Published in final edited form as:

Anal Chem. 2005 September 1; 77(17): 5662–5669.

Electron-Transfer Ion/Ion Reactions of Doubly Protonated Peptides: Effect of Elevated Bath Gas Temperature

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Abstract

In this study, the electron-transfer dissociation (ETD) behavior of cations derived from 27 different peptides (22 of which are tryptic peptides) has been studied in a 3D quadrupole ion trap mass spectrometer. Ion/ion reactions between peptide cations and nitrobenzene anions have been examined at both room temperature and in an elevated temperature bath gas environment to form ETD product ions. From the peptides studied, the ETD sequence coverage tends to be inversely related to peptide size. At room temperature, very high sequence coverage (~100%) was observed for small peptides (≤ 7 amino acids). For medium-sized peptides composed of 8–11 amino acids, the average sequence coverage was 46%. Larger peptides with 14 or more amino acids yielded an average sequence coverage of 23%. Elevated-temperature ETD provided increased sequence coverage over room-temperature experiments for the peptides of greater than 7 residues, giving an average of 67% for medium-sized peptides and 63% for larger peptides. Percent ETD, a measure of the extent of electron transfer, has also been calculated for the peptides and also shows an inverse relation with peptide size. Bath gas temperature does not have a consistent effect on percent ETD, however. For the tryptic peptides, fragmentation is localized at the ends of the peptides suggesting that the distribution of charge within the peptide may play an important role in determining fragmentation sites. A triply protonated peptide has also been studied and shows behavior similar to the doubly charged peptides. These preliminary results suggest that for a given charge state there is a maximum size for which high sequence coverage is obtained and that increasing the bath gas temperature can increase this maximum.

Mass spectrometric analysis of peptides produced by enzymatic digestion is common to most protein identification work currently being performed. Tandem mass spectrometry of enzymatically produced peptides, either via the generation of “sequence tags”¹ or via automated analysis of uninterpreted data,^{2,3} is particularly important when protein mixtures are being analyzed. By far, the most common enzyme used for digestion is trypsin, which cleaves proteins at positions C-terminal of lysine and arginine residues. As a consequence of the basic nature of these two amino acids, and the typical size of the peptides produced, most tryptic peptides produce doubly charged ions when subjected to electrospray ionization (ESI). This makes the analysis of doubly charged peptides, and particularly tryptic peptides, important for most LC/MS/MS-based methods for protein identification because electrospray is the most common ionization method used in coupling on-line condensed-phase separations with mass spectrometry. The most prevalent MS/MS methods in use for peptide/protein identification involve the collision-induced dissociation (CID) of protonated peptide cations, which generally leads to cleavage of the peptide backbone amide (C_0-N) bond to produce b-type and y-type sequence ions. Unfortunately, in many cases, the information gained from CID is insufficient to identify the peptide, as the fragmentation is limited to small-molecule loss or concentrated in only a few specific backbone cleavages.⁴ Another limitation often encountered with CID arises with posttranslationally modified peptides/proteins. Several important and common

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posttranslational modifications tend to be lost when the modified peptide is subjected to CID.^{5,6} This complicates localization of the site at which the modification was originally attached and often limits the range of informative backbone cleavages.

A technique that has been shown to complement CID of multiply protonated peptides is electron capture dissociation (ECD).^{7,8} In ECD, multiply protonated polypeptide cations are stored in a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer and exposed to low-energy electrons. Capture of electrons by the peptide cations results in charge reduction and fragmentation of the peptide backbone at the amine (N-C α) bond to produce c-type and z-type sequence ions. ECD generally results in cleavage of a wider range of peptide backbone bonds than CID with less dependence on peptide composition. It also shows a preference for the cleavage of peptide backbone bonds, even in the presence of potentially labile posttranslational modifications, such as phosphorylation and glycosylation.⁸ ECD also shows a preference for cleavage of disulfide bonds, another common posttranslational modification, which can be advantageous in some cases.⁹ Unfortunately, ECD is largely limited to FT-ICR mass spectrometers, and other types of mass spectrometers are not well suited for the technique, although there have been some recent reports of efforts to adapt electrodynamic ion traps for ECD.^{10, 11}

