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Dipolar DC Induced Collisional Activation of Non-Dissociated Electron-Transfer Products

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Abstract

The application of electron transfer and dipolar direct current induced collisional activation (ET-DDC) for enhanced sequence coverage of peptide/protein cations is described. A DDC potential is applied across one pair of opposing rods in the high-pressure collision cell of a hybrid quadrupole/time-of-flight tandem mass spectrometer (QqTOF) to induce collisional activation, in conjunction with electron transfer reactions. As a broadband technique, DDC can be employed for the simultaneous collisional activation of all the first-generation charge reduced precursor ions (e.g., electron transfer no-dissociation or ETnoD products) from electron transfer reactions over a relatively broad mass-to-charge range. A systematic study of ET-DDC induced collision activation on peptide/protein cations revealed an increase in the variety (and abundances) of sequence informative fragment ions, mainly *c*- and *z*-type fragment ions, relative to products derived directly via electron transfer dissociation (ETD). Compared to ETD which has low dissociation efficiency for low-charge-state precursor ions, ET-DDC also showed marked improvement, providing a sequence coverage of 80–85% for all the charge states of ubiquitin. Overall, this method provides a simple means for the broadband collisional activation of ETnoD ions in the same collision cell in which they are generated for improved structural characterization of polypeptide and protein cations subjected to ETD.

Keywords

Dipolar DC Collisional Activation; Electron Transfer Dissociation; ETnoD; Quadrupole ion trap; Multiply-protonated polypeptides

Introduction

Over the last few decades, tandem mass spectrometry (MS/MS) has become the technique of choice for generating primary structural information from gaseous peptide/protein ions.^{1,2,3} The most commonly used method for activating peptides/proteins for fragmentation is collision-induced dissociation (CID)⁴, which is available on almost all commercially

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Supporting Information

Additional information on reaction set-up and comparisons mass spectral data for ETD and ET-DDC reactions.

available mass spectrometers. CID involves energetic collisions of ions with an inert gas species, predominantly cleaving the thermally labile amide bonds ($C(=O)-N$) of the polypeptide backbone, generating *b*- and *y*-type fragment ions.⁵ Electron-based dissociation techniques, such as electron capture dissociation (ECD)⁶ and electron transfer dissociation (ETD)⁷, have also found increasing application as an alternative fragmentation technique for peptides/protein identification and characterization. In both ECD and ETD, the N-C bond between the amide nitrogen and its alpha carbon is cleaved as the major backbone fragmentation channel, giving rise to sequence informative *c*- and *z*-type fragment ions. Such electron-based dissociation methods have attractive feature of less sequence dependence on the cleavage sites, and preservation of thermally labile post-translation modifications (PTMs).^{8,9} Both ECD and ETD often provide structural information that is complementary to that provided by CID alone.¹⁰

The efficiency of ETD in generating fragment ions is compromised by competing proton transfer (PT) reactions as well as non-dissociated (intact) electron transfer products (ETnoD). The latter issue also applies to ECD.¹¹ The extent to which proton transfer competes with electron transfer is determined both by characteristics of the analyte cation¹² and the anion reagent¹³ used for the ET reaction. Over the years, several reagents have been used to minimize proton transfer, but no reagent as yet has been identified to completely avoid proton transfer.^{12,14} Electron transfer/capture can also lead to the formation of a long-lived, charged-reduced species that does not directly generate fragments (i.e., ETnoD/ECnoD products). The extent of formation of ETnoD products is dependent upon the charge state of the precursor ion,¹⁵ as well as the identities of the protonated sites¹¹. Particularly for polypeptides/protein ions in low charge states, ECnoD and ETnoD products are often prominent, resulting in low sequence coverage.^{16,17} Several supplemental activation techniques have been reported to disrupt or limit the formation of ETnoD products to enhance the total yield of fragment ions. For example, the use of elevated bath gas temperature to increase the internal energy of both precursor ions and ETnoD products has been reported to increase the total ETD product ion yields.¹⁴ Ledvina *et al.* have shown that the infrared photoactivation during an ETD reaction can minimize protein folding, which has been found to increase direct fragmentation from ETD reactions.¹⁸ This approach was termed activated ion ETD (AI-ETD) and has been extended to protein ions of up to 70 kDa in mass.¹⁹ Both low-energy resonance excitation CID (ETcaD)²⁰ and high-energy resonant-excitation CID (ET/CID-MS³)²¹ of charge-reduced precursor ions have also been shown to yield enhanced formation of *c*-/*z*-ions. The application of a tailored-waveform to electron transfer products in a linear quadrupole ion trap demonstrated a broadband collisional activation approach to increasing the yields of *c*-/*z*-ions in an ETD experiment.²² Likewise, application of beam-type collisional activation to ETnoD ions has been shown to be effective in enhancing *c*- and *z*-ion yields.²³ The injection of all the unreacted precursor and charge-reduced precursor ions into a pressurized ion trap, commonly referred as EThcD, has also been extensively utilized to obtain dual fragment ion series, i.e., both *b*-/*y*- and *c*-/*z*- type fragment ions in a single spectrum, for enhanced sequence coverage from ETD reactions.²⁴

