Higher-energy Collision-activated Dissociation Without a Dedicated Collision Cell*

Graeme C. McAlister‡, Douglas H. Phanstiel‡, Justin Brumbaugh‡, Michael S. Westphall‡, and Joshua J. Coon‡§¶

Beam-type collisional activation dissociation (HCD) offers many advantages over resonant excitation collision-activated dissociation, including improved identification of phosphorylated peptides and compatibility with isobaric tag-based quantitation (e.g. tandem mass tag (TMT) and iTRAQ). However, HCD typically requires specially designed and dedicated collision cells. Here we demonstrate that HCD can be performed in the ion injection pathway of a mass spectrometer with a standard atmospheric inlet (iHCD). Testing this method on complex peptide mixtures revealed similar identification rates to collision-activated dissociation (2883 versus 2730 IDs for iHCD/CAD, respectively) and precursor-product-convolution efficiency comparable to that achieved within a dedicated collision cell. Compared with pulsed-Q dissociation, a quadrupole ion trap-based method that retains low-mass isobaric tag reporter ions, iHCD yielded isobaric tag for relative and absolute quantification reporter ions 10-fold more intense. This method involves no additional hardware and can theoretically be implemented on any mass spectrometer with an atmospheric inlet. Molecular & Cellular Proteomics 10: 10.1074/mcp.O111.009456, 1–6, 2011.

Beam-type collisional dissociation (HCD)1 is the primary means to effect vibronic dissociation. The approach involves energetic injection of selected precursor cations into a collision cell filled with inert gas (>1 mTorr). These dedicated collision cells are central components of numerous hybrid MS systems, e.g. Q-TOF, QQQ, Orbitrap, etc. Ion trap MS systems, however, accomplish collisional activation via resonant excitation of a trapped precursor population (ion trap collision-activated dissociation (CAD)). Rather than imparting one or two higher-energy collisions, as in HCD, CAD slowly heats precursor ions with hundreds of low energy collisions. The result is a slightly different product ion distribution (from HCD), which lacks low m/z products because of a low-mass cutoff imposed by the magnitude of the radio frequency (RF) trapping field. Yet the ubiquity of ion traps has made CAD a widely used dissociation method (1); it has resulted in some of the most comprehensive proteomic analyses to date, including the identification of the entire yeast proteome and over 30,000 phosphorylation sites in mice (2, 3).

The recent introduction of quadrupole linear ion trap (QLT) hybrids with dedicated collision cells has allowed a direct comparison of CAD to HCD (4–7). As it is able to access higher-energy fragmentation channels, HCD results in more peptide identifications than CAD, particularly for phosphorylated peptides (4, 8). Beyond that, HCD offers several advantages. First, CAD is not subject to the low-mass cutoff inherent to CAD, and is therefore compatible with isobaric tagging strategies for multiplexed quantitation (e.g. isobaric tag for relative and absolute quantification (iTRAQ) and TMT) (9–11). Second, acquisition of collision-based dissociation spectra in a standard form would greatly facilitate cross-platform comparison and data transferability. For example, downstream selected reaction monitoring experiments, which utilize HCD (i.e. QQQ), depend on prior knowledge of precursor-to-product transitions from upstream discovery data (12, 13). Third, HCD generates immonium ions and other secondary fragments that can aid both sequence determination and post-translational modification detection (14).

The dedicated collision cell required to implement HCD, however, increases instrument complexity and cost, especially for standalone QLT systems. Here we demonstrate that HCD can be performed both efficiently and quickly in the pre-existing atmospheric inlet region (inlet HCD or iHCD). iHCD requires no hardware modification and brings HCD capability to any MS system having an atmospheric pressure (AP) inlet. Typical MS operation involves ion transmission from an AP inlet to a mass analyzer, a QLT in our case, using a collection of electrostatic and electrodynamic ion optics and quadrupole ion guides (15). These elements pass ions through differentially pumped vacuum regions, the high pressure sections (>1 mTorr) of which can provide an in situ collision cell. Following injection and isolation, the resulting precursor population can be transmitted back along the ion injection pathway with kinetic energy sufficient to induce dissociation. The products of these collisions, which are trapped in that space, can be either injected...
As the ions pass into the higher pressure regions near the AP inlet, the isolated precursor population is transmitted back along the ion injection pathway with a high degree of kinetic energy. As the ions pass into the higher pressure regions located near the atmospheric pressure inlet, the ions collide with the neutral gas molecules leaking in from the AP inducing beam-type CAD. Generated products, trapped in that space, are then injected (C) into the QLT for m/z analysis (D).