While the trapping of electrons and peptide/protein cations simultaneously is difficult in electrodynamic ion traps, cations and anions can be stored together readily,¹² and it has recently been reported that ion/ion reactions can provide a way to access ECD-like results in electrodynamic ion traps. Hunt and co-workers have reported results for some anionic reagents that react with peptide cations by electron transfer, producing ECD-like fragmentation to yield c-type and z-type sequence ions in a process termed electron-transfer dissociation (ETD).^{13, 14} It has subsequently been shown that electron-transfer ion/ion reactions also share ECD's preference for disulfide bond cleavage¹⁵ and that ETD experiments combined with CID experiments can yield complementary information about peptide structure and glycan structure, respectively, for a glycopeptide in a quadrupole ion trap,¹⁶ in analogy to that provided by ECD and infrared multiphoton dissociation in an FT-ICR mass spectrometer.^{17, 18} It has been noted, however, that the peptide structural information that can be obtained via ETD can be highly charge-state dependent.¹⁹ A number of peptides that could form both doubly and triply protonated cations were studied, and it was found that, while the triply charged cations gave almost complete sequence coverage, the doubly charged cations gave much poorer sequence coverage, with fragmentation often limited to one or both ends of the peptide. The ions studied were model peptides, not tryptic peptides, and generally had nontryptic structures (lysine and arginine residues at positions other than the C-terminus), so generalization to tryptic peptides was not necessarily warranted. This work was motivated, in part, by this observation because of its potential relevance to the use of ETD involving bottom-up proteomics strategies that employ tryptic digestion. Another motivation was to determine whether the use of elevated bath gas temperatures in the electrodynamic ion trap could affect the utility of ETD for doubly charged peptides (both tryptic and nontryptic).

Several techniques have been used to improve the performance of ECD as applied to whole protein ions. While ECD has been very effective for smaller proteins, cleaving, for example, 67 of 75 possible bonds in ubiquitin (8.6 kDa), as the size of the protein increases, the sequence coverage of ECD decreases, showing evidence for cleavage of only 33 of 152 bonds in myoglobin (17 kDa), and yielding no fragments in larger proteins.^{7,9,20} It has been posited that peptide backbone bonds are actually cleaved in the larger systems but that noncovalent interactions prevent fragments from separating. Methods to disrupt noncovalent interactions prior to electron capture have therefore proven to be effective in increasing the extent of observed fragmentation. In "activated ion" (AI) ECD, for example, cations and electrons are admitted to the ICR cell at the same time, following a gas pulse.²¹ This results in collisional

activation of the cations, which presumably disrupts noncovalent interactions and increases the sequence coverage obtainable. Using this approach, for example, evidence for cleavage of 99 out of 152 amine backbone bonds in myoglobin has been noted²¹ as has cleavage of proteins as massive as 42 kDa. "Plasma" ECD is a variation of the AI-ECD approach, differing largely in the timing of the events, with the electrons being admitted with the gas pulse, which is then followed by admission of the cations.²² Plasma ECD improved on the performance of AI-ECD with carbonic anhydrase (29 kDa), AI-ECD resulted in 116 of a possible 258 bonds being cleaved while plasma ECD resulted in 183 of the possible 258 bonds being cleaved. Both AI-ECD and plasma ECD occur as the cations are admitted to the ICR cell and, thus, in the absence of means for ion isolation external to the ICR cell,²³ do not allow for isolation of a specific charge state of the protein of interest inside the ICR cell. Thus, the fragmentation products are the integration of all those formed from all the charge states formed by ESI. A method that does allow for the isolation of a charge state of interest is to increase the temperature in the ICR cell during ECD experiments.^{24,25} This has been done with both ubiquitin and cytochrome *c* to study the gas-phase folding and unfolding of these two proteins. The results vary with the charge state chosen, with, for example, +6 and +7 ubiquitin showing a fairly sizable increase in sequence coverage while +8 and +9 parent ions show much smaller gains.^{24,25} This has been interpreted within the context of the three-dimensional protein structure. A protein that is more tightly folded will tend to have more noncovalent interactions and be more resistant to separation of covalent bond cleavage products than a less tightly folded structure. The initially more tightly folded ion can therefore be expected to show a larger increase in observed dissociation upon heating than a protein that is initially less tightly folded. Another approach to disrupting noncovalent interactions is to use an infrared laser to heat and unfold cations either prior to reaction with electrons or simultaneous with exposure to electrons.^{24–26} What all these methods have in common is some form of elevating the internal energy of the cations of interest to break noncovalent bonds, allowing for the observation of a wider range of backbone bond cleavages.

