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Electron Transfer Dissociation of iTRAQ Labeled Peptide Ions

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Abstract

Triply and doubly charged iTRAQ (isobaric tagging for relative and absolute quantitation) labeled peptide cations from a tryptic peptide mixture of bovine carbonic anhydrase II were subjected to electron transfer ion/ion reactions to investigate the effect of charge bearing modifications associated with iTRAQ on the fragmentation pattern. It was noted that electron transfer dissociation (ETD) of triply charged or activated ETD (ETD + supplemental collisional activation of intact electron transfer species) of doubly charged iTRAQ tagged peptide ions yielded extensive sequence information, in analogy with ETD of unmodified peptide ions. That is, addition of the fixed charge iTRAQ tag showed relatively little deleterious effect on the ETD performance of the modified peptides. ETD of the triply charged iTRAQ labeled peptide ions followed by collision-induced dissociation (CID) of the product ion at m/z 162 yielded the reporter ion at m/z 116, which is the reporter ion used for quantitation via CID of the same precursor ions. The reporter ion formed via the two-step activation process is expected to provide quantitative information similar to that directly produced from CID. A 103 Da neutral loss species observed in the ETD spectra of all the triply and doubly charged iTRAQ labeled peptide ions is unique to the 116 Da iTRAQ reagent, which implies that this process also has potential for quantitation of peptides/proteins. Therefore, ETD with or without supplemental collisional activation, depending on the precursor ion charge state, has the potential to directly identify and quantify the peptides/proteins simultaneously using existing iTRAQ reagents.

Introduction

The relative quantitation of proteins expressed in cells or tissues of different states (e.g., healthy versus diseased) is of major interest in proteomics. Extensive effort has been devoted to the development of stable isotope-labeling methods both to identify proteins and to determine the concentration ratios of the proteins in different states by mass spectrometry (MS). There are a number of stable isotope technologies that exist today for protein quantification using MS.^{1–4} Among them is the Isotope Coded Affinity Tag (ICAT)¹ approach, which is widely used for relative quantitation of proteins via MS. However, the ICAT technique employs cysteine-specific chemistry, which can limit its application, particularly for species with low cysteine content. In addition, most of the post-translational modification (PTM)-containing peptides are often lost in the affinity step because they lack a cysteine residue. Furthermore, the ICAT approach is designed to examine only two samples at the same time.

Isobaric tagging for relative and absolute quantitation (iTRAQ)³, a proteomic approach used to differentiate protein levels quantitatively via tandem mass spectrometry (MS/MS), has been developed to address the issues mentioned above. This approach fulfills the requirements for broad proteome coverage while retaining PTM information, allows for multiplexing, enables absolute quantitation by adding an internal standard, and can provide improved quantitation

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statistics by expanding multiplexing to include duplicates or triplicates in the design. The iTRAQ reagent contains a reporter group, a balance group and an amino-active group, which modifies the N-termini and the lysine side chains of the peptides. It is well known that the charged reporter group can be generated upon collision-induced dissociation (CID)⁵ and is unique to each of the iTRAQ reagents. The reporter product ions ($m/z = 114-117$) are used to quantify their respective modified samples. In addition, CID of iTRAQ labeled peptide ions also gives rise to a series of distinguishable b- and y-type ions for peptide identification. The approach is especially important for identifying and quantifying native non-tryptic peptides such as those found in plasma, saliva⁶, or serum. This iTRAQ technique has been demonstrated for quantitation of protein levels through direct labeling of their tryptic peptides^{3, 7} or tagging of the intact proteins followed by trypsin digestion,⁸ respectively.

Electron capture dissociation (ECD) and electron transfer dissociation (ETD) are dissociation methods that are similar to one another and complementary to CID. The former has been mostly restricted to Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometers^{9, 10} whereas the latter has been implemented on electrodynamic ion traps.^{11, 12} In both ECD and ETD, N-C α bond cleavages along the peptide backbone yield mostly even-electron c- and odd-electron z-type fragment ions, providing information for peptide/protein sequencing. Compared to CID, ECD and ETD are less dependent on the identities of the amino acid side-chains in determining the extent of sequence information and they preserve the PTMs, which allows characterization of peptide/protein modifications.^{13, 14} It has been reported that ETD of doubly charged peptide ions with 10–16 amino acid residues usually gives rise to relatively low ETD efficiency and therefore limited sequence information.^{15, 16} A number of approaches have been established to increase the ETD efficiency and to improve the sequence coverage by converting the intact charge reduced electron transfer survivor ion to sequence informative c- and z-type species through supplemental activation of the survivor ion^{15,17,18,19}.