**EXPERIMENTAL PROCEDURES**

*Cell Growth and Lysis—*Human embryonic stem cells (line H1) were maintained in a feeder-independent system, as previously described (18). Upon reaching 70% confluency, cells were passaged enzymatically using dispase (Invitrogen, Carlsbad, CA) at a 1:4 splitting ratio. Cells were harvested by individualizing for 10 min with an adequate volume of prewarmed (37 °C), 0.05% TrypLE (Invitrogen, Carlsbad, CA) to cover the culture surface. Following cell detachment, an equivalent volume of ice-cold Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Carlsbad, CA) was added before collecting the cells. Cell pellets were subsequently washed twice in ice-cold phosphate-buffered saline and stored at −80 °C. Approximately 10^6 cells were collected for each analysis.

Samples were lysed via sonication in lysis buffer containing 40 mM NaCl, 50 mM Tris, 2 mM MgCl₂, 50 mM NaF, 50 mM b-glycer-aldhyde phosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mini EDTA-free protease inhibitor (Roche Diagnostics, Indianapolis, IN), and 1 phosSTOP phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN).

*Digestion and iTRAQ Labeling—*Cysteine residues were reduced with dithiothreitol, alkylated using iodoacetamide, and digested in a two-step process. Proteinase Lys-C (Wako Chemicals, Richmond, VA) was added (enzymatic:protein ratio = 1:100) and incubated for ~2 h at 37 °C in lysis buffer. Samples were then diluted with 50 mM Tris pH 8 until the urea concentration was 1.5 M and digested with trypsin (Promega, Madison, WI) (enzyme:protein ratio = 1:50) at 37 °C overnight. Reactions were quenched using trifluoroacetic acid. Samples were dried to completion and purified using C18 solid phase extraction columns (SepPak, Waters, Milford, MA). iTRAQ labeling was performed according to manufacturer-supplied protocols. Once mixed, samples were dried to completion and purified by solid phase extraction.

*Mass Spectrometry—*Experiments were performed on three systems: LTQ, LTQ-Orbitrap, LTQ-Orbitrap XL (Thermo Fisher Scientific, San Jose, CA). Fig. 1 depicts the scan cycle. In brief, following injection and isolation of a particular precursor m/z peak (see Fig. 1A), the isolated precursor population is transmitted back along the ion injection pathway with a high degree of kinetic energy (see Fig. 1B). As the ions pass into the higher pressure regions, near the AP inlet, the ions collide with the neutral gas molecules leaking in from the atmospheric source. The precursor ions collide with the background gas molecules and fragment. The products are then trapped. Following fragmentation and trapping, product ions can either be further processed or mass analyzed (see Figs. 1C and 1D). All the associated voltages and times for this scan cycle were optimized prior to any large-scale analysis (vide infra). In these experiments, we fragmented precursor ions and trapped the resulting products in quadrupole 0 (Q0).

**Liquid Chromatography-Tandem MS (LC-MS/MS) Analysis—**LC-MS/MS analysis was performed using a NanoAcquity UPLC system (Waters, Milford, MA) coupled to a LTQ-orbitrap XL (Thermo Fisher Scientific, San Jose, CA). Peptide samples were loaded onto a pre-column (75 μm ID, packed with 5 cm C₁₈ particles (Alttech, Deerfield, IL)) for 10 min at a flow rate of 1 μL/min. Peptides were then eluted from a reversed-phase LC column (50 μm ID, packed with 15 cm C₁₈ particles (Alttech)) with a 120 min linear gradient from 1% to 35% acetonitrile (0.2% formic acid) at a flow rate of 300 nL/min. An additional 30 min were used for column washing and equilibration. The column making procedure was previously described (5).

MS¹ spectra were acquired at a resolving power of 60,000 and an automated gain control target of 1,000,000. Following MS¹ analysis, the 10 most intense precursors were selected for data-dependent activation using iHCD. Precursors having either unassigned or ≤2 charge states were rejected. A 60 s dynamic exclusion window was employed (repeat count of 1). MS²/MS AGC targets were set to 40,000.