The aforementioned studies were directed largely to whole protein ions rather than to dications of the size range typical for tryptic peptides. It is not clear that intramolecular noncovalent interactions can be expected to play as important a role with tryptic peptide ions as they might with whole protein ions. However, it has been recently shown that fewer ECD channels are noted for peptide ions when the temperature of the ICR cell is reduced.²⁷ It was suggested that this was due to the lesser structural heterogeneity available to the peptide ions at reduced temperature, which resulted in less variety in the sites of charge solvation. This interpretation is consistent with evidence that suggests that it is the sites of charge solvation that determine where cleavage is observed in ECD, with structural heterogeneity determining how widely varied these sites are.^{27–31} In this work, we examine the ETD behavior of cations derived from 27 peptide species, 22 of which were derived from tryptic digestion, that range in size from 5 to 18 residues in a 3D quadrupole ion trap, both at room temperature and at an elevated bath gas temperature. Of particular interest is both the overall efficiency of ETD, in terms of parent ions converted to product ions, and the sequence coverage represented by the observed ETD fragments. The results have relevance both to understanding the overall ETD process and to a potentially important application of ETD in bottom-up proteomics.

EXPERIMENTAL SECTION

Methanol, acetonitrile, and glacial acetic acid were purchased from Mallinckrodt (Phillipsburg, NJ). Cytochrome *c*, ubiquitin, myoglobin, lectin from glycine max (soybean lectin), angiotensin I, bradykinin, RKRARKE, TPCK-treated trypsin, ammonium bicarbonate, and nitrobenzene were obtained from Sigma-Aldrich (St. Louis, MO). Trifluoroacetic acid was obtained from Pierce (Rockford, IL). GAILAGAILR and KGAILAGAILR were synthesized by SynPep (Dublin, CA).

Before digestion, the proteins were heat denatured in water at 95 °C for ~5 min. Immediately following denaturation, the proteins were chilled on an ice bath. Each protein (~0.5–0.75 mg) was dissolved to a volume of 0.5 mL of aqueous 200 mM ammonium bicarbonate. TPCK-treated trypsin (5 µL of a 1 mg/mL aqueous solution) was added to the protein solution to effect digestion. The solution was incubated at 38 °C for ~12 h. After digestion, peptides were fractionated by reversed-phase HPLC (Agilent 1090, Palo Alto, CA) using an Aquapore RP-300 (7-µm pore size, 100 × 4.6 mm i.d.) column (Perkin-Elmer, Wellesley, MA) operated at 1 mL/min using a previously described gradient.¹⁶ The fractions were lyophilized and then dissolved in 50/50/1 (v/v/v) methanol/water/acetic acid to ~10 µM for nano-ESI.^{32,33} Nontryptic peptides were also dissolved in 50/50/1 (v/v/v) methanol/water/acetic acid to ~10 µM for nano-ESI.

All experiments were performed on a Hitachi (San Jose, CA) M-8000 quadrupole ion trap mass spectrometer that has been modified for ion/ion reactions.³⁴ Nitrobenzene anions were generated using an atmospheric sampling glow discharge ionization³⁵ source and were injected into the trap through a hole in the ring electrode. Headspace vapors of nitrobenzene in air were admitted into the glow discharge source at a pressure of ~900 mTorr and a software TTL trigger connected to a fast high-voltage pulser (GRX-1.5K-E, Directed Energy Inc., Fort Collins, CO) was used to pulse the discharge. Peptide cations were formed using nanoelectrospray ionization. The samples were loaded into nanospray emitters pulled from borosilicate capillaries (1.5-mm o.d., 0.86-mm i.d.) using a P-87 Flaming/Brown micropipet puller (Sutter Instruments, Novato, CA). A stainless steel wire was inserted into the back of the capillary, and a potential of 1.5–2 kV was applied to the wire for ionization. For high-temperature experiments, the instrument's end cap heaters were controlled by a home-built external temperature controller. The heaters were run at a nominal temperature of 150 °C, resulting in a helium bath gas temperature of 160–165 °C, based on a calibration using the thermal dissociation rate of the +3 bradykinin ion as a thermometer reaction.³⁶

For a typical experiment, cations were injected for ~1 s. Isolation was accomplished using the Hitachi's filtered noise field (FNF)^{37,38} waveforms and by raising the amplitude of the radio frequency signal applied to the ring electrode of the ion trap to eject unwanted ions (~50 ms). Nitrobenzene anions were injected for ~100 ms into the ion trap during which time a FNF waveform was applied to eject anions other than nitrobenzene $[M - H]^-$ and M^- ions. Approximately 200 ms was allowed for ion/ion reactions to occur. Residual anions were ejected after the reaction time by raising the rf level of the trap, and cations were then analyzed by resonance ejection. Spectra shown here are averaged over ~5 min (~250 scans).

