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analytical chemistry feature

Collisions or Electrons? Protein Sequence Analysis in the 21st Century

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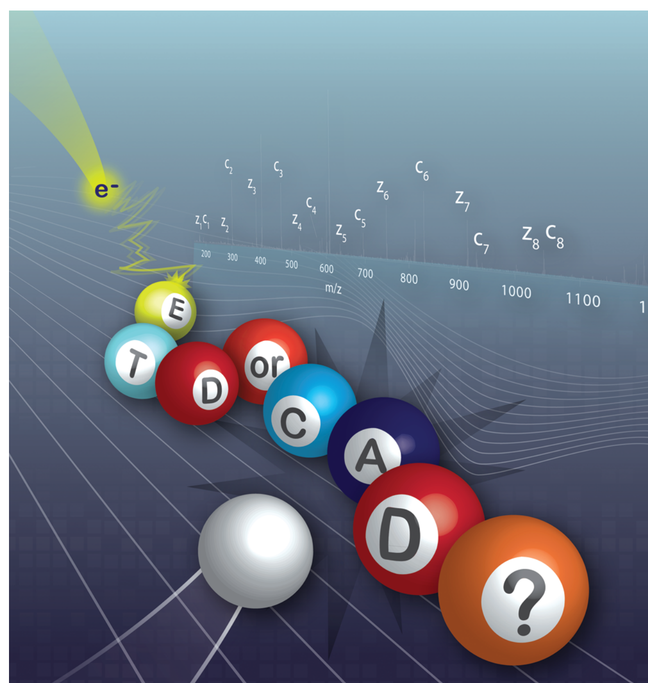
How dissociation is effected determines the upstream sample handling whereas the spectral features it produces regulate the downstream informatics approach. (To listen to a podcast about this feature, please go to the *Analytical Chemistry* website at pubs.acs.org/journal/ancham.

For decades now, the choice method to impart dissociation has been the collision of peptide cations with rare gas atoms in a process known as collision-activated dissociation (CAD).[1−3] This method is particularly simple and effective—in a very real sense, it is the scaffold upon which today's proteomic methodologies are built. Just 10 years ago, a very different fragmentation technique was discovered, one that relies on the capture of electrons rather than on collisions.[4−7] The technique, electron capture dissociation (ECD), has been widely acclaimed, though it is mainly restricted to instruments that use magnetic fields for ion confinement. Five years ago, another discovery was made: ECD-like fragmentation could be induced by reaction of peptide cations with radical anions.[8,9] This technique, electron transfer dissociation (ETD), can be readily implemented on the more common ion trap mass spectrometers. Today, ETD has been made commercially available by several vendors.[10−12] But exactly what does ETD bring to the proteomics table? How does it fit into the current workflow? In addition to discussing ETD and its attributes, this article aims to provide a basis for defining the role ETD will play in protein sequence analysis.



RYAN LYNCH AND RACHAEL LARSEN

TANDEM MS

The objective of MS/MS is to produce a collection of peptide fragment ions that differ in mass by a single amino acid; this allows researchers to read the amino acid sequence of the precursor peptide.[13] In CAD, these fragments are generated by subjecting a population of isolated precursor cations to collisions with gas atoms. These collisions supply sufficient internal energy to induce covalent bond breakage. Cleavage of the amide linkage—the favored CAD target—gives rise to b- and y-type fragment ions (Figure 1, top). ETD, on the other hand, follows from the reaction of multiply protonated peptide cations with small-molecule anions. These reactions proceed by transfer of an electron from the anion to the peptide cation. The result is an odd-electron cation that undergoes free-radical-driven cleavage, just as in ECD.[4] Unlike CAD, ETD takes aim at the $N-C_\alpha$ bond, generating even electron c-type and odd electron $z^\bullet$-type product ions (Figure 1, bottom).

From a pragmatic perspective, product ions resulting from amide bond cleavage are worth no more or less than those derived from $N-C_\alpha$ bond dissociation. That is, for spectral interpretation, there is no apparent advantage of using b- and y-type versus c- and $z^\bullet$-type product ions, or vice versa. Note that the formation of a product ion pair when one series is odd-electron and the other is even does offer special opportunities when product ion masses are measured at very high accuracies.[14]

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Sarepta Exhibit 1019, page 1