It has been reported that the charge bearing sites play an important role in both ETD and ECD performance. For example, a relatively high tendency for side-chain losses from arginine has been noted in an ETD study as well as tendency for formation of intact electron transfer products from peptides in which histidine is expected to be a charge bearing site.²⁰ ECD experiments involving the fixed charge derivatives and radical trap derivatives have shown decreased backbone cleavages and increased side-chain cleavages, which significantly reduced the sequence coverage relative to ECD of their untagged counterparts.^{21–24}

Compared to other fixed charge derivatives, possible advantages of the iTRAQ modification technique under ECD or ETD conditions are two-fold: (1) the isobaric tag is not a particularly efficient radical trap, which otherwise might sequester the H radical upon electron capture or transfer resulting in inhibition of backbone dissociation; (2) the tag is attached to the peptide through a normal peptide bond, which is expected to be easily cleaved by ECD or ETD. Therefore, it is of interest to determine the effect of the modifications of the N-terminus and lysine side chains of the peptide, which is fundamental to the iTRAQ technique, on ETD behavior. In this study, the ETD behavior of iTRAQ labeled peptide ions is examined to determine if charge bearing modifications used in the iTRAQ technique affect the extent to which both sequence and quantitative information can be obtained by application of ETD to peptides labeled with existing iTRAQ reagents.

Experimental

Materials

Methanol and glacial acetic acid were purchased from Mallinckrodt (Phillipsburg, NJ). Azobenzene was obtained from Sigma-Aldrich (St. Louis, MO). The 4-plex iTRAQ labeled tryptic peptide mixture of bovine carbonic anhydrase II was provided by Applied Biosystems

(Foster City, CA). All materials were used without further purification. The iTRAQ labeled peptide mixture was diluted to 0.5 μ M in 49.5/49.5/1 (v/v/v) methanol/water/acetic acid for positive nano-electrospray ionization (nano-ESI).

Mass Spectrometry

All experiments were performed using a prototype version of a Q TRAP 2000 mass spectrometer²⁵ (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) modified for ion/ion reactions.²⁶ The Q TRAP electronics were modified to superpose auxiliary RF signals on the containment lenses of the Q3 quadrupole array, which allows mutual storage of ions of opposite polarity in the low pressure Q3 linear ion trap (LIT). The Q3 LIT is pressurized with nitrogen bath gas at around 3.5×10^{-5} Torr. The frequency and amplitude of the auxiliary RF signals applied to the containment lenses were optimized for the electron transfer ion/ion reaction experiments. A home-built pulsed dual source, nano-ESI for generating multiply charged peptide cations and atmosphere pressure chemical ionization (APCI) for producing azobenzene radical anions, was coupled directly to the interface of the Q TRAP mass spectrometer.²⁷ All experiments were controlled by Daetalyt 3.14, a research version of software provided by MDS SCIEX.

In a typical experiment performed in this study, the iTRAQ labeled mixture was sprayed and the ion of interest was isolated under RF/DC mode in the Q3 LIT, followed by performing either ion trap CID or ETD on the isolated iTRAQ labeled peptide ion. The experimental procedure for the electron transfer ion/ion reactions performed herein has been reported in detail elsewhere.²⁷ In brief, a typical experiment (electron transfer ion/ion reaction, followed by collisional activation) employed in this work comprised the following steps: (1) application of a high voltage (+1.0–1.5 kV) on the nano-ESI emitter and injection of positive ions into the Q3 LIT with relatively low kinetic energies (Q3 DC offset was 7 V attractive relative to Q0 DC offset); (2) switching off of the high voltage on the nano-ESI emitter while the analyte ions were cooled in Q3; (3) isolation of the ions of interest under RF/DC mode in Q3; (4) cooling of the isolated ions; (5) pulsing of the high voltage (–2.5 kV) applied to the APCI needle and injection of azobenzene radical anions which were mass selected by Q1 in the mass-resolving mode into the Q3 LIT; (6) mutual storage of oppositely charged ions in the Q3 LIT; (7) cooling of the product ions for 50 ms in Q3; (8) application of an auxiliary AC to collisionally activate the product ions of interest in Q3; (9) cooling of the ions for 50 ms in Q3 and (10) mass analysis of ions in Q3 via mass selective axial ejection (MSAE)²⁸ using a supplementary RF signal with a frequency of 380 kHz. For a typical electron transfer ion/ion reaction without supplemental activation, steps 7 and 8 employed for collisional activation were not required. Q3 isolation was used instead of Q1 isolation in order to remove any CID fragments formed during the passage of the ions through the high collision cell (Q2) of the triple quadrupole/linear ion trap mass spectrometer. The spectra shown here were typically the averages of 30–200 individual scans.

