Improved Sequencing of Oxidized Cysteine and Methionine Containing Peptides Using Electron Transfer Dissociation

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Oxidative modifications to the side chains of sulfur-containing amino acids often limit the number of product ions formed during collision-induced dissociation (CID) and thus make it difficult to obtain sequence information for oxidized peptides. In this work, we demonstrate that electron-transfer dissociation (ETD) can be used to improve the sequence information obtained from peptides with oxidized cysteine and methionine residues. In contrast to CID, ETD is found to be much less sensitive to the side-chain chemistry, enabling extensive sequence information to be obtained in cases where CID fails to provide this information. These results indicate that ETD is a valuable technique for studying oxidatively modified peptides and proteins. In addition, we report a unique and very abundant product ion that is formed in the CID spectra of peptides having N-terminal cysteine sulfinic acid residues. The mechanism for this unique dissociation pathway involves a six-membered cyclic intermediate and leads to the facile loss of NH₃ and SO₂, which corresponds to a mass loss of 81 Da. While the facile nature of this dissociation pathway limits the sequence information present in CID spectra of peptides with N-terminal cysteine sulfinic acid residues, extensive sequence information for these peptides can be obtained with ETD. (J Am Soc Mass Spectrom 2007, 18, 1499–1506) © 2007 American Society for Mass Spectrometry

Mass spectrometry (MS) is widely used for sequencing and identifying amino acid modifications in peptides and proteins. Identifying modifications to proteins is important for a variety of reasons. Post-translational modifications (PTMs) of proteins are necessary for a wide range of cellular functions such as protein trafficking, protein-protein interactions, and transcription. Identifying and pinpointing these modification sites are important for more deeply understanding protein function, both normal and abnormal. In this context, PTMs such as phosphorylation, acetylation, glycosylation, sulfonation, and methylation are important to characterize. Oxidation is another important protein modification that is typically associated with oxidative stress [1–4], but recent work has also shown that protein oxidation can play a regulatory role as well [5]. Furthermore, an increasing number of techniques make use of oxidative labeling to study protein structure. These methods use radicals (e.g., [•]OH) to modify solvent-exposed [6–9] or metal-bound

amino acids [10–17], and MSⁿ to identify oxidatively modified residues, typically in conjunction with proteolytic digestion.

Very often side-chain modifications to peptides can make sequencing by collision-induced dissociation (CID) difficult. Perhaps the most well known example is the effect of phosphorylation on peptide ion dissociation. The CID spectra of phosphorylated peptides are commonly dominated by a neutral loss of H₃PO₄, often with little other sequence information present. Similarly, side-chain oxidation can dramatically affect peptide dissociation patterns and limit sequence information that is available by CID. For example, oxidation of cysteine to cysteine sulfinic acid leads to selective dissociation on the C-terminal side of this residue when the peptide charge state does not exceed the number of arginine residues in the peptide [18–23]. Also, methionine oxidation to methionine sulfoxide can cause peptide CID spectra to be dominated by a neutral loss of methane sulfinic acid (CH₃SOH), which can limit sequence information [24–28]. Very recently, histidine oxidation was also found to notably affect the dissociation pattern of peptides ions [29], complicating attempts to correctly sequence these peptides.

To overcome the limited sequence information that is often found in the CID spectra of modified peptides,

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alternate dissociation techniques have been used. Electron capture dissociation (ECD), implemented on a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer, has been very successfully applied to sequence peptides containing labile PTMs [30–35]. Very recently, electron-transfer dissociation (ETD) on a linear ion trap was demonstrated by Hunt and coworkers [36] and subsequently by others [37, 38]. This technique is similar to ECD but uses radical anions instead of free electrons to dissociate protonated peptides. Like ECD, ETD results in c- and z-type ions that often provide abundant sequence information [36–43]. Studies indicate that ETD is particularly well suited for the characterization of peptides containing PTMs [43], but the utility of this approach for oxidized peptides has not been carefully studied.

