactions of ubiquitin 12+ (*m*/*z* 714) with the different reagent anions of fluoranthene by altering the ionization chamber voltage: (a) ETD MS/MS alone and (b) ETD followed by PTR. The subsets (i) and (ii) shows arbitrarily selected fragment ions that are not resolved in the ETD MS/MS-data and were isotopically resolved after the subsequent charge reduction step as shown in the subsets (iii), (iv), (v) and (vi).

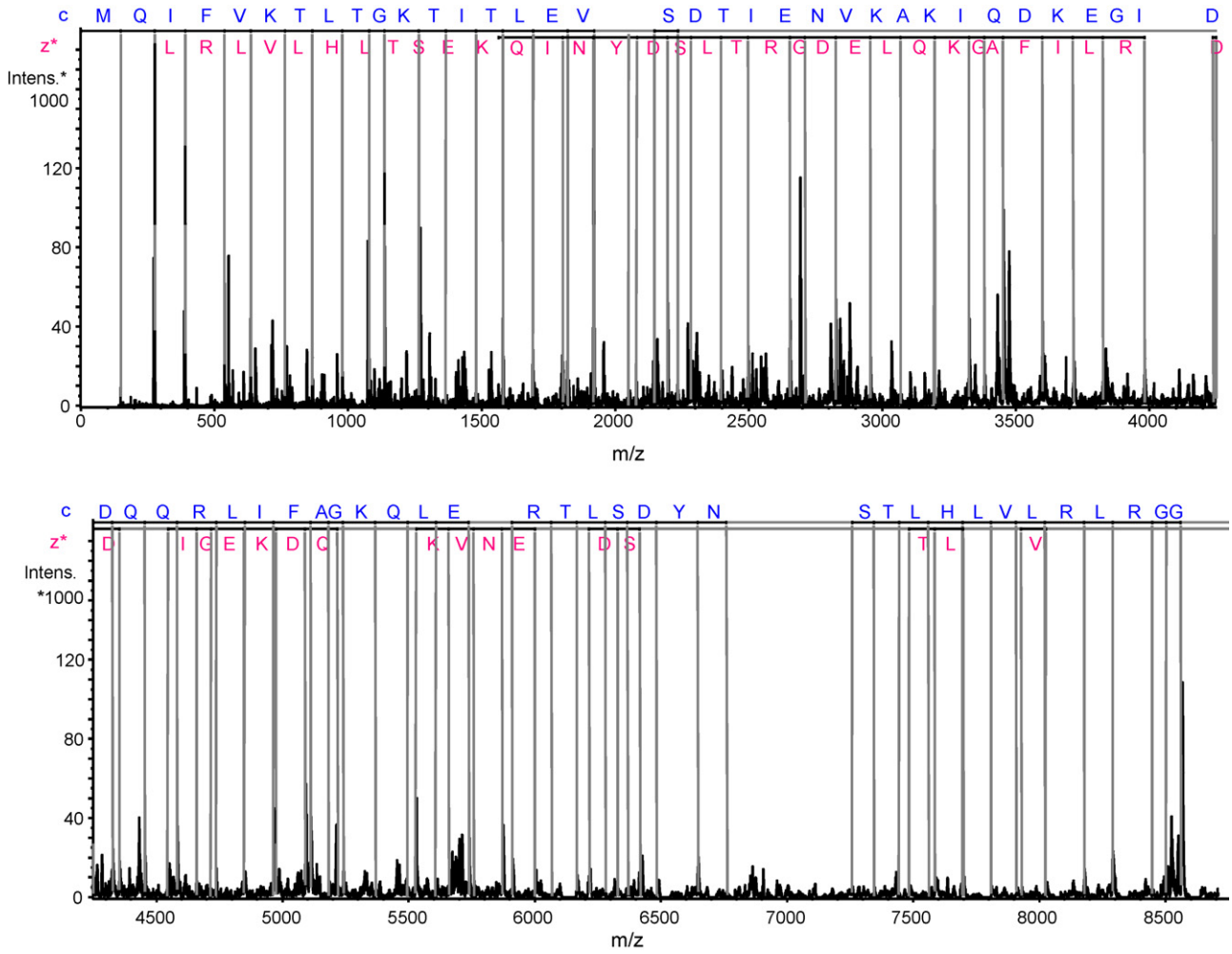


Fig. 7. Deconvoluted ETD/PTR product ion spectrum of ubiquitin 12+ (m/z 714). All identified c- and z^*-ions including the amino acid sequence are labeled.

3.4. High accurate mass measurements confirming the molecular formula of reagent anion

In order to confirm the close shell configuration of the proposed naphthyl anion 203, we analyzed the different fluoranthene anions with a modified Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. The ETD-capabilities of this instrument for the protein characterization have recently been presented [28]. The design of the chemical ionization source as well as the orthogonal introduction of the reagent anion into the octapoles of the main transmission device is the same as the nCI-source implementation in the 3D ion trap mass spectrometer used in the current investigation.

With the mass accuracy of less than 1 ppm and the mass resolving power of 1,000,000 the exact mass of the different reagent anions is easily determined (Fig. 5).

With the nCI-source at −5 V, the radical anion at m/z 202.0788 is generated. The decrease of the ionization chamber voltage results in the formation of the anion at m/z 203.0876 which does have a mass difference towards the radical anion of 1.0079 Th. This is consistent with the additional mass of a hydrogen atom, as