Peptide Activation

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Infrared Photoactivation Reduces Peptide Folding and Hydrogen-Atom Migration following ETD Tandem Mass Spectrometry**

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Electron capture dissociation (ECD)[1]—an experiment generally performed within the high magnetic field of a Fourier transform ion cyclotron resonance mass spectrometer—results from the mutual storage of thermal electrons with multiply protonated peptide cations. The technique is particularly useful, as it generates random backbone cleavage with little regard to the presence of posttranslational modifications (PTMs), amino acid composition, or peptide length. Electron transfer dissociation (ETD),[2] the ion–ion analogue of ECD, is conducted in radio-frequency (RF) quadrupole ion trap devices, in which radical anions serve as electron donors. Since it can be implemented on virtually any mass spectrometer with an RF ion transfer or storage device, ETD has become an increasingly widespread dissociation method.

The capture of an electron can trigger a free-radical-driven rearrangement that results in N–C_α backbone cleavage and the production of *c*- and *z*•-type fragment ions. Sometimes, however, the precursor cation captures the electron and forms a long-lived, charge-reduced species that does not separate (an ECnoD or ETnoD product).[3] This phenomenon becomes more probable as the mass-to-charge (*m*/*z*) ratio of the precursor increases. As the charge density decreases, the magnitude of intramolecular noncovalent interactions increases, so that the newly formed *c*- and *z*•-type fragment ions often remain bound following electron capture and cleavage—an obstacle of higher consequence for ETD, which is conducted under conditions of elevated pressure.[4] McLafferty and co-workers reported that photon bombardment of the precursor cation prior to ECD (activated-ion ECD, AI ECD) decreased nondissociative electron capture,[5] presumably by destroying the secondary structure of the peptide cation prior to electron capture.

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ETD = electron transfer dissociation.

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ETD is conducted at pressures that are approximately 10^6 times higher than those used in ECD (which is carried out at approximately 0.13 Pa). Therefore, precursor cations undergoing ETD are considerably cooler, and preactivation either with photons or through collisions is expected to produce only short-lived (< 1 ms) unfolding. Recently, we examined the use of collisions to coerce the ETnoD products into dissociating through a technique coined ETcaD (ETD in conjunction with collisional activation).[3] The method increased the number and intensity of N–C_α backbone cleavages; however, the majority of the newly formed fragment ions displayed evidence of hydrogen-atom rearrangement to produce even-electron *z*-type fragments and odd-electron *c*•-type products. ECD practitioners propose that such rearrangements occur because the *c*- and *z*•-type fragment ions are held in close proximity, so that an H atom can be abstracted from the *c*-type and directed to the *z*•-type product (this hydrogen-atom transfer occurs prior to the separation of the two fragment ions).[6,7] For large-scale sequencing applications, these rearrangements are problematic, as the mass window needed to define a possible fragment becomes too large.

We considered subjecting the precursor cations to collisional activation through resonant excitation during the entire ion–ion reaction period to inhibit collisional cooling and refolding prior to electron transfer. A side effect of resonant excitation, however, is that the precursor cations undergo an increase in velocity. Ion–ion reaction rates are governed by the velocity of the participants, and such increases in velocity inhibit ion–ion reactions.[8] McLuckey and co-workers reported the use of elevated bath-gas temperatures to increase the degree of ETD fragmentation, but did not discuss the impact of the method on hydrogen-atom abstraction.[9] We reasoned that infrared photons (10.6 μm) could be used, in conjunction with ion–ion reactions, to continuously increase precursor-ion internal energy, destroy noncovalent interactions, and limit hydrogen-atom migration. Recent results of Kaplan[10] show that the photoactivation of peptide cations undergoing ETD can increase direct fragmentation; however, hydrogen-atom migration was not evaluated. To test our hypothesis, we modified a dual cell linear ion trap system in such a way that an IR beam irradiated both linear ion traps along their axial length. Peptide cations and reagent (azobenzene) anions were generated at atmospheric pressure (AP) and introduced through a single inlet.