As first demonstrated by Tolmachev *et al.*, the application of a dipolar direct current (DDC) field between opposing electrodes in a pressurized linear quadrupole ion trap allows for broadband collisional activation.²⁵ The DDC field displaces all ions from the center of the

ion trap into regions with higher RF field where more RF heating is induced, thereby resulting in an increase in ion internal energy via collisions with the bath gas. We have adapted this approach to a 3-D ion trap system^{26,27} as well as to linear quadrupole ion traps in hybrid QqTOF instruments.^{28,29} To first approximation, this approach leads to m/z -independent levels of ion heating²⁴ and therefore makes it particularly appealing as a means for activating the ETnoD ion populations across a relatively wide m/z range. In this work, we explore the use of DDC in the quadrupole collisional cell of a QqTOF instrument, in conjunction with ET reactions, as a means of collisional heating of charge-reduced precursor ions from ET reactions, referred to as ET-DDC. All the ETnoD products can be simultaneously activated across a broad mass range, leading to a significant increase in sequence informative fragment ions, especially c - and z -type fragment ions. We also show that ET-DDC provides enhanced comprehensive sequence coverage (80–85%) for all the precursors ion charge states investigated for ubiquitin.

Experimental Section

Materials.

All the reagents and solvents were purchased from commercial sources and were used without further purification. Ubiquitin (bovine), cytochrome *c* (bovine), myoglobin (equine heart), TPCK-treated trypsin, azobenzene, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol (MeOH), and Optima LC/MS-grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tryptic digestion was performed on cytochrome *c*, followed by reversed-phase high-performance liquid chromatography separation based on the procedures described in detail elsewhere.²¹ Solutions of peptides/proteins were prepared to be 10 μ M in 50/49/1 (v/v/v) methanol/water/formic acid for analysis via positive nano-electrospray ionization (nESI).

Mass Spectrometry.

All experiments were performed on a TripleTOF 5600 quadrupole/time-of-flight mass spectrometer (SCIEX, Concord, ON, Canada), modified to perform ion/ion reactions.³⁰ The instrument geometry comprises three quadrupoles (RF-only ion guide (q0), an RF/DC mass resolving quad (Q1), and an RF-only collision cell (q2), which is the “q” in the generic QqTOF designation) and a reflectron time-of-flight (TOF) analyzer. The q0 and q2 were modified to allow application of dipolar DC across a pair of opposing rods in both the q0 and q2 quadrupole.²⁶ A home-built pulsed dual source consisting of nano-electrospray ionization (nESI) and atmospheric pressure chemical ionization (APCI) source was coupled with the instrument to generate ions of both polarities.³¹ The nESI source was used for the formation of protein cations and the APCI source was used to generate radical anions (a schematic of the ion source setup is shown in Supporting Information, Figure S1). Azobenzene was used as the electron transfer reagent as it readily forms radical anions under atmospheric conditions and provides moderately high electron transfer efficiencies.¹² Electron transfer ion/ion reactions were implemented in the q2 quadrupole in mutual storage mode, as previously reported in detail elsewhere.^{12,28} The electron transfer ion/ion reactions typically includes precursor ion (cation) injection to q2 LIT (100 ms) with isolation, anion injection into q2 (100 ms) with isolation, and mutual cation/anion storage (20 ms). For ET-

DDC collisional activation experiments, electron transfer products were cooled (10 ms) in q2, subjected to collisional heating by applying DDC potential across a rod set of q2 in the presence of bath gas (6–8 mTorr N₂), cooled with the DC removed (30 ms), and ejected into the TOF for mass analysis. The values for the drive RF voltage (V_{RF}) and DDC potential are listed in the text below. The number of spectra for averaging is the same for both normal ETD and ET-DDC spectra shown.

Data Analysis.