RESULTS AND DISCUSSION

The spectra for reaction of doubly charged bradykinin with nitrobenzene anions, at both room temperature and elevated temperature, are shown in Figure 1. Bradykinin provides an example where additional fragmentation can be seen under heated ion trap conditions. The nonheated ion/ion reaction (Figure 1A) produces fragmentation of the peptide backbone at one of five possible cleavage sites (N-terminal proline cleavage is not observed in either ECD or ETD because it requires breaking two bonds). The heated ETD data (Figure 1B) show cleavages at every possible N-C_α bond. Here the fragment ions seen are predominantly c ions, suggesting that the remaining proton in this reaction is likely located in the vicinity of the N-terminal arginine. Note that, in contrast with CID of doubly charged peptides, which often gives rise to the appearance of complementary ions, ETD can only give one fragment ion per cleavage. The appearance of two charged fragments from cleavage of a particular bond can be helpful in spectral interpretation, but not surprisingly, this is much less common in the ETD of doubly charged peptides than in CID because one of the charges is neutralized by electron transfer. In addition to c/z bradykinin sequence ions, neutral losses from the arginine side chain are

observed. The a_8^+ and y_7^+ ions are also observed from this reaction, which could arise from either thermal dissociation or ETD (observation of a/y ions has been noted in ECD⁷). Given that neither are observed as prominent products from the thermal dissociation of doubly charged bradykinin,³⁶ ETD is perhaps a more likely explanation. Table 1 shows a summary of the sequence coverage obtained for several model peptides for nonheated and heated ETD.

For ease of discussion, the tryptic peptides in this study have been organized into tables by the number of amino acids they contain and numbered as shown in Tables 2 (7 or fewer amino acids), 3 (8–11 amino acids), and 4 (more than 14 amino acids). For example, the peptide IFVQK in Table 2, from the tryptic digest of cytochrome *c*, is denoted as peptide 1. While heating appears to improve the sequence coverage for many of the peptides studied, there are instances when ETD of the peptide under nonheated conditions already produces cleavage at every possible N–C $_{\alpha}$ bond. Figure 2 shows the reaction of peptide 1 with nitrobenzene under (a) nonheated and (b) heated conditions. In this example, the heated reaction shows no additional sequence ions as compared to the nonheated reaction. While it has been previously reported that doubly charged peptides often produce poor sequence coverage from ETD, some of the results from tryptic peptides suggests that the amount of sequence coverage observed may be dependent on peptide size. In the case of peptide 1, the peptide has a relatively small size (5 amino acids) and shows fragmentation at every N–C $_{\alpha}$ bond. In addition to sequence coverage, there are several other noteworthy observations when comparing the nonheated versus heated data. The reaction of nitrobenzene anions with peptide cations has been shown to produce adducts of $m/z \approx 31$ on z ions.¹⁶ Figure 2A shows such adducts on several of the z ions. When the same reaction is performed under heated conditions, these adducts are noticeably absent. Currently the reasons for this are unclear, and the origin of these adducts is under investigation. Another important observation is the presence of thermal dissociation products in a heated environment, which may complicate spectral interpretation. In Figure 2B, several thermal dissociation products (y_3^+ , y_4^+ , b_2^+) can be observed. While these ions complicate the spectrum, it has been suggested that there are advantages for seeing both b/y ions and c/z ions in a spectrum. Horn et al. have suggested that for ECD, “golden sets” of ions (fragments from the same pair of amino acids between adjacent bonds) can be used to distinguish whether fragments contain the N- or C-terminus of a peptide. y and z' ions between the same pair of adjacent amino acids give a $\Delta m = 16.02$ Da while b and c ions give a $\Delta m = 17.03$ Da.³⁹ These spacings are dependent on the fact that ECD predominantly gives fragmentation of the N–C $_{\alpha}$ bond to give c/z' ions. However, in some cases, c'/z ions have been observed.²⁰ ETD may give similar fragmentation to ECD, but the frequency of c/z' versus c'/z ions has not yet been determined. The formation of c'/z ions would lead to spacings of $\Delta m = 15.02$ Da between y and z ions and $\Delta m = 16.02$ between b and c' ions. By examining the spacing between peaks, N- and C-terminal fragments can potentially be distinguished, although further investigation into the identities of the fragmentation products in ETD is needed to assess the general utility of this approach.

