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Activated-Ion Electron Transfer Dissociation Enables Electron-Based Dissociation Following Proton Transfer Charge Reduction

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ABSTRACT

Purpose: Demonstrate the applicability of AI-ETD to low charge density ions generated by proton transfer charge reduction (PTCR) purification.

Methods: Several intact protein standards were analyzed on a Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer modified to perform AI-ETD. Proteins were first charge-reduced with PTCR and then fragmented by either ETD or AI-ETD. Sequence coverage was evaluated to determine the effectiveness of each strategy.

Results: Activated ion ETD represents a useful fragmentation technique following proton transfer charge reduction (PTCR) purification. While standalone ETD generates little to no sequence informative data following PTCR, AI-ETD is able to generate substantial fragmentation following PTCR, allowing the use of electron-based fragmentation in methods incorporating PTCR purification.

INTRODUCTION

PTCR has recently emerged as a powerful technique for analyzing complex mixtures of intact proteins.[1,2,3] Under normal circumstances, protein charge state envelopes will tend to be superimposed on one another, complicating mass assignment.[1,2] However, PTCR can sufficiently separate species that would ordinarily remain unresolved, enabling better intact mass determination and clean re-isolation for fragmentation.[1,2] While this strategy is attractive, it limits the kinds of dissociation strategies that can be used to analyze owing to the resulting low charge states.

Electron-based dissociation strategies like electron transfer dissociation (ETD) have been demonstrated to be particularly effective for the fragmentation of intact proteins.[4] However, these techniques have a significant dependence on charge density; charge-dense precursor ions abundantly generate c and z• fragment ion when ETD is performed, but charge-depleted precursor ions typically generate few sequence informative ions and almost exclusively undergo electron transfer with no dissociation (ETnoD).[4] This property severely limits their utility following PTCR reactions. Fortunately, activated ion ETD (AI-ETD) has enabled a strategy to reduce the charge dependence seen in ETD. Concurrent infrared (IR) irradiation serves to heat ions, unfolding the protein and improving ETD fragmentation efficiency even at lower charge states.[5]

MATERIALS AND METHODS

Sample Preparation

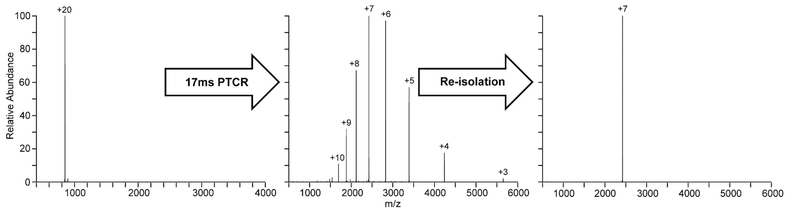
Purified apomyoglobin, carbonic anhydrase, and enolase were acquired from Sigma Aldrich. All proteins were prepared in a solution of 60% water, 40% acetonitrile, 0.1% formic acid, Lyophylized powders of ubiquitin, apomyoglobin, and carbonic anhydrase were reconstituted in this solution and analyzed with no further sample processing. Enolase was cleaned up with an Amicon® Ultra regenerated cellulose molecular weight cutoff filter prior to reconstitution. Samples were then directly infused injection into a modified Orbitrap Eclipse mass spectrometer.

Test Methods

Proteins were analyzed on a modified Orbitrap Eclipse Mass Spectrometer capable of AI-ETD activation. A Synrad Firestar CO2 continuous wave IR laser (10.6 um, 60W) was coupled to the instrument such that the beam laser beam passed through the center of the dual cell quadrupole linear ion trap (q-LIT) mass analyzer. Instrument control software modifiations were made to the instrument to allow concurrent IR irratiation during an ETD reaction.

The PTCR procedure prior to fragmentation is illustrated in Figure 1. The most abundant precursor in the original charge state distribution was isolated using the quadrupole mass filter. Isolated ions subjected to PTCR in the linear ion trap until the most abundance charge state appeared at ~2500 m/z. This charge state was isolated in the linear ion trap and subjected to either ETD or AI-ETD.

Figure 1. Procedure for PTCR purification prior to fragmentation.



MATERIALS AND METHODS (Continued)

ETD reaction time were selected such that ~80% of the precursor ions were depleted using ETD. Laser powers of to ~7W, ~8W, and ~6W were used for apomyoglobin, carbonic anhydrase, and enolase, respectively. The resulting ions were then analyzed in the Orbitrap at 240,000 resolution in full profile mode. 100 scans were averaged to generate each of the final scans and 5 microscans were used when analyzing enolase. An initial precursor target of 5e5 was used for all scans.