A research version of software developed by AB SCIEX was used for instrument control and data acquisition. MS/MS spectra were processed using THRASH deconvolution algorithm written in MATLAB (MathWorks, Natick, MA, USA), with an S/N threshold setting of three.³² The peak list obtained from the deconvolution algorithm was loaded into ProSight Lite³³ to determine the number of matched fragments. The BY and CZ* fragmentation methods in ProSight Lite, which matches *b*-/*y*- and *c*-/*z*-type product ions, were used for the majority of the analysis for ETD and ET-DDC product ion spectra.

Results and Discussion

Electron Transfer-Dipolar Direct Current (ET-DDC) for Polypeptide/Protein Ions.

For initial proof of concept, doubly protonated MIFAGIKK obtained from tryptic digestion of cytochrome *c* was used as a test case for this study. Figure 1(a) shows the ETD product ion spectrum, where the $[M+2H]^{2+}$ precursor ions of the peptide (m/z 454.2) were isolated and reacted with radical anions (m/z 182) derived from azobenzene, with a mutual storage time of 20 ms. Seven sequence-informative product ions were observed (c_3 , c_7 , z_5 - z_7 , and y_5 - y_6), giving a sequence coverage of 57.1%. Note that the protein sequence coverage throughout this study is defined as the number of cleaved inter-residue positions in the mass spectrum divided by the total number of possible inter-residue cleavage positions. Along with fragment ions, a dominant charge-reduced precursor at m/z 907.4 was also observed. Figure 1(b) shows the ET-DDC product ion spectrum generated using 30 V DDC (i.e., +15V/−15V applied to the opposing rods) and 548 V_{RF} applied for 100 ms to all the ions shown in Figure 1(a). Ten sequence informative ions were observed (c_3 , c_7 , z_2 - z_7 , and y_5 - y_6), increasing the sequence coverage to 100%. This result is consistent with other approaches to activating ETnoD ions. The DDC approach, however, does not require a laser (in contrast to IRMPD), resonant excitation frequency tuning (in contrast to resonant excitation ion trap CID), injection into another collision cell (in contrast with beam-type CID approaches such as EThcD), or tailored wave-forms. In this respect, the ET-DDC approach is a relatively simple approach to activating ETnoD products.

The generation of ETnoD products from peptide ions of tryptic digestion is a general phenomenon in bottom-up proteomics. It is also extensive in top-down proteomics and is increasingly problematic as the precursor ion charge state decreases.¹⁵ The ET-DDC approach could, therefore, prove to be particularly useful in top-down applications of ETD. To examine this possibility, ubiquitin (~8.6 kDa) was tested as a model protein system. A relatively low charge state of ubiquitin, viz., the $[M+7H]^{7+}$ ion (m/z 1224.4), was subjected to ETD (Figure 2(a)). Only 33 structurally informative fragments (*c*-/*z*- and *b*-/*y*-ions) were

obtained directly from the ETD process, yielding a sequence coverage of 30.2%. In addition to ET fragments, the product ion spectrum includes multiple dominant charge-reduced intact ubiquitin ions, including both electron transfer and proton transfer products. A striking increase in both the diversity and abundances of fragment ions was observed upon supplemental activation by ET-DDC (30 V DDC, 1475 V_{RF}, 100 ms) as compared to the ETD-only experiment (Figure 2(b)). Deconvolution of the product ion spectrum resulted in the identification of 121 structurally informative fragments, yielding a sequence coverage of 84.2%. Note that the abundances of all the charge-reduced ubiquitin ions decreased after ET-DDC compared to the ETD spectra (Supporting Information, Figure S2). This, along with the appearance of more *c*- and *z*-ions, is consistent with DDC activated dissociation of ETnoD products. We note that the amplitude of the DDC was low enough to avoid extensive fragmentation of proton transfer products that would tend to generate *b*- and *y*-type ions. The zoomed-in regions of fragment ions in Figures 2(c) (ETD experiment) and 2(d) (ET-DDC experiment) demonstrate that the overall fragmentation efficiency and the number of sequence ions have significantly increased using ET-DDC compared to ETD. Various studies have shown that the long-lived electron capture or transfer dissociation products ions experience a hydrogen-atom migration from the even-electron *c*-type ions (*c*⁺) to the odd-electron *z*-type ions (*z*⁺) forming an even electron *z*-type ions (*z*⁺) and an odd-electron *c*-type ions (*c*⁺).^{20, 34} Similar phenomena of hydrogen-atom migration were also observed with some of the newly formed fragments ions from the ET-DDC process.

Experimental variables for ET-DDC collisional activation.