**Database Search and False Discovery Rate (FDR) Analysis—**The resulting data files were searched using the Open Mass Spectrometry Search Algorithm (OMSSA) version 2.1.4 (19). Pre- and postsearch processing was performed using the Coon OMSSA Proteomic Analysis Software Suite (COMPASS) (20). Data was searched against the International Protein Index (IPI; http://www.ebi.ac.uk/IPI/) human database version 3.57, which had been concatenated with a reversed version of the same database (21). Full enzymatic specificity was required, allowing up to three missed cleavages. Carbamidomethylation of cysteines, iTRAQ 4-plex on the N terminus, and iTRAQ 4-plex on lysines were set as fixed modifications, whereas oxidation of methionines and iTRAQ 4-plex on tyrosines were set as variable modifications. An average mass tolerance of ±4.5 Da was used for the precursor, whereas a monoisotopic mass tolerance of ±0.5 Da was used for fragments ions. The resulting peptide spectral matches were trimmed to a 1% FDR using both e-value and precursor mass accuracy (20). Peptides were grouped into proteins following the rules previously established (22). P-scores for unique peptides that were grouped together by a common protein were multiplied to obtain the protein P-scores. Proteins were filtered by this score to achieve a 1% FDR.
RESULTS AND DISCUSSION

**iHCD Implementation**—As shown in Fig. 1 we elected to implement iHCD in Q0, which operates at a pressure comparable to dedicated collision cells (~1–5 mTorr). The iHCD scan cycle involves four major steps: (A) injection and isolation of selected precursor m/z peak (QLT), (B) activation in the high pressure region near the AP inlet (Q0), (C) injection of product ions into the system for further processing (e.g. product isolation, ion/ion reaction, etc.) and/or (D) m/z analysis in either the primary or secondary analyzer, if present (e.g. QLT or orbitrap, respectively). For maximum sensitivity steps B and C required extensive optimization (i.e. high precursor-to-product conversion efficiency). Initial evaluation of voltages and times was performed using triply charged angiotensin cations.

Fig. 2 presents the results of these procedures as performed on three generations of QLT instruments (LTQ, LTQ-Orbitrap, and LTQ-Orbitrap XL). Fig. 2A details one such analysis; here the product ion current produced upon iHCD of triply protonated angiotensin cations was measured as a function of the voltage offset between Q0 and the QLT (i.e. the optimal collision energy) for producing a sequence-informative MS/MS spectrum as a function of precursor m/z and charge state are shown in D.

**Fig. 2.** iHCD parametric evaluation. A, the relationship between the maximum product ion signal and the voltage offset between Q0 and the QLT. B, the time required for the ejection of product ions out of Q0 and into the QLT. As judged by data from three platforms, a standalone LTQ, an LTQ-Orbitrap Classic, and an LTQ-Orbitrap XL, the optimal conditions are identical. A schematic overview of the conditions necessary to effect iHCD on triply protonated angiotensin ions is presented in C. Finally, plots relating the optimal offset voltages between Q0 and the QLT (i.e. the optimal collision energy) for producing a sequence informative MS/MS spectrum as a function of precursor m/z and charge state are shown in D.
the time is extended to 20 ms most product ions are effectively ejected. This extended ejection time is expected as Q0 is 85 mm long and has no axial gradient. Fig. 2C shows the optimal voltages (all ion optics) for the interrogation of triply charged cations at m/z 433. With these conditions we estimate the overall precursor-to-product conversion efficiency of iHCD at ~40%—a yield typical of collision cell HCD.

Ideal activation parameters, of course, vary with precursor charge (z) and mass (m); more specifically, the optimal voltage offset between the QLT and Q0 is precursor z and m dependent. To counter this we developed a normalized collision energy algorithm that automatically tails iHCD parameters to precursor characteristics. To generate the algorithm we repeatedly interrogated a yeast whole-cell lysate that was enzymatically digested with LysC by nLC-MS/MS. During each experiment we varied one instrument parameter (e.g. the voltage offset between Q0 and the QLT), producing over 20,000 peptide spectral matches. Next, we empirically determined what instrument settings produce the most identifications for a given z and m/z range—e.g. for precursors between 300 and 400 m/z and with a charge state of 2 the offset that produced the maximal number of peptide spectral matches was ~20V (Fig. 2D). This process was repeated for each m/z range to generate a line of best fit. The trend shows an increasing amount of kinetic energy must be applied with increasing m/z. Trends very similar to those depicted in Fig. 2D have been described for other collision-activated dissociation methods (23–28). We then incorporated the normalized collision energy algorithm into the instrument control code to automatically calculate optimal iHCD voltage offsets for any selected precursor.

Comparison to Existing Fragmentation Methods—To compare iHCD to existing collision-based methods we infused angiotensin and activated the 3 precursor using CAD, PQD, HCD, and iHCD. Because they result from beam-type frag-

Fig. 3. MS/MS spectra resulting from either iHCD (A), CAD (B), PQD (C), or HCD (D) of triply protonated angiotensin cations.