predicted. When both types of anions – radical anion 202 and naphthyl even electron anion 203 – are produced, the hybrid instrument easily separates the signal at m/z 203.0822 for the ^{13}C isotope of the radical anion from the signal at m/z 203.0876 for the naphthyl anion. This result underlines the high performance of the FTICR mass spectrometer for applications where high resolving power and high mass accuracy are required.

3.5. Multiple ion/ion reaction: ETD followed by PTR

The following multiple ion/ion reactions have been performed using different reagent anions derived from fluoranthene. When multiply charged proteins are fragmented by ETD without a subsequent charge state reduction step, the MS/MS spectra result in a complex mixture of several hundred fragment ion signals having multiple charge states. Fig. 6a shows the ETD MS/MS from ubiquitin precursor $[M+12H]^{12+}$. The charge reduced molecular ions and a plethora of unresolved peaks are observed in the m/z-range of 500–1500. The detected fragment signals match to several overlaid multiply charged fragment ions induced by the electron transfer. For few lower charged fragment ions, the predicted molecular mass for selected ETD-fragments can be confirmed; however a much larger portion of the obtained signals are hardly assigned and the resulting protein sequence information is rather poor.

The consecutive proton transfer reaction of the highly charged ETD-fragments with the PTR-reagent anions significantly reduces the complexity of the ETD MS/MS data (Fig. 6b). For this sequential ion/ion reaction approach different sets of nCI-source voltages are applied. First, nCI-source voltages for the generation of the fluoranthene radical anion are utilized and the resulting ETD-reagent anion