The major experimental parameters associated with DDC for a given ion are the DDC amplitude (V_{DDC}) and drive RF amplitude (V_{RF}), which determines the extent of RF-heating via displacement of the ions from the center of the ion trap, and the DDC activation (heating) time (Supporting Information, Equation S1-S3), which determines how long the ion population is subjected to activation.^{23, 27} Tuning these parameters allows fragmentation of the relatively unstable ETnoD products without extensive fragmentation of the first generation fragment ions generated by ETD. Figure 3 demonstrates the effect of V_{DDC} with constant V_{RF} (1475 V) and heating time (100 ms). Raising the DDC voltage from 0 to 30 V results in significant increases of *c*-, *z*-, and *y*-type fragment ions with an improvement in the signal-to-noise ratio by as much as a factor of 15 (Figure 3(c)). However, when the DDC voltage is increased to 35 V, the abundances of *b*-/*y*-type complementary fragments, such as b₁₈²⁺, begins to increase significantly relative to *c*- and *z*-type product ions, as shown in figure 3(d). The former ions likely arise from fragmentation of the relatively stable proton transfer products. Increasing the V_{DDC} to 40 V (Figure 3(e)) results in an increase in total fragment ion abundance but the overall sequence coverage is slightly reduced relative to the V_{DDC} = 35 V data. This observation likely results from the loss of some first-generation fragmentation products due to further fragmentation. A further increase in V_{DDC} results in loss of total ion signal as the ion displacement is becoming sufficiently high to compromise trapping efficiency. Figure 3(f) shows the overall plot for sequence coverage at variable DDC amplitude at 100 ms activation time. The highest sequence coverage (87%) was obtained at 35 V DDC due to maximal contributions from both *c*-/*z*- and *b*-/*y*-type product ions (Supporting Information, Figure S3). The DDC amplitude of 30 V was found to maximize the abundances of the *c*-/*z*-type informative product ions relative to the *b*-/*y*-type

ions, presumably due to preferential cleavage of the less stable ETnoD products versus the proton transfer products.

Figure 4 demonstrates the effect of variable DDC activation time using 30 V DDC amplitude. The spectra show an increase in yield and types of fragment ions with activation time up to 100 ms. Further increasing the activation time did not make a significant contribution to the total number of *c*-/*z*-type fragment ions. However, some subtle changes are noted in the relative abundances of the fragment ions as the DDC activation time is extended (e.g., 1500 ms) (Figure 4(d)). These data demonstrate that it is possible to establish DDC activation conditions that allow for extensive fragmentation of ETnoD products without significant contributions from dissociation of proton transfer products and with minimal further fragmentation of first-generation *c*-/*z*-type products.

Charge State of Precursor Ions and ET-DDC.

It is well-established that the efficiency of ECD or ETD fragmentation of multiply protonated proteins decreases with the precursor ion charge state.^{14–16} Figure 5 compares the performance of ET-DDC with that of ETD for different precursor charge states (+11, +9, and +7) of ubiquitin. The number of fragment ions for the +7 charge state of ubiquitin (33 fragments) is significantly lower than the +11 charge of ubiquitin (103 fragments) using ETD, demonstrating the charge-state dependency. Compared to ETD, ET-DDC improves the dissociation efficiency for all the precursor charge states, even for precursors with high charge states (e.g. 121 fragments for +7 charge state). In terms of sequence coverage, the ET-DDC maintains a high overall coverage (80–85%) compared to ETD (30.2–68.5%) across all the charge states investigated (Supporting Information, Figure S4). ET-DDC also results in a slight increase in the total number of *b*-/*y*-type fragment ions compared to normal ETD (Supporting Information, Figure S5) under the conditions used here, which likely arises from DDC of the proton transfer product(s).

For a given operating RF frequency and quadrupole inscribed radius, the upper *m/z* limit for storing ions under DDC conditions is inversely related to V_{DDC} and directly related to the square of V_{RF} (see equation S3). For the $V_{\text{RF}} = 1475$ V and $V_{\text{DDC}} = 30$ V used here, the upper *m/z* limit is approximately *m/z* 12590. The size of the protein that can be subjected to an informative ET-DDC experiment is not generally limited by the upper *m/z* limit associated with DDC, as it is usually straightforward to generate precursor and product ions of sufficiently high charge to fall within the upper *m/z* limit. It is not the ability of DDC to fragment ETnoD ions that is a limiting factor in determining the upper protein mass. Rather, it is the ability of mass analyzer to determine the masses of the product ions. The THRASH approach used here depends upon the ability of the analyzer to resolve isotopic peaks to determine the charge. For the analyzer used in this study, with an approximate resolution of 30,000 FWHM, it is challenging to identify large fractions of product ions for proteins larger than roughly 20,000 Da in mass, because product ions that overlap in *m/z* can complicate the determination of the charge states of one or all of the overlapping ions. A protein the size of apomyoglobin (16,950 Da) provides an example in which most of the product ions generated in an ET-DDC experiment can be identified via the THRASH approach used in this work. Figure 6 compares the ETD results (Figure 6(a)) with ET-DDC results (Figure 6(b)) for