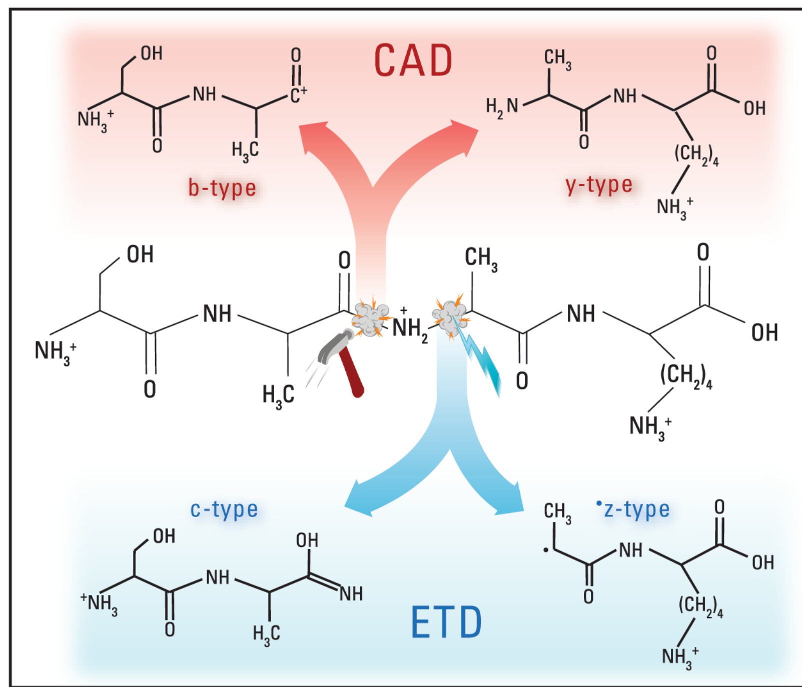

Figure 1. Fragment ion types produced following either CAD or ETD.

Setting that aside for now, I will focus my discussion on the defining features of each technique.

SEQUENCE COMPATIBILITY

The ideal dissociation method would randomly cleave peptide backbone bonds regardless of peptide length, z, m/z, and amino acid composition or order and would be indifferent to the presence of posttranslational modifications (PTMs); however, no such method currently exists. Some of these attributes are related, but their collective effect on fragmentation has sculpted virtually every aspect of contemporary proteomics. During collisional activation, energy is deposited on a relatively slow timescale (picoseconds to microseconds) and is redistributed throughout the peptide precursor cation to induce cleavage of the weakest bond(s), the protonated amide linkages. Ideally, amide linkages are randomly protonated within the precursor population so that upon collision, a variety of fragments are made, each differing from the next by the mass of an amino acid. The key to making this happen is to encourage random protonation along the amide backbone linkages; typically this is done by employing the protease trypsin, which cleaves proteins at lysine and arginine residues to yield peptides that contain no internal basic amino acids. That is, upon digestion with trypsin, the resulting peptides will not have internal amino acids that are capable of blocking random protonation.[15,16] A side effect of trypsin use is that the resultant peptides are small, averaging ~10 residues in length.

Figure 2 depicts the utility of CAD for sequencing three broad categories of peptides: short (tryptic), PTM-containing (long or short), and large. Imagine that the short peptide undergoing CAD is a small car colliding with a wall in a high-speed crash test—an admittedly imperfect but nonetheless useful analogy. From such an experiment, we expect the car to incur significant damage by breaking into various pieces. But what happens if we hold the trypsin and apply a different protease? Numerous other enzymes will produce longer peptides, on average, than trypsin does. This increase in size is beneficial, because there are now fewer peptides to sequence per protein; however, many of these peptides contain internal basic residues—remember that internal basic residues sequester charge and prevent random backbone cleavages. Back to the car analogy, we can easily recognize that as the size of the vehicle grows, it suffers less damage following an encounter with the wall. A tractor-trailer undergoes only minor damage, while the body of the vehicle remains intact (Figure 2). For this reason trypsin is the preferred enzyme for proteomics.

Even if we use trypsin to force the generation of well-behaving, CAD-friendly peptides, complications still arise. Perhaps the most considerable of these obstacles is the presence of protein PTMs. When peptides contain PTMs (e.g., phosphorylation, glycosylation, sulfonation), the preferred CAD dissociation pathways often change.[17,18] In phosphorylated peptides, for example, the phosphoryl group competes with the amide bonds of the backbone as the preferred site of protonation so that upon CAD, phosphoric acid is displaced, leaving the backbone bonds intact. Imagine ramming a car with a bicycle attached to the roof into the wall (Figure 2). The first thing to come off is the weakly attached bicycle. Similarly, CAD spectra of other PTM-containing peptides are frequently devoid of the consecutive backbone cleavages necessary for sequence identification.

Figure 3 compares CAD and ETD fragmentation methods as applied sequentially to an eluting phosphopeptide. The top panel displays the first MS/MS scan acquired using CAD; the spectrum is characterized by a single m/z that corresponds to the loss of H_3PO_4, as graphically depicted with the bicycle in Figure 2. Sequence assignment with this spectrum is therefore impossible. The very next scan utilized ETD and generated the spectrum shown in the lower panel. Upon application of ETD, every possible backbone fragment is observed, defining the unknown peptide sequence as RKpSILHTIR (pS denotes a