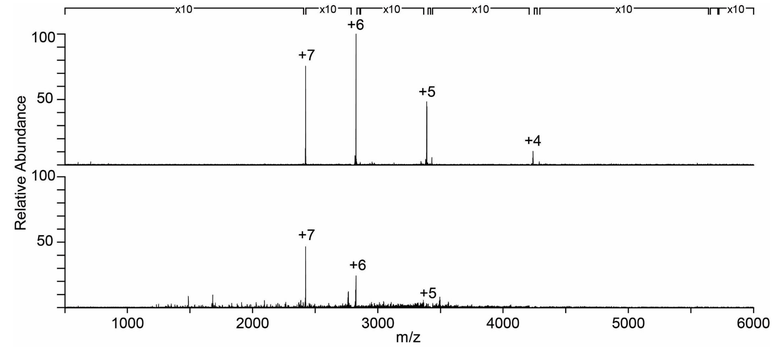
Data Analysis

The resulting averaged scans were analyzed via TDValidator version 1.0. Spectra were annotated for sequence coverage in TDValidator using a S/N cutoff of 5, max ppm tolerance 20 ppm, sub ppm tolerance 15 ppm, cluster tolerance 0.35, minimum score of 0.6, charge states ranging from 1 to each precursors charge state, and using the distribution generator Mercury7. Peaks incorrectly assigned near charge-reduced precursor and neutral loss peaks were removed.

RESULTS AND DISCUSSION

A comparison of the standalone ETD vs AI-ETD fragmentation spectra for apomyoglobin is depicted in Figure 2. Even superficially, the resulting MS^3 spectra differ substantially. The standalone ETD spectrum contains very few identifiable product ions aside from charge-reduced precursor ions. Further, the actual distribution of charge-reduced precursor peaks differ as well, being significantly lower intensity in the AI-ETD spectrum. This is a clear depiction of how AI-ETD reduces the occurrence of ETnoD during the reaction, thereby leading to more signal being distributed into fragment ions rather than successive charge-reduced products.

Figure 2. ETD (top) and AI-ETD (bottom) fragmentation spectra from fragmenting the +7 charge state of apomyoglobin. Fragment ions have been magnified 10x in both spectra.



The improvements associated with AI-ETD can be clearly seen in the comparison of sequence coverage illustrated in Figure 3. ETD fragmentation generated no sequence informative fragment ions. Although not indicative of its effectiveness on charge-dense precursors, this clearly illustrates the limitations of using ETD following PTCR. Nonetheless, AI-ETD is capable of generating a plethora of sequence informative fragment ions even at very low charge states. The ubiquity of c and z• fragment ions indicate this is principally from enhancing the electron transfer process, likely as a result of IR induced unfolding the protein and breaking noncovalent interactions within the molecule.[4]

Figure 3. Sequence coverage maps from performing ETD only (left) and AI-ETD (right) to apomyoglobin. At low charge states, ETD alone generates essentially no sequence informative fragment ions, but AI-ETD enables the reaction to still produce useful information. Note: c/z fragments are red, b/y fragments are blue.

RESULTS AND DISCUSSION (Continued)

The performance improvement in apomyoglobin largely extended to the other proteins investigated as well. Table 1 illustrates a breakdown of both the sequence coverage and fragment ions generated. Across all proteins, ETD fragmentation generated minimal sequence coverage at the charge states generated by PTCR. This severely limits the utility of ETD for analyzing complex protein mixtures as PTCR purification becomes more necessary. However, AI-ETD restored the sequence coverage to a level representative of standalone ETD on a highly charged analyte.

Notably, some b and y ions are present in the AI-ETD spectrum but not the ETD spectrum. While y ions can potentially occur as an ETD product ion, the b ions are indicative of some low-level infrared multiphoton dissociation occurring during the reaction. When optimizing the laser power for these experiments, it was found that the settings used generated minimal fragmentation when performing IRMPD alone, yet b ions are present in the AI-ETD spectrum and the precursor ion intensity is slightly lower in the AI-ETD spectrum, further supporting the presence of low level IRMPD. While this process could be eliminated by reducing the laser power, it was found that lower laser intensities not only reduced the overall sequence coverage as a result b/y fragment ions; the numbers of c/z• fragment ions also decreased substantially. This behavior is currently under investigation.

Table 1. Summary of fragmentation performance of ETD vs AI-ETD for all standard proteins investigated. Each evaluated metric is presented as ETD/AI-ETD.

	Molecular Weight (Da)	Sequence Coverage	c ions	z• ions	b ions	y ions
Apomyoglobin	16,941	>1% / 82%	0 / 81	1 / 57	0 / 39	1 / 15
Carbonic Anhydrase	29,007	3% / 34%	11 / 31	3 / 47	1 / 5	1 / 10
Enolase	46,642	>1%% / 34%	0 / 89	1 / 26	0 / 15	0 / 14

Given these results, AI-ETD represents a powerful strategy for coupling the high sequence coverage characteristic of electron-based fragmentation techniques to the sensitivity gains associated with PTCR. This performance is only expected to improve as techniques like ion parking improve the PTCR process by concentrating ion current into a single channel.[2]

CONCLUSIONS

- Standalone ETD generally fails to generate useful sequence information following PTCR purification due to the low charge states of the resulting product ions.
- Activated-ion ETD minimizes the charge-dependence associated with ETD, enabling high sequence coverage to be obtained even following significant charge reduction with PTCR.
- The ability to couple ETD to PTCR improves the prospect of using ETD for the analysis of complex mixtures of intact proteins.[1]
- The success of this strategy will likely improve when paired with other techniques like ion parking which maximize the number of ions retained in the PTCR purification step.[3,6]

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