following an electron transfer reaction involving the $[M+14H]^{14+}$ ion with expanded portions of the m/z range (i.e., m/z 890–1030). A total of 107 sequence informative fragment ions were observed using ET-DDC, compared to 52 observed with ETD process only, which increases the sequence coverage from 34.2% to 66.4%. (A breakdown of the number of b -/ y - and c -/ z - ions observed in the ETD and ET-DDC experiments for this precursor ion is given in Figure S6.)

Conclusions

Dipolar DC applied across opposing rods in a quadrupole collision cell is an effective means for broadband collisional activation with relatively uniform ion heating across a relatively wide m/z range. The DDC amplitude at fixed RF amplitude and DDC time can be tuned to maximize the collection of products from relatively weakly-bound ions while minimizing contributions from cleavages of relatively stable ions. These characteristics make DDC collisional activation particularly attractive for peptide/protein ion characterization following an electron transfer reaction. Under appropriate conditions, the relatively weakly-bound ETnoD products generated by electron transfer can be dissociated, thereby generating both additional and more abundant c -/ z -type ions, without significant sequential fragmentation of first-generation product ions. The overall process is denoted here as ET-DDC. As the DDC voltage is increased, it is also possible to fragment the proton transfer products that are also always present in an ETD experiment, thereby generating additional b -/ y -type ions. However, sequential fragmentation of first-generation product ions can become significant as the DDC voltage increases. Ion ejection at high DDC voltages ultimately limits the energy that can be imparted to trapped ions. Using ubiquitin cations for illustration, a significant increase in the yield of ET fragments was obtained in all cases using ET-DDC compared to the standard ETD approach, resulting in enhanced structural characterization. The enhancement is particularly striking when the precursor ion charge state decreases, as in consistent with other approaches with supplemental excitation of ET product ions. The ET-DDC approach requires no laser or injection of ions into another collision cell and is less tuning intensive than single frequency resonance excitation of ET-noD products. It can be implemented on any quadrupole ion trap provided a dipolar DC voltage can be applied across opposing electrodes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of tandem mass spectra for (a) ETD and (b) ET-DDC (30 V DDC, 548 V_{RF}, 100 ms) reactions of the doubly protonated (+2) peptide, MIFAGIKK (m/z 454.2), obtained from tryptic digestion of cytochrome *c* with azobenzene radical anions. The sequence inset shows the cleavage positions for the peptide, with the sequence coverage in parentheses. Peaks labeled with asterisks were present during the isolation.

Figure 2. Comparison of tandem mass spectra for (a) ETD and (b) ET-DDC (30 V DDC, 1475 V_{RF}, 100 ms) reactions for +7 precursor charge state of ubiquitin with azobenzene radical anions. (c) Zoomed-in region of fragment ions from panel (a). (d) Zoomed-in region of fragment ions from panel (b).

Figure 3.

Zoomed-in region of fragment ions from ET-DDC for +7 charge state of ubiquitin with 100-ms heating time, 1475 V_{RF} , and variable DDC amplitude (m/z range 1040–1060). (a) 0 V DDC, (b) 20 V DDC, (c) 30 V DDC, (d) 35 V DDC, and (e) 40 V DDC. The numbers in parentheses are the sum intensity of each fragment ion for 500 scans. (f) Plot for sequence coverage at variable DDC amplitude.

Figure 4. Zoomed-in region of fragment ions from ET-DDC for +7 charge state of ubiquitin with 30 V DDC, 1475 V_{RF}, and variable heating time (m/z range 1450 – 1520). (a) 10 ms, (b) 50 ms, (c) 100 ms, and (d) 1500 ms.

Figure 5. Total number of sequence informative fragment ions generated by ETD and ET-DDC for different precursor charge state of ubiquitin.

Figure 6.

Comparison of tandem mass spectra for (a) ETD and (b) ET-DDC (32 V DDC, 1462 V_{RF}, 100 ms) reactions for $[M+14H]^{14+}$ of apomyoglobin with azobenzene radical anions. (c) Zoomed-in region of fragment ions from panel (a). (d) Zoomed-in region of fragment ions from panel (b).