Table 2 shows a summary of the tryptic peptides composed of seven or fewer amino acids examined in this study. For all these peptides, with the exception of peptide 2, both heated and nonheated ETD showed 100% sequence coverage. Nonheated ETD of peptide 2 (data not shown) gave a c_3 ion that was not observed in the heated ETD data. In the nonheated experiment, the abundance of the c_3 ion is relatively low. Heating the ion trap gives rise to an increase in detector noise such that, given its low abundance in the nonheated data, it is possible that the c_3 ion is present in the heated experiment but cannot be observed above the noise. Regardless of the reason for the lack of a c_3 ion in the heated bath gas experiments, the results shown in Table 2 suggest that peptides composed of a relatively low number of amino acids produce extensive fragmentation, even without heating.

The tryptic peptides studied here are grouped into three tables according to size because their ETD behavior tends to correlate with size. The peptide ions within each table tend to show more similarity to one another than to the peptides in the other tables. For the peptides in Table 2, as mentioned above, the sequence coverage, defined here as percent of possible N-C α cleavages excluding cleavage N-terminal to proline, was near 100% for all peptides at both temperatures. For peptides composed of 8–11 amino acid residues (Table 3), the average sequence coverage from a nonheated ETD experiment was 46%, while peptides composed of 14 or more amino acids (Table 4) showed an average sequence coverage of 23% in nonheated ETD experiments. These results suggest that, for doubly charged tryptic peptides, there is a size dependence for the amount of sequence coverage produced by ETD. When heated, the sequence coverage from ETD for the peptides in Table 3 increased to an average of 67%, while heated ETD experiments improved the average sequence coverage for peptides in Table 4 to 63%. Although sequence coverage increased for both sets of peptides, 100% sequence coverage was not achieved.

The location of fragmentation from ETD is interesting to note for many of the peptides studied. In many cases, particularly those of the larger peptides in Table 3 and the peptides in Table 4, fragmentation occurs primarily at the ends of the peptide, particularly under nonheated conditions. This observation may well be related to the sites of protonation in the peptide prior to electron capture. For example, in peptide 13, the two protons are expected to be located primarily on the N-terminus and the C-terminal lysine residue. The electron-transfer reaction presumably occurs at these charged sites and causes fragmentation in their vicinity. When the peptide is heated, more cleavage sites are represented but the new fragmentation largely appears to extend from the ends of the peptide. This behavior might be rationalized on the basis of increased proton mobility within the reacting peptide population as the temperature increases. The increasing internal energy allows a wider range of protonation sites and structures to be accessed by the ions, resulting in a wider variety of sequence ions being observed. Nevertheless, the middle of the peptide remains the least likely location for charge due to electrostatic repulsion. This is consistent with the fact that the regions that show no clear evidence for cleavage in the tryptic peptides at either temperature tend to be in the middle of the peptide.

Comparison of the data derived from peptides 17 and 19 also highlights the possible importance of protonation site and the extent to which temperature affects where charge is distributed in the peptide. The two peptides have the same amino acid sequence with the exception that peptide 19 has an additional lysine residue on the N-terminus. Dongré et al. have shown that the placement of a lysine residue in a peptide sequence affects the surface-induced dissociation efficiency.⁴⁰ A peptide with a lysine residue at the N-terminal end shows a higher collision energy for the onset of fragmentation than a peptide with lysine at the C-terminal end, and as the onset collision energies correlate strongly with basicity, this suggests that an N-terminal lysine has a larger proton affinity than a C-terminal lysine. While there is no independent evidence that this is the case here, it is probably safe to assume that the proton is bound more strongly to the N-terminal region of peptide 19 than it is to peptide 17. At low temperature, ETD of peptide 17 occurs at two locations on the N-terminal end of the peptide and only one location on the C-terminal end. Peptide 19 shows one cleavage site at the N-terminal end of the peptide and one cleavage site at the C-terminal end. When heated, the ETD results in more extensive fragmentation for both peptides 17 and 19. However, peptide 17 shows several more z-type ions toward the middle of the peptide than peptide 19. Data collected at both temperatures are consistent with a proton being more tightly sequestered at the N-terminal end of peptide 19 than in peptide 17, with the difference being somewhat more apparent in the data collected at the higher temperature.