Sarepta Exhibit 1019, page 2

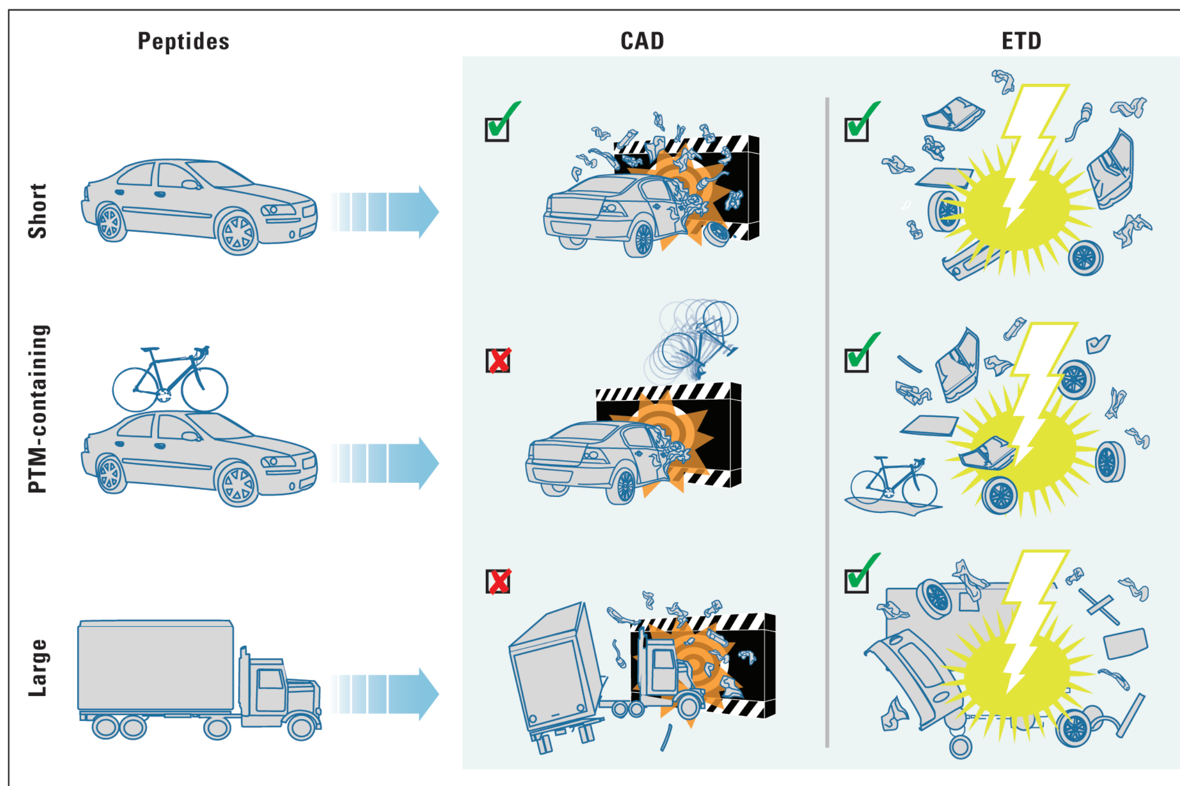

Figure 2. Analogy illustrating a typical outcome of dissociation for three categories of peptide precursors: short, PTM-containing, and large. CAD is highly effective for short peptides but is generally less effective for those that are either large or PTM-containing. ETD is more or less indifferent to peptide length or the presence of PTMs.

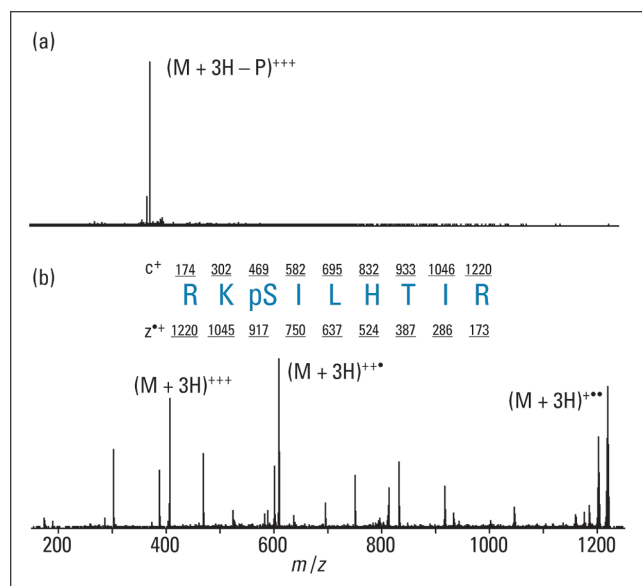

Figure 3. Tandem mass spectra obtained from a phosphopeptide eluted during a nHPLC/MS/MS experiment. In (a), the tandem mass spectrum following CAD is shown. The spectrum is dominated by a single *m/z* corresponding to loss of a phosphoric acid moiety. No peptide backbone cleavage is observed. Sequence identification is impossible. In (b), the tandem mass spectrum following ETD fragmentation is shown. Every single backbone cleavage product is observed. The sequence is easily assigned as RKpSILHTIR.

phosphoserine). Even in cases where CAD does provide enough backbone cleavages for sequence identification, site localization can be difficult. Cleavage between each inter-residue linkage is critical in this regard. Many recent reports describe the use of ETD for phosphorylation, glycosylation, and sulfonation analyses.[12,19−33]