The successful analysis of oxidized proteins, however, is very important, both broadly and in specific contexts. Some estimates indicate that about 10% of all cellular protein material in humans is oxidatively damaged, and this amount can increase 2 to 5-fold during aging [44, 45]. Protein oxidation is also implicated in the etiology of a variety of diseases. Notable among these are neurodegenerative diseases such as Alzheimer's, which is marked by significant protein oxidation in the neuritic plaques that are the pathological hallmarks of these diseases [46, 47]. A variety of other diseases, including rheumatoid arthritis, muscular dystrophy, and cataractogenesis are also associated with elevated levels of oxidatively-damaged proteins [45]. In this work, we report the use of ETD for sequencing oxidized cysteine- and methionine-containing peptides. Cysteine and methionine are the most readily oxidizable of the naturally occurring amino acids. Given the prevalence of protein oxidation in a variety of important contexts, analysis of peptides containing these oxidized residues is expected to be somewhat commonplace. We demonstrate that ETD provides much more reliable and abundant sequence information than CID for peptides with these oxidized residues because it overcomes the facile neutral losses typically observed in their CID spectra.

Experimental

Materials

Hydrogen peroxide (30%), formic acid, tris(hydroxymethyl)-aminomethane (Tris), and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) were obtained from EM Science (Gladstone, NJ). Dithiothreitol (DTT), sodium ascorbate, ascorbic acid, copper(II) sulfate, and ammonium acetate were purchased from the Sigma-Aldrich Corporation (St. Louis, MO). Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Endoproteinase Asp-N was obtained from Roche Diagnostics (Indianapolis, IN), and trypsin was obtained from Promega (Madison, WI). All reagents were used as provided. Distilled, deionized

water was generated with a Millipore (Burlington, MA) Simplicity 185 water purification system.

The peptides CLRRASLG, SLRRSSCFGGR, CDN-QIKKM, CDPGYIGSR, CTFPGHSALMK, HCKFWW, CQDSETRTFY, RRWQWRMKKLG, YGGFMKR, and KKSRRGDYMTMQIG were obtained from Sigma-Aldrich. The peptides TMVVHEKPDIDLGR and TMVVHEKPD-DLGRGGNEESTK are proteolytic fragments of the protein Cu/Zn superoxide dismutase (SOD) from bovine erythrocytes, which was obtained from Sigma-Aldrich. To digest Cu/Zn SOD, a 115 μ L solution of the protein buffered at pH 7.4 with 12 mM ammonium acetate was mixed with 250 μ M DTT, and 25% (vol/vol) acetonitrile, and the mixture was incubated at 37 °C for 30 min. After this 30 min incubation, 4 μ g of trypsin and 0.4 μ g of endoproteinase Asp-N were each dissolved in 100 μ L of ammonium acetate (12 mM, pH 8.0) and added to the dissolved SOD mixture. This final mixture was incubated at 37 °C for 18 h. The reaction was terminated by changing the pH of the solution to $\sim$ 2 by the addition of acetic acid.

Peptide Oxidation

All the peptides and proteins were oxidized using metal-catalyzed oxidation (MCO) reactions as described previously [13, 14, 17]. MCO reactions with all peptides were performed at room-temperature in aqueous solutions containing 250 μ M peptide, 250 μ M CuSO₄, 10 mM ascorbate, 2 mM H₂O₂, and 50 mM Tris-HCl/Tris, buffered to a pH of 7.4. For Cu/Zn SOD, MCO reactions were performed at 37 °C in solutions containing 40 μ M Cu/Zn SOD, 100 mM ascorbate, 2 mM H₂O₂, and 100 mM Tris/Tris-HCl buffered to pH 7.4. In all cases, the reactions were initiated by the addition of ascorbate, H₂O₂, or both. Reactions were stopped after 15 or 30 min by the addition of 1% (vol/vol) glacial acetic acid.