KGAILKGAILR, GAILAGAILR, and peptides 7, 10, 12, 16, and 18 all have an arginine residue, the most basic of all amino acid residues, at the C-terminus. Under nonheated ETD

conditions, they all exhibit similar fragmentation behavior. The peptides show fragmentation at the N-terminal end and little or no cleavage in the vicinity of the arginine residue (only peptide 7 shows fragmentation). Upon heated ETD, these peptides in many cases give more extensive fragmentation toward their N-terminal ends, but only one fragment on the C-terminal end (with the exception of KGAILKGAILR and peptide 7, which give more than one). This behavior is different from the peptides containing C-terminal lysine residues, where upon heating, a wider range of fragments was observed adjacent to the lysine residue. The arginine residue appears to limit the extent of fragmentation more than the lysine, which is further evidence for a role for temperature-dependent charge site location. That is, the arginine residue is likely to be more capable of keeping the proton sequestered than lysine as temperature increases.

Although size appears to be an important factor for the amount of sequence coverage observable from ETD when charge state is held constant, it is not the only determinant of a peptide's ability to cleave from ETD. In this study, a triply charged peptide composed of 18 amino acids has also been subjected to ETD (triply charged version of peptide 22). The nonheated ETD reaction of peptide 22 (Figure 3A) shows cleavage at 12 of 16 possible cleavage sites, and 12 of the possible 26 c and z ions were observed (the other 6 c and z ions were below the mass range analyzed). The heated ETD reaction (Figure 3b) shows cleavage of all possible N-C α bonds and 25 of the 26 possible c and z ions were observed, meaning that in addition to the increase in sequence coverage many more complementary fragment pairs were observed. The triply charged peptide shows more fragmentation than its doubly charged counterpart in both cases (Table 4). In most cases reported thus far,^{13,14,19} triply charged peptides have been shown to produce almost 100% sequence coverage from ETD. However, this peptide contains the largest number of amino acids for which ETD results have been reported to date, which suggests that the mass of the peptide influences the ETD sequence coverage for triply charged peptides as well. The heated ETD results for this peptide also suggest that the same underlying processes that can increase sequence coverage for doubly protonated peptides by elevating the bath gas temperature can also be in effect with larger and more highly charged ions.

While it is of interest to examine sequence coverage of peptides, which reflects the extent of available structural information, it is also of interest to examine the degree to which ion/ion reactions lead to fragmentation products. Electron transfer competes with proton transfer in all of the reactions discussed here, and it is important to examine how changing the nature of the peptides might affect this competition. One means to do this is with ETD efficiency calculations. The percent ETD efficiency is defined here as

$$\frac{\sum \text{c, z, neutral side chain losses}}{\sum \text{post I/I products}} \times 100 \quad (1)$$

This calculation provides an estimate of the percentage of dissociation reactions that occur by electron transfer relative to the total number of ion/ion reaction product ions. It does this by summing the abundances of all the known ETD product ions (c-type and z-type ions, as well as neutral side-chain losses) and comparing it to the sum of the abundances of all reaction products, including those that do not reflect fragmentation. Unfortunately, eq 1 is deficient in several respects for this purpose. A common fragment observed in ECD is loss of a hydrogen,²⁹ but this gives a product that is indistinguishable from the proton-transfer products, so the contribution from this potential ET fragmentation channel cannot be determined. Another difficulty is that it is possible for electron transfer to occur without dissociation, and unless this product is fairly abundant, it will be hidden under the isotopic envelope of the proton-transfer products. The presence of these nondissociative electron-transfer products has been demonstrated with CID experiments following the ion/ion reaction.¹⁹ Both of these difficulties lead to an underestimation of the extent of electron transfer by the percent ETD calculation,