INSTRUMENTATION

Since the first description of ETD in 2004, numerous instrumental configurations have been reported. In the initial implementation, a linear ion trap mass spectrometer was modified by adding a source of reagent anions, and the linear ion trap was altered to allow concurrent confinement of both anions and cations.[8,9] Such systems offer an advantage in that the unoccupied end of the linear ion trap is accessible and readily modified to accommodate an anionic reagent source, chemical ionization (CI) in this case. That design is now the basis for the LTQ XL ETD. ETD can also be realized within 3D ion trap systems in a similar way, except that both anion and cation populations are injected through a single ion pathway, with one exception.[34] Two vendors have made commercial 3D ion trap ETD systems by addition of a CI source.[11,12] And in the last few years, McLuckey and co-workers have pioneered the development of methods to generate ETD-inducing anions under atmospheric-pressure conditions so that the downstream MS system requires minimal modification.[35−37] The major aims of such approaches are to generate an intense beam of highly efficient ETD-inducing reagents and to not impact peptide cation production. These approaches show genuine promise and may allow for a more simplified, inexpensive pathway to impart ETD on virtually any MS system.

Linear ion trap mass spectrometers have many advantages over 3D ion traps for peptide analysis, and the ETD application is no

Sarepta Exhibit 1019, page 3

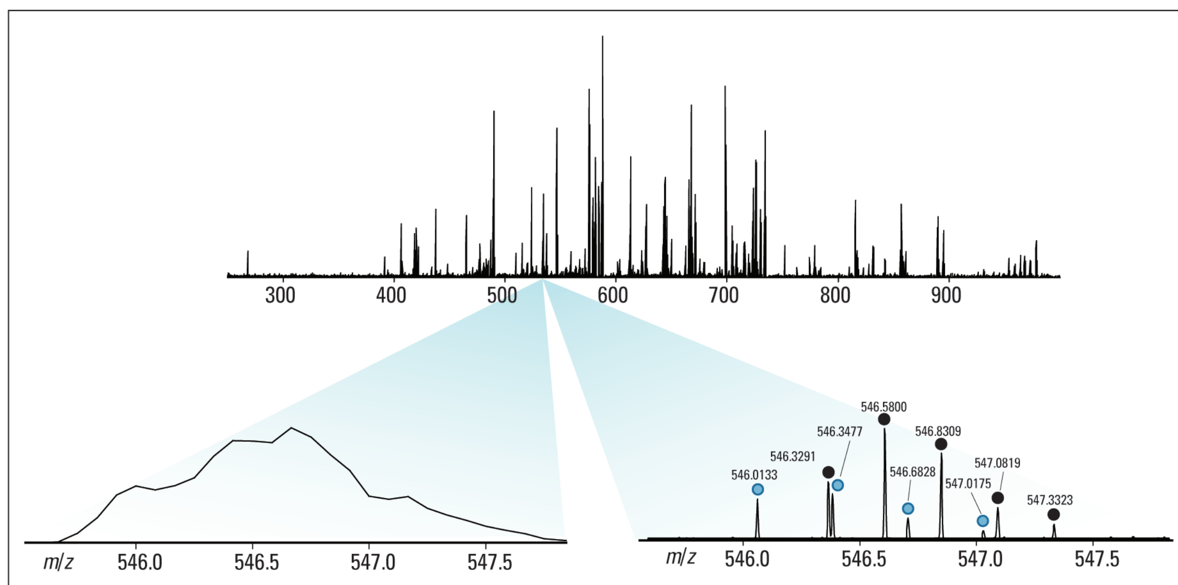

Figure 4. ETD product ion spectra following dissociation of a 24-residue peptide. Numerous c- and z•-type fragment ions are generated and detected. The expanded views display a small *m/z* region when the analysis of these products is performed with an ion trap and an Orbitrap. The mass resolution offered by the Orbitrap allows for unambiguous identification of two closely spaced isotopic clusters of z•-type fragment ions. Blue circles, ${z_{14}}^{3+} = 546.0147$; black circles, ${z_{18}}^{4+} = 546.3304$. (Adapted from Ref. 40.)

exception. Linear ion traps offer higher ion capacities, injection efficiencies, and detection efficiencies and are readily coupled with other mass analyzers (e.g., TOF, Orbitrap, and FT ion cyclotron resonance).[38] For ETD, all of these benefits are relevant. First, ETD is capable of dissociating large peptides and even whole proteins. Several dozen or even hundreds of c- and z•-type ion fragments are generated; thus, the ability to start with the largest precursor cation population possible is critical. For example, if you started with 10,000 precursor cations and divided that signal into 200 fragments, assuming all were in one charge state, you would have ~50 ions per fragment *m/z* bin. Now imagine starting with 100,000 precursor cations and doing the same experiment. You no longer have 50 ions per fragment *m/z* bin but have 500, a 10-fold improvement in signal. This means that linear ion traps, which offer 10- to 50-fold improvement in ion storage capacity over 3D systems, can allow for the direct analysis of large peptides and whole proteins in single scans, whereas 3D systems will require spectral averaging to compensate for lower-S/N spectra.