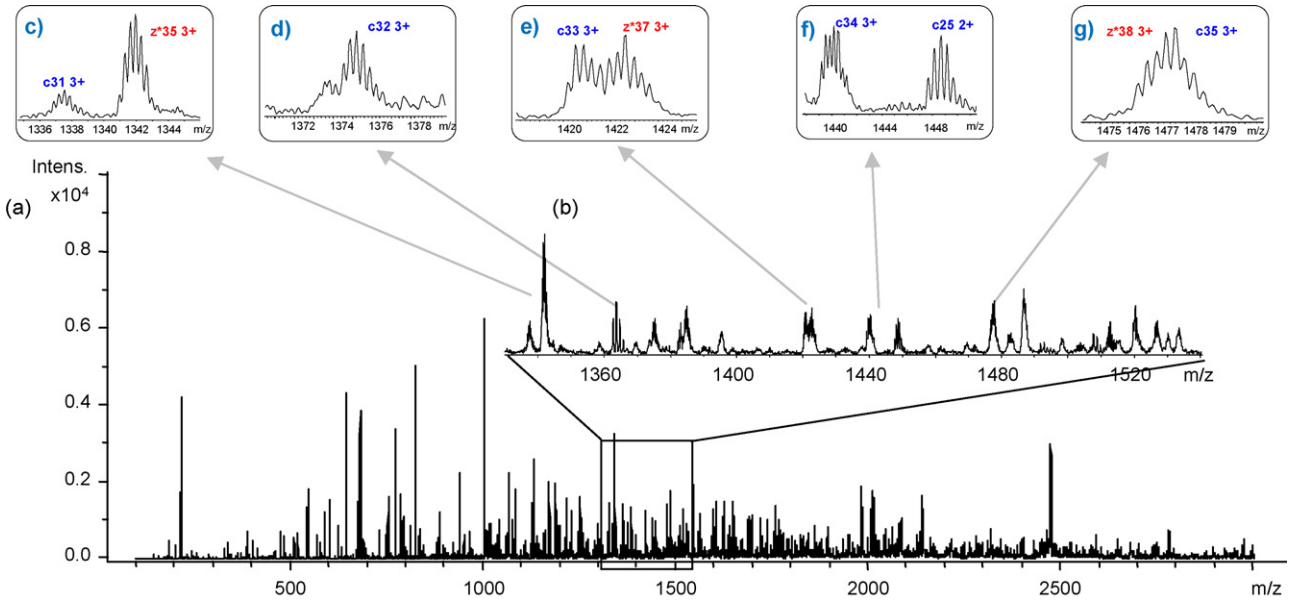


Fig. 8. ETD/PTR product ion spectrum of cytochrome *C* 16+ (*m*/*z* 774) (a). The subset (b) shows the mass range from *m*/*z* 1320–1560 including the triply charged c31–c35 ion. As demonstrated by the additional subsets, each of these fragment ions is isotopically resolved (c)–(g).