and, to the extent that they vary from peptide to peptide, can make comparisons of the extent of electron transfer between peptides problematic. While these difficulties make the conclusions that can be drawn about the extent of electron transfer less certain, eq 1 remains the best measure currently in place for making this estimation and, in any case, is directly relevant to the analytical utility of ETD. Figure 4 shows how the percent ETD efficiency varies with peptide mass for both heated and nonheated experiments. The results are shown for 22 of the 27 peptides used in this study, the other 4 suffered from overall low signal-to-noise ratios, as well as overlaps of c/z ions with b/y ions that made accurate quantitation difficult in the heated experiments. For lower molecular weight peptides, like those in Table 2, the nonheated ETD efficiency is generally larger than 30%. As peptide molecular weight increases, both the heated and the nonheated ETD efficiency decreases, falling into the 15–25% range for the midsize peptides and down to 10% or lower for the larger peptides. So, there seems to be a consistent trend of decreasing ETD efficiency as peptide mass increases. However, there does not seem to be a consistent trend for the difference between heated and non-heated efficiencies. For the smaller peptides, it is generally true that nonheated ETD resulted in a better efficiency than heated ETD, but for the midsize and larger peptides, some show an increase in efficiency with heating, some show a decrease in efficiency with heating, and some stay about the same. For higher mass peptides, the relatively low ETD efficiency may not be a cause for concern because these peptides are more likely to form triply charged ions, which, in general, show more fragmentation than their doubly charged counterparts. Two of the peptides examined here, peptide 16 and peptide 22, were observed as triply charged ions as well as doubly charged ions. The efficiencies for the triply charged ions were roughly a factor of 4 larger than the efficiencies for the doubly charged ions, with peptide 16 improving from 15.4% with the doubly charged ion to 55.7% with the triply charged ion, and peptide 22 going from 2.9 to 13.2%. So it appears that, like sequence coverage, the efficiency depends on both the size and charge of the peptide.

The fact that there does not appear to be a consistent trend in percent ETD with increasing temperature for the larger peptides, despite the fact that there is generally an increase in sequence coverage with increasing temperature, is noteworthy. The use of elevated bath gas temperatures apparently does not increase the number of peptide ions that undergo ETD, at least not on a consistent basis. Rather, elevated bath gas temperature affects the fragmentation channels open to the ions that do undergo ETD. This observation is inconsistent with an interpretation that elevated bath gas temperature significantly increases the number of peptides that fragment and that the new fragments observed arise from those species that did not undergo ETD at room temperature. The observed behavior is consistent, however, with the notion that an increase in bath gas temperature can increase the distribution of charge within the peptide ions such that the ETD fragmentation is more broadly distributed than at room temperature.

CONCLUSIONS

From the peptides studied here, most of which were tryptic peptides, it is evident that the sequence coverage obtained via ETD of doubly protonated species tends to be inversely related to peptide size. The percent ETD also decreases with peptide size. Previous results,¹⁹ combined with those observed here for +3 peptide 22, suggest that there is likely to be an optimum size range for triply charged peptides as well, although it has not been precisely defined yet. It seems likely that for any given charge state this observation will be true. Elevation of the bath gas temperature tends to yield an increase in sequence coverage, particularly in cases in which sequence coverage is low at room temperature. This allows for extension of the range of peptide size and charge that gives relatively high sequence coverage, but sequence coverages approaching 100%, as noted for small peptides, was not achieved. Nevertheless, from a practical standpoint, the use of elevated bath gas temperature in trypsin-based bottom-up proteomics has merit as 100% sequence coverage is by no means a necessity for peptide

identification. The use of elevated bath gas temperatures does not, however, appear to improve percent ETD, even for those species that show relatively low percent ETD at room temperature. The increase in sequence coverage without a concomitant increase in percent ETD warrants further study. However, this result, along with the tendency for cleavages to be localized at the ends of the tryptic peptides, is consistent with a major role for site of protonation within the doubly charged peptide in influencing which channels contribute in ETD. The results reported here are rationalized on the basis of elevated bath gas temperature correlating with a wider charge distribution in the peptide ion population that undergoes electron transfer.

Acknowledgements

The authors thank Jason Hogan for generating the soybean lectin digest samples and for the construction of a computer program to generate sequence ion masses. They also thank Jim Zimmerman and Ethan Badman for their assistance in the construction of the temperature controller. This research was sponsored by the National Institutes of Health, under Grant GM 45372.