A second critical attribute that renders linear ion traps particularly useful for conducting ETD experiments is that anionic and cationic populations can be processed and stored separately until the reaction period is initiated. This characteristic is important because spatial segregation affords complete control of the reaction duration and allows for the subsequent reaction with a different type of anion. The LTQ XL ETD system, for example, has a linear ion trap that is divided into three separate sections. Direct current offsets to each section are controlled independently such that anions and cations can be moved from one section to the other without interaction. This means that the isolated cation precursor can be stored and maintained separately while the opposite end of the device is being loaded with anionic reagents, during an anion purification step, if necessary. Then, when both populations are ready, the ion–ion reaction is allowed to commence. Three-dimensional systems are operated by first capturing and isolating the cation precursor and then filling with the reagent anion. The difference is that during the filling, the anions that arrive first already start reacting with the precursor. Thus, there is no way to separate the filling and reacting time into discrete steps.

A final benefit of linear ion trap systems is that they are easily coupled with a secondary mass analyzer, usually one that provides higher resolving power and mass accuracy. The first coupling of ETD with a hybrid system was described by McLuckey et al.[36] ETD product ions were mass analyzed in a downstream TOF analyzer. Since that time, both linear ion trap FT ion cyclotron resonance MS and Orbitrap hybrids have been modified to perform ETD.[39−41] So far, only the LTQ Orbitrap XL ETD hybrid is commercially available, but I suspect the aforementioned systems will likewise come to market in the near future. Figure 4 displays ETD product ion spectra of a 24-residue peptide that were acquired either with a linear ion trap or on an Orbitrap analyzer. From the expansions, it is obvious that the resolving power of the Orbitrap can be highly advantageous for assignment of spectra from even modest-sized peptides. And, as I discuss later, the benefit of coupling ETD with such an analyzer goes well beyond spectral assignment.

ETD FOR SHOTGUN PROTEOMICS

Shotgun proteomic experiments rely on enzymatic digestion of protein mixtures to create highly complex samples containing hundreds of thousands of peptides.[42−44] These peptides are chromatographically separated and ultimately sampled by the mass spectrometer for MS/MS analysis. Given the exciting attributes of ETD, many proteomics groups are now seriously considering whether to invest in ETD-capable instrumentation. For those in search of PTMs, the case for ETD is quite clear; but what about for large-scale shotgun proteomics experiments, where the name of the game is identifying as many unique proteins as