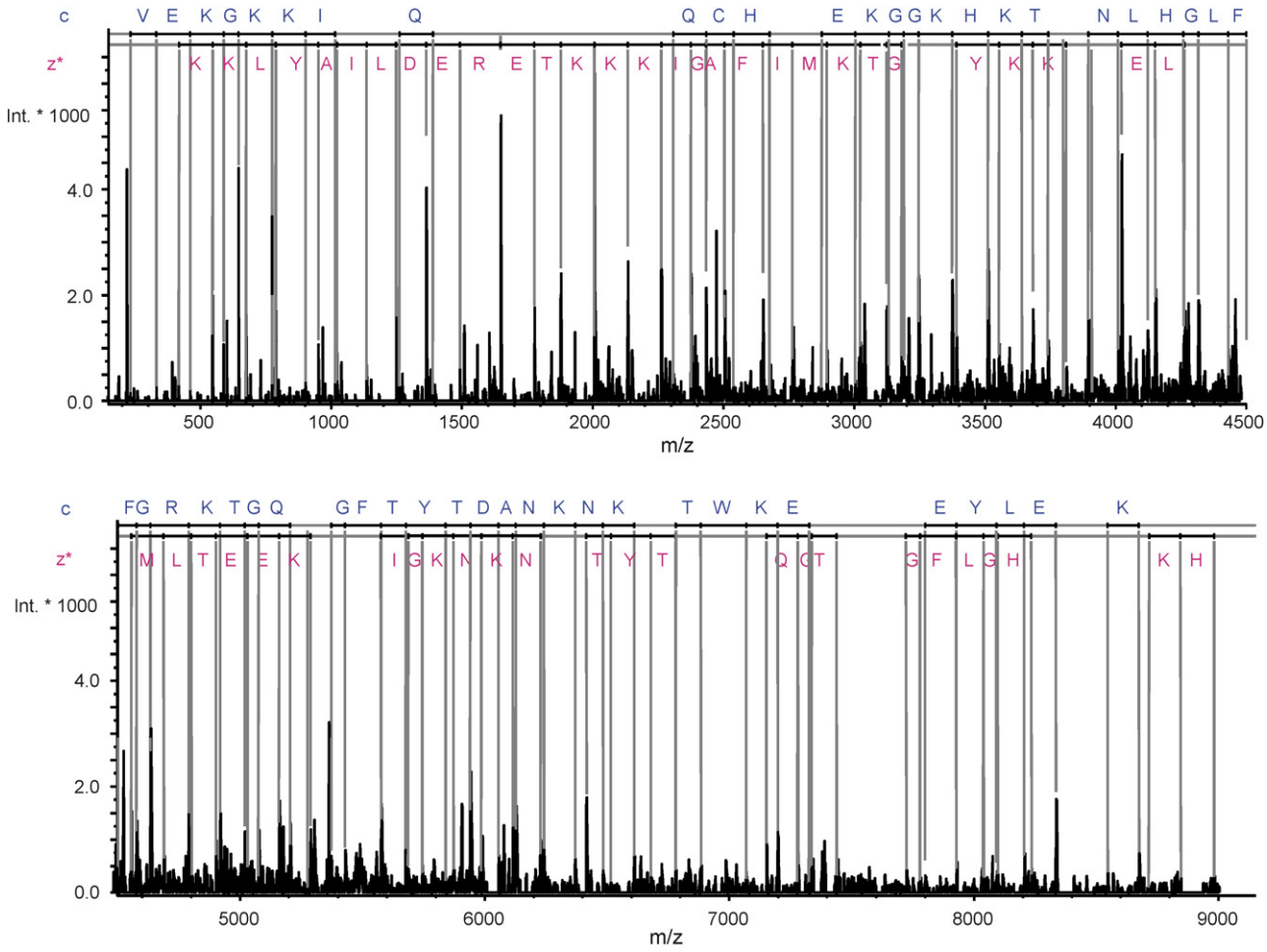


Fig. 9. Deconvoluted ETD/PTR product ion spectrum of cytochrome *C* 16+ (*m*/*z* 774). All identified c- and z*-ions including the amino acid sequence are labeled.

is introduced into the ion trap for the initial ETD-step. During the ETD reaction time, the nCI-source voltages are switched to the settings for which the PTR-anions are generated. After the ejection of the fluoranthene radical anions out of the ion trap, the PTR-anions are accumulated inducing proton transfer from the fragment ions towards the PTR-reagent anions.

For MS-analysis with radiofrequency ion trap, the peak width is independent of the mass position and the mass resolution increases with m/z. With each proton getting abstracted from the fragment ion, the m/z-value of the resulting ion gets higher. The performance of the mass analysis benefits from the charge state reduction as well as from the shifting of the fragment to higher m/z-value. For example, a previous quintuply charged fragment ion at 696 Th, that is not resolved in the ETD MS/MS data (subset (i) Fig. 6a), is shifted towards higher m/z after the PTR-step. The resulting doubly charged fragment at m/z 1737.5 is isotopically resolved and matches to the c-31 fragment (subset (iii) in Fig. 6b).