Results and Discussion

Triply charged 4-plex iTRAQ labeled peptides

Ion trap CID and ETD—Ion trap CID and ETD spectra of the triply charged peptide cation with a sequence of *EPISVSSQQL*K (*denotes the iTRAQ labeled sites) from the 4-plex iTRAQ labeled mixture are shown in Figure 1. In the CID spectrum (Figure 1a), only one reporter ion at m/z 116 is observed, which indicates the peptide is tagged only by the 116 iTRAQ reagent in the 4-plex labeled sample. The species at m/z 145 is the isobaric tag shown in Scheme 1, which is identical for all the 4-plex iTRAQ reagents. A highly abundant peak at m/z 162 generated by ETD of the triply charged peptide ion (Figure 1b) is believed to be caused by the N-C cleavages (solid lines) next to the modification sites, as shown in Scheme 1. The

neutral loss of 161 Da is presumably due to cleavages of the same bonds with charge retention by the larger fragment. The presumed structure of the m/z 162 species is shown in Figure 1b. Higher sequence coverage (91%) due to 10 out of 11 possible peptide backbone bond cleavages is achieved by ETD compared to the observation of 4 out of 11 possible backbone amide bond cleavages obtained by CID (36% sequence coverage), where sequence coverage is defined as a percentage of possible backbone bond cleavages. However, the abundant m/z 162 peak observed in the ETD spectrum does not provide any quantitative information directly for the peptide because this species contains both the reporter group and the balance group, which is indistinguishable for each of the 4-plex iTRAQ reagents.

ETD + supplemental CID of the m/z 162 product—Collisional activation of the m/z 162 ion was performed to determine if any reporter ions would be generated. Figure 2 shows ETD mass spectra of the triply charged peptide *EPISVSSQQL*K ion without (Figure 2a) and with (Figure 2b) collisional activation of the m/z 162 ion following the electron transfer ion/ion reaction. Note that the data in Figure 1b were collected under conditions in which more of the precursor ions were allowed to undergo electron transfer ion/ion reactions than was the case for the experiments leading to the data in Figure 2. Therefore, the spectra in Figure 2, acquired under similar conditions, are shown for direct comparison. In Figure 2b, the reporter ion at m/z 116 is observed in the ETD spectrum after the m/z 162 ion was activated, which is consistent with the reporter ion directly produced by CID of the same triply charged precursor ion. Therefore, the reporter ion at m/z 116 formed via the sequential activation process is expected to provide quantitative information for the peptide/protein similar to that obtained directly from CID.

ETD of the triply charged 4-plex iTRAQ labeled peptide ion with sequence *EPISVSSQQ(M-ox)L*K also produced a highly abundant peak at m/z 162 and provided 91% sequence coverage for the peptide (data not shown). In the ETD spectra of both triply charged *EPISVSSQQL*K and *EPISVSSQQ(M-ox)L*K species, the neutral losses of 103 Da and 161 Da from the intact charge-reduced electron transfer product ions were observed. The mechanism for formation of the 103 loss is discussed below.

Doubly charged 4-plex iTRAQ labeled peptides

CID, ETD and activated ETD—In the CID spectrum of the doubly charged iTRAQ labeled peptide ion with the sequence *DGPLTGTYR from the 4-plex labeled sample (Figure 3a), the only reporter ion noted is at m/z 116. This result is consistent with the CID of the triply charged peptide ions, indicating that the peptide is labeled only by the 116 iTRAQ reagent. However, the peak at m/z 162 is not seen in the ETD spectrum of this doubly charged peptide ion (Figure 3b). The sequence coverage generated from ETD (25%) is much lower than that derived from CID of the same ion (87.5%). However, the ETD efficiency and the sequence information acquired from ETD of doubly charged peptide ions can be improved by activating the singly charged electron transfer survivor ion following the ion/ion reaction. The resulting ETD mass spectrum of doubly charged *DGPLTGTYR ion after activating the singly charged survivor product species is shown in Figure 3c. The ETD efficiency is improved from 32% to 49% upon collisional activation on the intact singly charged electron transfer product ion that survived the ion/ion reaction, where the ETD efficiency is defined as:

$$\% \text{ETD} \equiv \frac{\sum c,z, \text{neutral losses}}{\sum \text{post Ion/Ion products (residual } 2^+ \text{ excluded)}} \times 100 \quad (1)$$

for doubly charged ions. The sequence coverage for this peptide increases from 25% to 87.5% without decreasing the intensity of the 103 neutral-loss peak when the intact charge-reduced

electron transfer species was activated. Therefore, the same percentage of sequence coverage for *DGPLTGTYR is acquired by either CID or activated ETD through applying auxiliary AC to the intact electron transfer product ion. Due to the complementary nature of CID and ETD, full sequence coverage of the peptide *DGPLTGTYR is achieved by combining the information obtained from both CID and ETD. However, the m/z 162 ion does not appear after collisional activation of the electron transfer survivor ion, probably due to the low-mass-cut-off = 200 in the supplemental collisional activation step.

The 103 Da neutral loss

The neutral loss of 103 Da is observed in all the ETD spectra of triply charged and doubly charged iTRAQ labeled peptide ions acquired in this study. A possible mechanism associated with formation of the 103 neutral-loss species is shown in Scheme 2. The 103 Da loss is directly related to the reporter ion at m/z 116 and is unique to the 116 iTRAQ reagent, which implies that the 103 loss channel can probably provide similar quantitative information as the signature ion 116 for the peptide/protein.

Conclusions

Electron transfer ion/ion reactions of triply charged and doubly charged 116 iTRAQ tagged peptide ions with azobenzene radical anions in a LIT were investigated. ETD of the triply charged peptide ions generates a series of c- and z-type fragments, which provides higher sequence coverage than did CID of the same ions. In addition, an abundant peak at m/z = 162 is produced in the reaction involving the triply protonated species. However, this m/z 162 fragment does not provide quantitative information directly because it contains both the reporter and balance groups of the iTRAQ modification. Supplemental collisional activation of the m/z 162 species following the ion/ion reaction yields the reporter ion with m/z at 116, which is expected to provide similar quantitative information to that directly derived from CID of the triply charged precursor ion. In ETD of all the triply and doubly charged iTRAQ labeled peptide ions studied here, a 103 Da neutral loss is observed, whereas the m/z 162 fragment was generated only from triply charged precursor ions. The formation of the neutral-loss species is directly related to the reporter group and is unique for the 116 iTRAQ reagent. Therefore, the species formed from the neutral loss of 103 Da might serve as a more suitable reporter group when ETD is employed because no supplemental activation is required and it is observed from both triply and doubly charged precursor ions. Additionally, ETD performance of doubly charged iTRAQ labeled peptide ions is enhanced by activating the charge reduced intact electron transfer product ions following the ion/ion reaction, thereby increasing sequence coverage. Therefore, it is concluded from this study that ETD is compatible with existing iTRAQ reagents in that both sequence and relative quantitation information can be derived. Two means for accessing reporter ions are apparent. One involves collisional activation of the ETD product at m/z 162 formed from triply charged ions whereas the other arises from a neutral loss of the iTRAQ reporter group, which is noted for both doubly and triply charged ions.

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Figure 1. Tandem mass spectra of the triply charged 4-plex iTRAQ labeled peptide **EPISVSSQQML*K* ion: a) ion trap CID in Q3 (55.22 kHz, 60 mVpp); b) electron transfer ion/ion reaction with azobenzene radical anions in Q3 LIT.

Figure 2.

ETD mass spectra of triply charged 4-plex iTRAQ labeled peptide *EPISVSSQML*K species: a) without activating the species at m/z 162; b) with activating the m/z 162 ion following the ion/ion reaction (152.72 kHz, 260 mVpp).

Figure 3. MS/MS mass spectra of doubly charged 4-plex iTRAQ labeled peptide ion with sequence **DGPLTGTyr*: a) ion trap CID; b) ETD; c) activated ETD by applying collisional activation on the singly charged intact electron transfer product ion following the ion/ion reaction (53.33 kHz, 80 mVpp).

Scheme 1.

Cleavage sites of the triply charged 4-plex iTRAQ labeled peptide ion with sequence *EPISVSSQQML*K under electron transfer ion/ion reaction.

Mass = 103 for the 116iTRAQ reagent

Scheme 2.

Formation of the 103 Da neutral-loss ion from the intact charge-reduced species