Sarepta Exhibit 1019, page 4

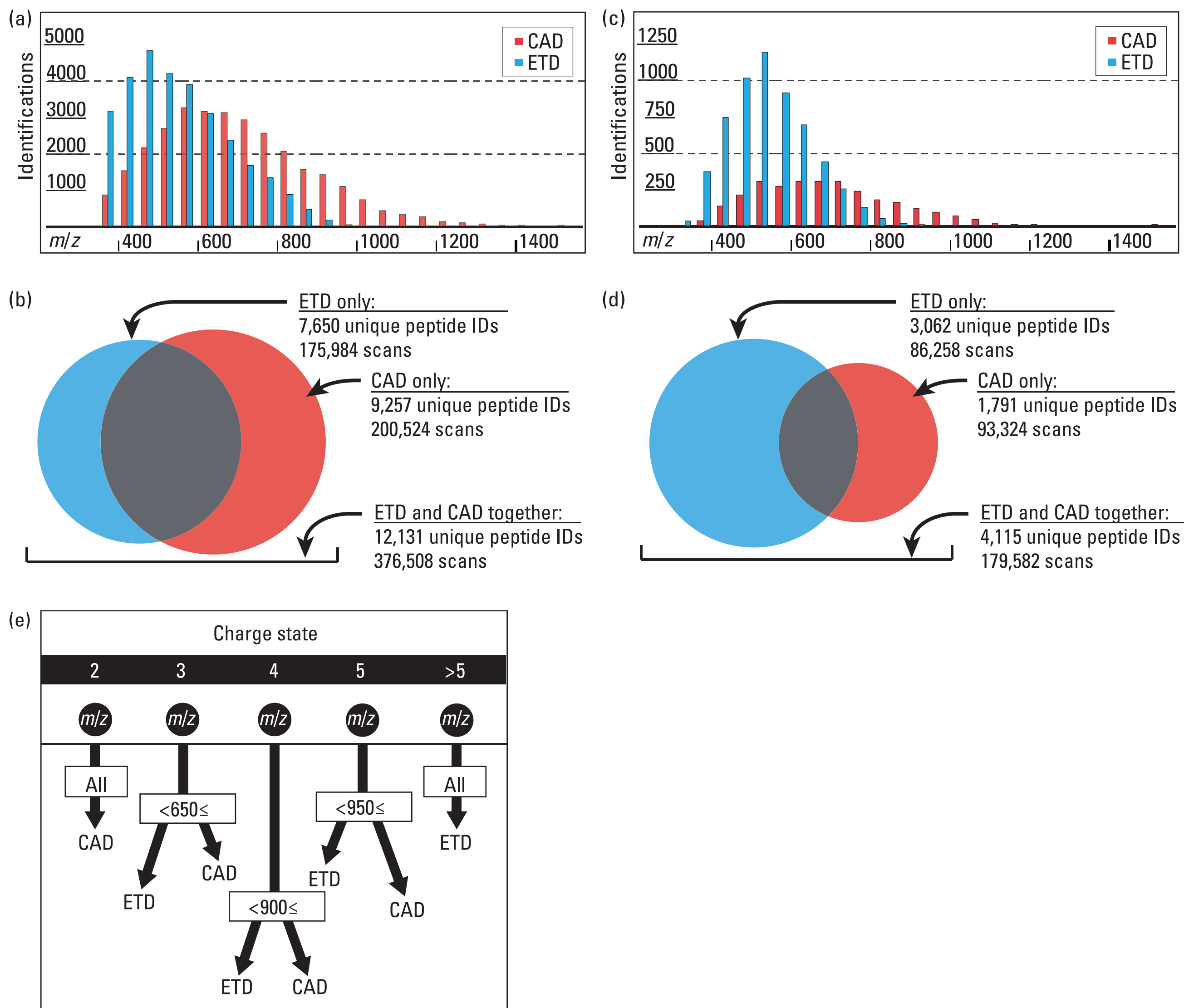

Figure 5. (a–d) Distribution of identified peptides using either the CAD (red) or ETD (blue) methods. The number of identified peptides for each method is displayed as a function of the precursor *m/z* ratio for (a, b) unmodified peptides from yeast and (c, d) phosphopeptides. (b, d) The redundancy and complementarity between the unique peptides identified during the CAD- and ETD-only analyses for the unmodified and PTM-containing datasets, respectively. (Adapted from Ref. 45.) (e) Schematic of the DT algorithm; *m/z* applies to the precursor selected for MS/MS interrogation.

possible? This question was the subject of a recent large-scale study performed by my laboratory.[45]

To test the impact of either CAD or ETD, peptides from a complex mixture of yeast were analyzed separately by each technique in triplicate (Figures 5a and 5b). First, we note that CAD identifies ~1500 more peptides than ETD, but that overlap is minimal. Further, as shown in Figure 5a, CAD has a preference for precursors with a higher m/z, whereas ETD tracks toward lower ones. This trend has been well documented and is discussed later.[46,47] Combination of one CAD and one ETD analysis, however, resulted in 7842 unique peptide identifications, topping either duplicate CAD (7359) or ETD (6006) analyses. Note that the statistics shown in Figures 5a and 5b are the sum of triplicate results. These trends were maintained when a complex mixture of phosphopeptides was analyzed (Figures 5c and 5d). The major difference here is that ETD has a much higher success rate for these peptides and accounts for nearly twice as many unique identifications as CAD.

The tendencies of CAD and ETD to favor differing precursor m/z ranges do offer interesting opportunities. On the hybrid ETD-enabled Orbitrap system, the high mass resolution can change the game. Namely, values of z and m/z are known before MS/MS sampling so that only the dissociation method with the highest probability of success is applied. The method, called data-dependent decision tree (DT; Figure 5e), was applied to the same yeast peptide and human phosphopeptide mixtures discussed earlier, and the results were excellent. Implementation of the DT method netted 93% and 95% of the identifications harbored by separate CAD and ETD methods, respectively, and did so with half the sample in half the analysis time. Duplicate application of the DT method on the yeast peptides landed 8939 unique peptide identifications versus the 7842 netted by sequential analysis by CAD and ETD, 7359 by duplicate CAD, and 6006 by duplicate ETD analysis. These data provide an exciting glimpse of the future, in which multiple dissociation methods will be useful for all large-