Fig. 4. The number of peptides identified from a yeast whole-cell lysate that was enzymatically digested with Lys-C and interrogated by HCD (FTMS), CAD (ITMS), and iHCD (ITMS) (A). B, the number of peptide identifications from a human embryonic stem cell lysate that was enzymatically digested with LysC and labeled with isobaric tags (iTRAQ).

Fig. 5. MS/MS spectra following dissociation of an iTRAQ-labeled peptide cation interrogated using either PQD or iHCD. In both cases, the resulting MS/MS spectra easily identified the corresponding peptide; however, the iTRAQ reporter ions are ~10-fold more intense in the iHCD spectrum. Additionally, secondary fragment ions are more prominent in the iHCD spectrum.
molecular tagging, iHCD and HCD closely resemble one another (Fig. 3). For example, both display pronounced histidine immonium ion \( m/z \) peaks and have an appreciable amount of lower \( m/z \) secondary fragment ions, which tend to accumulate in beam-type CAD MS/MS spectra. A notable difference between iHCD and CAD is the complete absence of the histidine immonium ion (\( m/z \) 100) in the CAD spectrum, because of the inherent low mass cutoff of the QLT. PQD, a variant of CAD designed to overcome low mass cutoff, yields intermediate immonium ion levels.

We next evaluated the ability of iHCD to identify peptides from a complex mixture (LysC digest) as compared with CAD and HCD. Under method-specific optimal conditions (e.g. preferred AGC targets, NCE values, etc.), iHCD outperformed HCD and was comparable to CAD (1% FDR; Fig. 4A). We conclude this performance gap between iHCD and HCD is mainly analyzer driven. A separate comparison of iHCD to CAD using the same analyzer yielded comparable identifications (2883 versus 2538 PSMs, respectively). Despite the reduced number of iHCD scans (9556, avg. rate 280 ms versus 9933, avg. rate 260 ms for CAD), iHCD returned slightly more identifications (2883 versus 2730). These results are typical of other comparisons we have performed (data not shown). We conclude that though somewhat less efficient and slightly slower, iHCD generates more sequence-informative MS/MS spectra than CAD.

Because compatibility with isobaric tagging strategies is one of the major differences between HCD and CAD, we next sought to characterize the ability of iHCD to identify and generate quantitative information from isobarically tagged peptides (e.g. iTRAQ/TMT). iTRAQ-labeled peptides, generated from a Lys-C digest of human ES proteins, were analyzed by PQD, HCD, and iHCD. We substituted PQD for CAD, which cannot retain the quantitative reporter ions. iHCD produced more than twice as many identifications compared with either PQD or HCD (Fig. 4B). As we have previously shown, reporter ion intensity directly correlates with quantitative accuracy (4, 29). To assess the ability of iHCD to produce iTRAQ reporter ions, we performed one analysis in which both PQD and iHCD were executed on every precursor. On average, reporter ions were ~10-fold more intense when interrogated by iHCD than by PQD (Fig. 5). iHCD and HCD generate comparable amounts of iTRAQ reporter signal. Taken together, these identification and quantification data indicate that iHCD is an excellent method for isobaric tag-based quantification.

CONCLUSION

We present a method for performing HCD in the mass spectrometer AP inlet region (iHCD). iHCD provides similar or better rates of identification compared with CAD and carries with it all the benefits associated with beam-type dissociation: (1) compatibility with isobaric tags for multiplexed quantitation, (2) direct transferability of precursor-to-product transitions from discovery to targeted analysis, (3) elimination of low-mass cutoff inherent to CAD, (4) improved sequencing capabilities, especially for post-translational modification (PTM)-containing peptides. iHCD eliminates the need for a dedicated collision cell, thereby reducing instrument complexity and cost. By doing so, iHCD brings beam-type fragmentation to virtually any MS system having an AP inlet (e.g. ion traps, single quadrupoles, etc.). Here we describe implementation of iHCD on an LTQ quadrupole linear ion trap mass spectrometer. Recent work has extended iHCD to the newer generation dual cell LTQ (Velos) with similar success (30). This result is significant because that system’s quite different AP inlet utilizes a stacked ring ion guide. In sum, iHCD is an effective, simple to implement technology that delivers beam-type CAD to any system having an AP inlet.

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† To whom correspondence should be addressed: Departments of Chemistry, University of Wisconsin, Madison, WI. E-mail: jcoon@chem.wisc.edu.

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