Instrumentation

All mass spectral measurements were carried out on a Bruker (Billerica, MA) HCTultra PTM Discovery quadrupole ion trap mass spectrometer equipped with an Agilent HPLC Chip Cube, G4240A (Santa Clara, CA). This mass spectrometer uses either CID or ETD to generate tandem mass spectra. For the CID experiments described in this work, an activation time of 40 ms was used. The amplitude of the resonance excitation voltage during the CID experiments was 1.0 V, and the SmartFrag procedure was used to ensure efficient ion dissociation. The SmartFrag procedure varies the resonance excitation amplitude from 30% to 200% of its value over the course of the activation period. ETD was performed with fluoranthene radical anions that were generated via negative chemical ionization with methane reagent gas. Samples were infused at a flow rate of 0.3 μ L/min via the Agilent G4240-61,001 MS calibration and diagnostic chip and a Cole Palmer syringe pump (Vernon Hills, IL). When performing ETD, low *m/z* cutoff

(LMCO) values ranging from 30 to 200 were used with reaction times ranging from 50 ms to 180 ms. The LMCO values and reaction times were chosen to give the most efficient precursor ion dissociation. The accumulation time for the fluoranthene radical anion was 3 ms. In some cases, collisional activation was used to aid the ETD of doubly-charged ions. In the context of the ETD experiment, this collisional activation process uses a low-amplitude (~ 0.3 V) resonance excitation voltage to resonantly excite any singly-charged radical cations that do not dissociate upon ETD of the doubly-charged precursor ion. This method, which is standard on the Bruker HCTultra PTM Discovery system, is similar to an approach described very recently [42].

Results and Discussion

Peptides with Cysteine Sulfinic Acid Residues

Oxidation of cysteine side chains can dramatically change the CID spectra of peptides containing this amino acid. Very often the CID spectra of such peptides contain less sequence information. This effect has been observed previously [18–23], and an example of this can be seen upon comparing the CID spectra of the doubly-charged ions of CLRRASLG and C*LRRASLG, where C* is cysteine sulfinic acid (Figure 1). The CID spectrum (Figure 1a) of unoxidized CLRRASLG, $[M + 2H]^{2+}$, contains numerous product ions such that the sequence of this ion can be determined. In contrast, the CID spectrum (Figure 1b) of the doubly-oxidized peptide $[M + 2O + 2H]^{2+}$ shows only two abundant product ions, one at m/z 414.3 and another at m/z 386.2. With only two product ions, not enough information is present to allow this peptide to be sequenced. The product ion at m/z 414.3 arises from a unique and previously unreported dissociation pathway that results in the loss of 81 Da. The proposed mechanism for this unique dissociation pathway will be discussed below. The product ion at m/z 386.2 is the y_7^{2+} product ion, and this product ion was expected based on previous work. Wang and coworkers demonstrated that the oxidation of cysteine to cysteine sulfinic acid results in facile cleavage of the peptide bond on the C-terminal side of the cysteine sulfinic acid residues upon CID [22]. This dissociation pathway arises due to the acidity of cysteine sulfinic acid, which allows it to donate a proton to facilitate the cleavage of nearby peptide bonds when the number of basic residues is greater than or equal to the number of protons on the ion. The proposed mechanism for this cleavage is similar to the mechanism proposed for the cleavage of the amide bond at the C-terminal side of aspartic acid residues [48]. This facile cleavage at the C-terminal side of the cysteine sulfinic acid typically leads to limited sequence information in CID spectra of cysteine sulfinic acid containing peptides.

To get more sequence information, an ETD spectrum of the doubly oxidized C*LRRASLG peptide ion

Figure 1. (a) CID of unoxidized CLRRASLG $[M + 2H]^{2+}$, (b) CID of doubly oxidized C*LRRASLG $[M + 2O + 2H]^{2+}$, and (c) ETD of doubly oxidized C*LRRASLG $[M + 2O + 2H]^{2+}$. The peaks labeled with an asterisk (*) in (c) are CID product ions.

Figure 2. (a) CID of doubly oxidized SLRRSSC*FGGR $[M + 2O + 2H]^{2+}$ and (b) ETD of doubly oxidized SLRRSSC*FGGR $[M + 2O + 2H]^{2+}$.

$[M + 2O + 2H]^{2+}$ was obtained (Figure 1c). The presence of unoxidized z_5 , z_6 , and z_7 product ions, and oxidized c_4 , c_5 , c_6 , and c_7 product ions allows the sequence to be determined and the site of oxidation to be identified. Clearly, oxidized cysteine does not limit the amount of sequence information obtainable from the ETD spectrum of this peptide like it does the CID spectrum.