Sarepta Exhibit 1019, page 5

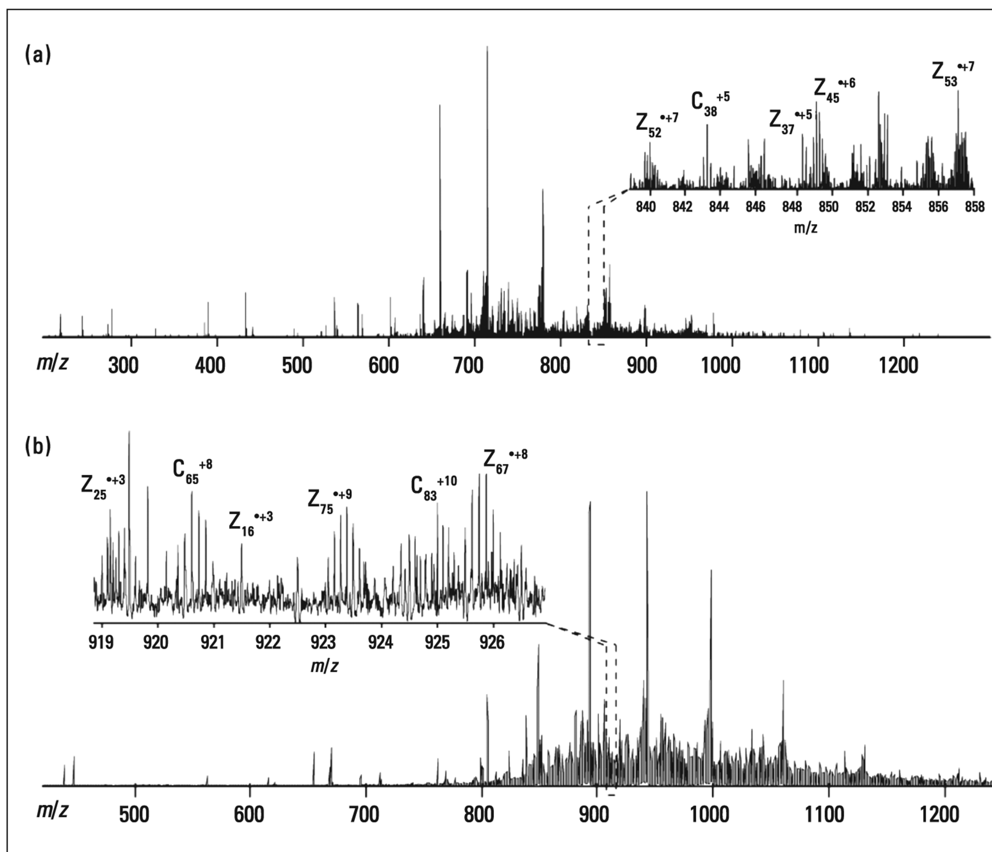

Figure 6. (a) An FT ETD-MS/MS single-scan spectrum of the ubiquitin +13 precursor (*m/z* 659). This spectrum was collected in 750 ms and harbored 112 of 150 possible backbone fragments to yield 88% sequence coverage. (b) The +20 charge state of myoglobin, a 17-kDa protein, was subjected to ETD followed by Orbitrap *m/z* analysis. Inspection of the spectra (50 averaged scans) revealed 127 of 304 possible backbone fragments, translating to 55% sequence coverage. (Adapted from Ref. 39.)

scale proteomic applications, and decisions about which method to apply can be tailored, in an automated fashion, to each precursor.

ETD FOR TOP-DOWN PROTEOMICS

Top-down sequencing, an emerging method of protein analysis, seeks to directly examine intact proteins by MS.[48−51] From an analytical perspective, this concept is highly attractive; for example, it allows PTMs to be examined within the context of one another, and alternative splice variants can be discovered.[52] Implementation, however, can be challenging because whole proteins are not as easily chromatographed as peptides. Likewise, tandem MS of intact protein cations is less straightforward than for peptides. Whole-protein cations possess at least 10-fold more backbone bonds to dissociate and literally hundreds or thousands of different fragment ions to analyze. Ten years ago, ECD was a great boon for the top-down approach, mainly for its ability to randomly cleave bonds across the entire length of small to modest-sized proteins.[53,54] ETD too has been highly effective for directly dissociating large peptides and whole proteins.[55] Figure 6 displays the direct dissociation of intact ubiquitin and myoglobin via ETD followed by *m/z* analysis with an Orbitrap mass analyzer.[39] For ubiquitin, 112 of 150 possible backbone bond cleavages were observed from this single-scan spectrum, which took ~750 ms to collect. Myoglobin, which is about twice the size of ubiquitin, required spectral averaging (50 seconds). Still, the resulting spectrum contained 127 of 304 possible cleavages.

High-resolution mass analysis is not required to identify the intact proteins following ETD. In fact, the high sensitivity of the ion trap mass analyzer can allow for the detection of proteins as large as 70 kDa in just a few seconds.[55] The lower resolving power of the ion trap, however, can make spectral identification challenging. To settle this, the highly charged c- and $z^{\bullet}$-type fragments generated from whole-protein dissociation via ETD can be deprotonated by a secondary reaction with a proton transfer anion. These sequential ion−ion reactions can be accomplished in ~100 ms and produce a very simple and straightforward ladder of fragment ions to interpret. Some very nice reports on using low-resolution mass spectrometers with ETD for whole-protein analysis have recently been published.[56,57] Whether coupled with low- or high-resolution mass analysis, ETD stands to play an important role in the developing field of top-down protein analysis.