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Figure 1.

Reaction spectra of bradykinin $[M + 2H]^{2+}$ with nitrobenzene anions under (A) nonheated and (B) heated conditions. Residual doubly charged peptide ions were ejected from the trap prior to mass analysis in (A) to avoid contributions to the spectra from CID products formed from the ejection of the doubly charged peptide during mass analysis.¹⁹

Figure 2.

Reaction spectra of peptide 1 $[M + 2H]^{2+}$ with nitrobenzene anions under (A) nonheated and (B) heated conditions.

Figure 3.

Reaction spectra of peptide 22 $[M + 3H]^{3+}$ with nitrobenzene anions under (A) nonheated and (B) heated conditions. For these spectra, residual doubly charged peptide ions were ejected from the trap prior to mass analysis. c/z fragment ions with m/z values lower than the $[M + 2H]^{2+}$ that were observed in spectra when the doubly charged peptide ions were not ejected are indicated in the peptide sequence with a *.

Figure 4.
Percent ETD efficiency vs peptide mass for nonheated and heated experiments.

Table 1

ETD Fragmentation Summary of Model Peptides

Peptide Name	Non-Heated ETD Fragmentation	Heated ETD Fragmentation
bradykinin	RPPGFSPFR	RPPGFSPFR
GAILAGAILR	GAILAGAILR	GAILAGAILR
KGAILKGAILR	KGAILKGAILR	KGAILKGAILR
angiotensin	DRVYIHPFHL	DRVYIHPFHL
RKRARKE	RKRARKE	RKRARKE

Table 2
ETD Fragmentation Summary of Doubly Charged Tryptic Peptides with Seven or Fewer Amino Acids

Peptide Number	Non-Heated ETD Fragmentation	Heated ETD Fragmentation
1 ^a	IFVQK	IFVQK
2 ^a	YIPIGIIK	YIPIGIIK
3 ^b	LIIFFACK	LIIFFACK
4 ^b	MOIIEVVK	MOIIEVVK
5 ^a	MIIFAGIIK	MIIFAGIIK
6 ^{a,c}	KYIPIGIIK	KYIPIGIIK

^aFrom cytochrome *c* tryptic digest.
^bFrom ubiquitin tryptic digest.
^cContains a missed cleavage.

Table 3
ETD Fragmentation Summary of Doubly Charged Tryptic Peptides with 8–11 Amino Acids

Peptide Number	Non-Heated ETD Fragmentation	Heated ETD Fragmentation
7 ^a	TSLPEWVR	TSLPEWVR
8 ^b	EDLIAYLK	EDLIAYLK
9 ^{b,d}	TGPNLHGLF	TGPNLHGLF
10 ^c	EGIPPDQQR	EGIPPDQQR
11 ^c	TLSDYNIQK	TLSDYNIQK
12 ^c	ESTLHLVLR	ESTLHLVLR
13 ^a	TTISWDLANNK	TTISWDLANNK
14 ^a	AETVSFSWNK	AETVSFSWNK
15 ^a	TSNILSDVDLK	TSNILSDVDLK
16 ^b	TGPNLHGLFGR	TGPNLHGLFGR

^aFrom soybean lectin tryptic digest.

^bFrom cytochrome

^cTryptic digest.

^cFrom ubiquitin tryptic digest.

^dChymotryptic peptide.

Table 4
ETD Fragmentation Summary of Doubly Charged Tryptic Peptides with 14 or More Amino Acids

Peptide Number	Non-Heated ETD Fragmentation	Heated ETD Fragmentation
17 ^a	TGQAPGFSYTDANK	TGQAPGFSYTDANK
18 ^b	VEADIAGHGQEVLR	VEADIAGHGQEVLR
19 ^{a,c}	KTGQAPGFSYTDANK	KTGQAPGFSYTDANK
20 ^d	TITLEVEPSDTIENVK	TITLEVEPSDTIENVK
21 ^a	GIITWGEETLMEYLENPK	GIITWGEETLMEYLENPK
22 ^{a,c}	GIITWGEETLMEYLENPKK	GIITWGEETLMEYLENPKK

^aFrom cytochrome *c* tryptic digest.

^bFrom myoglobin tryptic digest.

^cContains a missed cleavage.

^dFrom ubiquitin tryptic digest.