Taking advantage of the resolution in the enhanced full scan mode of the HCTultra, ETD fragments up to the charge state of four are assigned. Here, we control the PTR-step to obtain singly, doubly, triply as well as quadruply charged fragments at highest abundance by selecting appropriate number of PTR-reagent anions and quenching the proton stripping process after 150 ms. The charge state deconvoluted data of the multiple ion/ion reaction is given in Fig. 7. Sixty-nine c-ions out of 75 theoretical ETD fragments for the N-terminal site and 55 z*-ions out of 75 theoretical ETD-fragments for the C-terminal site are identified in the resulting virtual mass spectrum. With the enhanced scan mode of the 3D trap, triply charged fragments and to some extent even quadruple charged ions are isotopically resolved. After the charge state deconvolution, all multiply charged ions are shifted to singly charged higher masses in the resulting virtual mass spectrum. For example, the triply charge c-72 at m/z 2726 (subset (vi) Fig. 6b) is shifted to m/z 8176 in the virtual mass spectrum, that is close to the molecular weight of the intact protein. With the given resolution of the 3D ion trap and by the charge state deconvolution we receive the virtual mass range of 9000 Da, extending the m/z range of the instrument from 3000 Th by a factor of 3.

In order to elucidate the sequence of another protein with the present approach of multiple ion/ion reactions, we analyzed the 16-fold protonated precursor of cytochrome *C* with ETD-PTR MS/MS (Fig. 8a). For the subsequent proton transfer reaction, we had to increase the number of PTR-reagent anions in comparison to the ETD-PTR of ubiquitin because the charge states of ETD fragments of cytochrome *C* ions are on average higher than the fragment charge states of the ubiquitin ETD-MS/MS. For the cytochrome *C* ETD-PTR MS/MS data, we select the optimum conditions for the subsequent PTR-step by terminating the PTR-reaction, when quadruply, triply, doubly and singly charged fragment ions are obtained at the highest abundance.

In the insets c–g in Fig. 8, the triply charged fragment ions c-31 up to c-35 are displayed and all of them are isotopically resolved. All shown c-ions in the subsets c–g of the ETD-PTR MS/MS data in Fig. 8, are assigned to c-ions with post-translational modification of the heme-group still intact, which is known to be covalently bound to positioned Cys-14 and Cys-17. After charge state deconvolution, the mass range in the resulting virtual mass spectrum is extended by a factor of 3 enabling the sequence characterization of intact cytochrome *C*-protein, as depicted in Fig. 9. Nearly the entire sequence up to 9000 Da for both terminal sites are identified in the resulting deconvoluted data. ETD-fragments above 9000 Da are not observed, because these ions have to be detected as quadruply charged ions at higher m/z. The identification of these multiply charged fragments is limited by the resolving power of the instrument. In the deconvoluted data 59 c-ions out of 103 theoretical fragment and 63 z*-ions out of 103 theoretical fragments are assigned.

4. Conclusion

The present investigation on multiple ion/ion reactions extends the usability for new applications of 3D ion traps. Starting from only one volatile neutral compound in the nCI-source, two different types of reagent anions are produced selectively. Radical anions suitable for ETD are converted into even electron PTR-reagent anions just by changing the electrical potentials of the nCI-source.

The application of the sequential ion/ion-reactions of this method was demonstrated with the sequence characterization of intact ubiquitin where 69 out of 75 theoretical c-ion and 55 out of 75 z*-ion are observed in the ETD/PTR MS/MS data. This was then extended to cytochrome-*C*, resulting in the identification of nearly entire protein sequence up the 9000 Da.

The here presented selective anion production of ETD and PTR reagent from one compound in the nCI-source translates easily to routine applications of commercial MS-instruments. The combination of ETD followed by PTR opens new capabilities for proteomic studies where the top–down proteomic approach, i.e., the sequence determination of the intact protein, is of interest. Moreover, the elucidation of the sequence of intact proteins opens new methods for the identification of chromatographically separated proteins. These methods will need the development of new strategies for data dependant ETD/PTR-approaches.

As both types of ion/ion reactions can be applied independently, new applications of multiple ion/ion reactions can be devised. Under these new applications, the use of a preceding proton transfer reaction is of special interest, because this opens a way to investigate charge state dependent fragmentation of peptides as well as proteins, even if the precursor charge state of interest does not form under electrospray conditions.

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