INFORMATICS AND DATA PROCESSING

Translating raw spectra from tandem MS into a peptide sequence is a formidable task, no matter what the dissociation method. The standard approach, pioneered by Yates and co-workers, relies on the correlation of spectra to candidate sequences retrieved from protein sequence databases.[58] This technology has spawned

Sarepta Exhibit 1019, page 6

dozens of algorithms and is the basis for practically all high-throughput proteomic analysis. Each of these programs incorporates various models and methods for peak identification, peak scoring, and match probability assessment. But, for all these differences, they share a common theme—each was designed around the type of fragmentation observed in CAD. I raise this point to emphasize that virtually all ETD spectra have so far been analyzed by software that is not necessarily optimal. Whichever algorithm is used (SEQUEST, OMSSA, MASCOT, ProSight, etc.), ETD compatibility currently means switching from analysis of b- and y-type ions to c- and z$^{\bullet}$-type. As of today, virtually all ETD assessments are made on the basis of the outputs of these programs, including those discussed here. As we move forward, I predict newer search engines built around ETD fragmentation patterns, rather than adapted from a CAD point of view, will further improve ETD performance.

The reduced performance of ETD when applied to precursors of high *m/z* values is another area of informatic prospect. As precursor *m/z* values increase, a general shift toward nondissociative electron transfer occurs. This is why the DT discussed earlier is so effective—ETD and CAD have complementary performance across the broad range of observed precursor *m/z* values. Recently our laboratory reported on an automated method to gently activate (collisionally) the nondissociative ET product ion.[10] The method, coined ETcaD, is highly effective at inducing the formation of ETD fragments; however, those new fragments often experience H atom loss or gain. Specifically, c-type ions can lose a H atom to become c$^{\bullet}$-type, while z$^{\bullet}$-type fragments gain the H atom to generate z-type ions. The net result is that c-type fragments can be as predicted or 1 Da lighter, whereas z$^{\bullet}$-type products are possibly 1 Da heavier. When all these ions were considered, the mean sequence coverage for ETD went from $\sim$ 63% to $\sim$ 89% with ETcaD, which was even superior to CAD ($\sim$77%). However, the database search algorithms we used had difficulty with the 1-Da ambiguity of many newly generated fragment ions. I predict that with further development this search algorithm issue will likely be resolved, and we can expect the ETcaD method to offer excellent performance across a very broad precursor *m/z* range.

Recently our laboratory reported on a unique feature of c- and z$^{\bullet}$-type ions that will indeed offer new informatic opportunities.[14] Specifically, b-, c-, and y-type fragment ions contain an odd number of atoms with odd valence (e.g., N and H), whereas z$^{\bullet}$-type ions contain an even number of atoms with odd valence. We dubbed this phenomenon, an extension of Senior's rule, the valence parity rule, which dictates that no c-type ion can have the same chemical composition and, by extension, mass, as a z$^{\bullet}$-type ion.[59] To illustrate the importance of this, we asked how often peptide fragment ions (all possible amino acid combinations) would overlap if we could measure their masses with infinite accuracy. The result is striking. CAD products, even when measured with perfect accuracy, are often overlapping in *m/z*; however, the ETD products never have coinciding *m/z* values. At $\sim$1-ppm measurement accuracy, which is readily achieved on Orbitrap and FT ion cyclotron resonance systems, the majority of ETD product ions $<$1000 *m/z* can be readily identified as N- or C-terminal. The ability to easily identify fragment ion peaks by mass alone could be very powerful. These assignments can provide a method to directly determine amino acid composition, input for database search algorithms,[60] or a basis for de novo sequence analysis.

OUTLOOK

The response to the question posed in the title of this article is "both". A revised version should read, "Collisions and Electrons...".[61] And, with recent commercial development, researchers are now evaluating ETD for a variety of applications. ETD shows exceptionally strong performance when applied to specific categories of peptides (e.g., PTM-containing, basic, large). These strengths dovetail with the weaknesses of CAD that have long limited the methodology and applications amenable to MS-based proteomics. I predict practitioners of shotgun experiments will rely less heavily on trypsin and more on proteases that generate larger portions of protein. Such proteases will still produce short fragments that are perfect for CAD-based sequencing and larger ones for ETD. Complex mixtures will be less complex, and PTM motifs and patterns will be detected more readily. Dynamic range should likewise improve as the mass spectrometer scan time needed to sequence a 30-residue peptide is equivalent to that of a 10-residue one. In other words, if there are fewer peptides to sequence (because the pieces are longer), the mass spectrometer will have more time to sample lower-abundance precursors.

The commercial availability of ETD-enabled hybrid mass spectrometers should continue to expand. The high resolution afforded by these systems enables DT logic, which automatically tailors the dissociation method to the precursor before activation. The user will not need to possess a detailed knowledge of peptide fragmentation or need to toggle between ETD and CAD on each sample. Bioinformatic opportunities abound for ETD data sets, and I anticipate that algorithmic innovation over the next several years will improve ETD performance even further. These advances are likely to touch on all aspects of ETD application. Sequence tag and de novo sequence determination will become much more tractable, because ETD spectra tend to be rich in information and because of the valence parity rule that allows the separation of c- and z$^{\bullet}$-type products. The number of publications on ETD indicates that the technique is on the rise and will continue upward. The proteomics community's pursuit of the opportunities ETD affords—and its recognition of the technique's limitations—will determine how rapidly the advance of ETD continues.

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Sarepta Exhibit 1019, page 7

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Sarepta Exhibit 1019, page 8