Figure 1 A,B shows single-scan tandem mass spectra acquired following a 100 ms reaction of doubly protonated substance P cations (RPKPQQFFGLM, *m*/*z* 674) with radi-

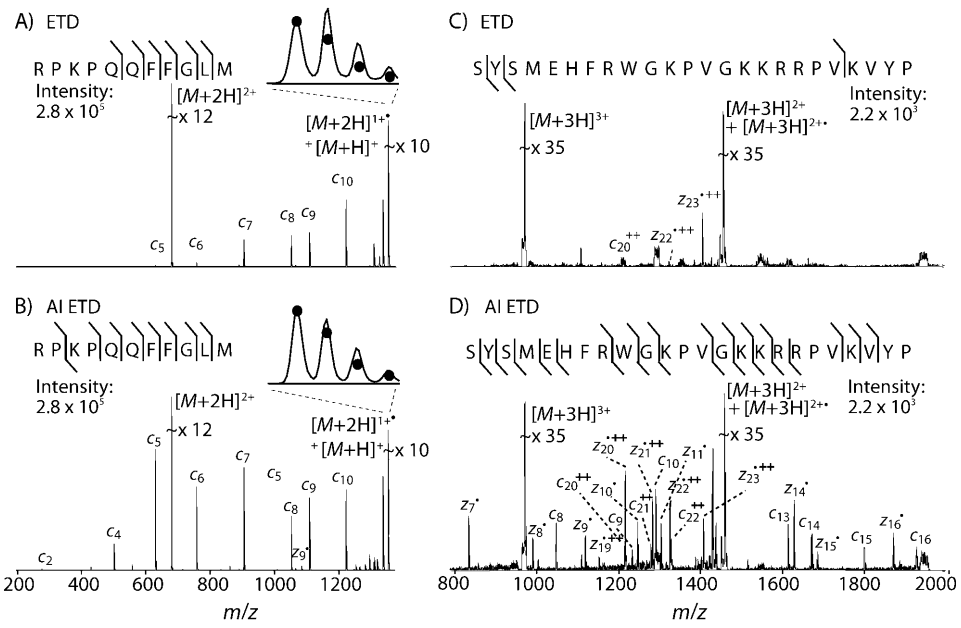

Figure 1. ETD and AI ETD spectra of A,B) RPKPQQFFGLM and C,D) ACTH for a 100 ms reaction with azobenzene radical anions. For AI ETD, the laser power was set at 99%.

cal anions of azobenzene either without (ETD, Figure 1 A) or with concomitant photon bombardment (AI ETD, Figure 1 B). ETD resulted in the production of six *c*-type product ions. Irradiation of the reactants during the ETD process resulted in the formation of nine *c*- and $z^{\bullet}$-type fragments, virtually all of which showed significant increases in intensity. The inset in Figure 1 A displays the isotopic envelope of the charge-reduced precursor, which comprises products of both ETnoD and proton abstraction. Proton abstraction is a side reaction that competes with electron transfer. The product composition is largely dependent upon the reagent anion:[11] azobenzene engages in more proton abstraction than our preferred reagent fluoranthene, but is conveniently produced under AP conditions. The inset also shows the theoretical isotopic envelopes of the proton-transfer charge-reduction product $[M+H]^+$ as filled circles. The heightened distribution of the ^{13}C isotopic cluster provides an estimate of the ETnoD population. From the spectrum in Figure 1 B, we concluded that AI ETD significantly lessens the degree of ETnoD, presumably by disrupting gas-phase secondary structure and increasing the probability of direct dissociation.

Figure 1 C,D shows the ETD and AI ETD mass spectra resulting from ETD activation of the triply protonated precursor of the peptide adrenocorticotropic hormone (ACTH; m/z 978). Similar results were observed, except in this case ETD produced only three fragment ions, whereas AI ETD resulted in 23 *c*- and $z^{\bullet}$-type products (for an expanded dataset and the effect of laser power, see Figure 1 in the Supporting Information). As the power was increased from 50 to 99% (maximum: 50 W), the intensity of the signal due to the $z_7^{\bullet}$-type product ion also increased. A simultaneous decrease in the amount of the hydrogen-atom-transfer product (i.e., *z*-type product) was observed, which is consistent with previous AI ECD studies.[7] As a result of the relatively high pressure of the system (ca. 0.63 Pa), IR activation did not induce detectable *b*-, *w*-, or *y*-type-fragment formation, even at the highest power settings.

We next explored the degree of hydrogen-atom abstraction, as this process was a significant limitation of our previous ETcaD approach. Figure 2 displays the product-ion distributions of the $z_6^{\bullet}$ and c_8 product ions formed following the dissociation of doubly protonated peptide cations with the sequence FSWGAEGQR by several methods: ETD with A) no other activation, B) concurrent photoactivation (AI ETD), C) postactivation by photons (ETirD), and D) postactivation through collisions (ETcaD). The intensity scale is identical for all parts of Figure 2; filled circles denote the theoretical isotopic distribution of the $z^{\bullet}$-type ion. Two principal conclusions can be drawn from these data: 1) AI ETD results in a marked improvement in intensity and a diminished proclivity for hydrogen-atom

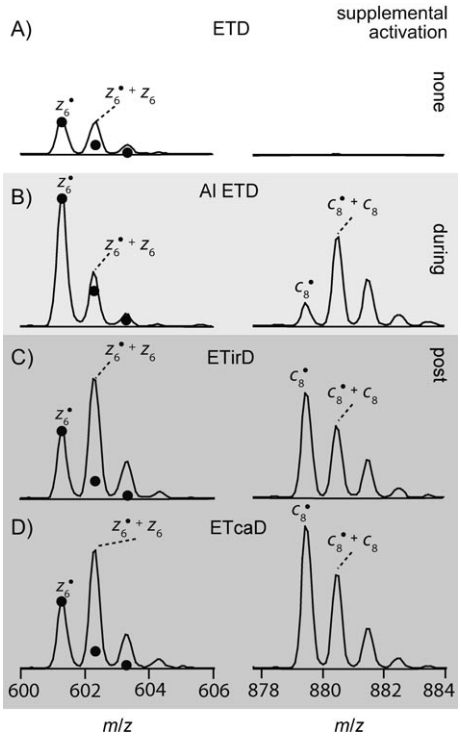

Figure 2. Isotopic distributions of the $z_6^{\bullet}$ and c_8 product ions generated from FSWGAEGQR by A) ETD, B) AI ETD, C) ETirD, and D) ETcaD in a 100 ms reaction with azobenzene radical anions. For AI ETD and ETirD, the laser power was set at 99%. Filled circles denote the theoretical distribution of typical ETD product ions.

abstraction as compared to ETD, and 2) remarkably, both postactivation methods (through photons and collisions) produce identical results. However, the increased product formation observed with the postactivation methods is accompanied by a large amount of hydrogen-atom abstraction. These results are consistent across all peptides examined.

To further confirm our guiding supposition—that concurrent photoactivation during the ion–ion reactions of precursor peptide/protein cations can induce gas-phase unfolding that is maintained in the high-pressure environment of the ion trap—we examined ubiquitin cations with charges ranging from +7 to +10. Studies by Clemmer and co-workers of ubiquitin conformations under similar pressures[12] revealed the +7 charge state to be in a compact conformation (cross-section ca. 1000 Å^2) during the time scale of our experiments (10 ms). As the charge state increases, so does the cross-section; thus, the +10 precursor was described as elongated (ca. 1500 Å^2). Direct ETD of the +7 precursor produced no detectable fragmentation, low-level dissociation was detected for the +8 precursor, and the +9 and +10 precursors underwent significant fragmentation (data not shown). Figure 3 in the Supporting Information shows selected fragments derived from the ETD of precursors with charge states between +7 and +10 and those produced by AI ETD of the +7 precursor. Numerous fragments were observed in the AI ETD spectrum that were found only in the ETD spectra of the +9 and +10 precursors. These data provide further evidence that concomitant photoactivation disrupts the secondary structure of the precursor cation during ETD reactions to yield increased fragmentation and diminished hydrogen-atom rearrangement.

Herein we have described AI ETD and demonstrated that it can substantially improve the utility of ETD for peptide-sequence analysis. The method shows particular promise to enable the use of ETD for low-charge-density peptide precursors (e.g., precursor $m/z > 800$) where gas-phase secondary structure prevents direct formation of *c*- and $z^{\bullet}$-type fragment ions, thereby permitting extensive hydrogen-atom migration. By limiting intramolecular interactions, AI ETD generates isotopic cluster peaks of lower complexity that more closely resemble theoretically predicted *c*- and $z^{\bullet}$-type product distributions than those derived from either ETD or postactivation strategies (ETcaD or ETirD). Another advantage of AI ETD over postactivation is that no additional time (above that needed for the ion–ion reaction) is required.

Experimental Section

A dual cell linear ion trap mass spectrometer was fitted with two atmospheric-pressure (AP) ionization sources: ESI for the generation of cations and AP chemical ionization (APCI) for the generation of reagent anions. Radiation at a wavelength of 10.6 μm was generated by the use of a 48-5 SYNRAD laser and was introduced on axis with the dual cell trap through a ZnSe window. Instrument firmware and hardware were modified so that the laser could be triggered externally to operate concurrently with the ETD reactions, which were conducted in the higher-pressure cell (ca. 0.63 Pa). For a more detailed description of the experiments, see the Supporting Information.

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