Another example in which ETD is able to provide more sequence information than CID for a peptide containing cysteine sulfinic acid involves the peptide SLRRSSC*FGGR. The CID spectrum of the $[M + 2O + 2H]^{2+}$ ion of this peptide is dominated by a loss of H_2SO_2 (Figure 2a). The loss of H_2SO_2 from cysteine sulfinic acid containing peptides has been demonstrated by several groups to be a major dissociation pathway for peptides containing cysteine sulfinic acid residues [22, 49]. The spectrum also shows modestly abundant y_4 and $[b_7 + 2O - H_2SO_2]^+$ product ions, which arise from the enhanced cleavages that typically occur at peptide bonds adjacent to cysteine sulfinic acid.

Here again the CID spectrum provides insufficient sequence information. The ETD spectrum (Figure 2b) of the same precursor ion, on the other hand, is much more informative. The spectrum shows unoxidized c_3 – c_6 ions, oxidized c_7 – c_{10} ions, unoxidized z_2 – z_4 ions, and oxidized z_8 and z_{10} ions, which provide almost complete sequence coverage and clearly indicate that Cys7 is oxidized. The greater sequence information present in the ETD spectra of this peptide and other peptides with cysteine sulfinic acid residues arises because of the nature of the dissociation process in ETD. While the details of the dissociation mechanism in ETD have not been fully characterized, ETD does not appear to rely on the mobilization of protons to dissociate peptides as is the case in CID. ETD has a unique propensity to randomly cleave N– C_α bonds of the peptide backbone with relatively little regard to peptide length, amino acid composition and PTMs [36]. So, just as ETD provides sufficient sequence information for phosphorylated peptides in cases where CID fails, so does it provide sufficient sequence information for cysteine sulfinic acid containing peptides. The limited sequence information that results from CID of peptides with cysteine sulfinic acid is fairly common when the number of charges on a peptide ion is equal to or less than the number of basic residues in the peptide; however, the increased sequence coverage possible for these peptides when ETD is used is also likely to be quite common. We have obtained results similar to those shown in Figures 1 and 2 for several other peptides containing cysteine sulfinic acid. These peptides include CDNQIKKM, CDPGYIGSR, CTFPGHSALMK, and HCKFWW.

Unique Dissociation Pattern of Peptides Having Cysteine Sulfinic Acid on the N-Terminus

As noted above, the loss of 81 Da from peptides with cysteine sulfinic acid on the N-terminus becomes a dominant peak in the CID spectra of peptides in which the number of basic residues equals or exceeds the peptide charge state (e.g., Figure 1b). This dissociation pattern usually occurs instead of the loss of H_2SO_2 (66 Da), which is usually diagnostic for cysteine sulfinic acid containing peptides. This new dissociation pathway has not been reported previously for any other cysteine sulfinic acid containing peptides. To investigate the mechanism of this unique dissociation pathway, we have studied several peptides containing cysteine sulfinic acid on the N-terminus. These peptides include CLRRASLG, CTFPGHSALMK, CQDSETRTFY, CDPGYIGSR, and CDNQIKKM. The CID spectra of these peptides show a predominant product ion that corresponds to a loss of 81 Da. Several pieces of experimental data provide insight into the likely mechanism for this neutral loss. These data include: (1) the mass of this neutral (i.e., 81 Da) is consistent with a loss of NH_3 and SO_2 ; (2) the loss of 81 Da is only seen for

Figure 3. CID spectrum of the doubly oxidized N-acetylated peptide C*LRRASLG [$M + 2H - H_2SO_2$] $^{2+}$.

peptides that have cysteine sulfinic acid on the N-terminus; peptides with internal or C-terminal cysteine sulfinic acid residues do not form this product ion (e.g., Figure 2a). (3) the b ions formed by MS 3 of the ion corresponding to the loss of 81 Da produce $b_n - 81$ ions, indicating this loss occurs from the N-terminus; (4) tandem mass spectra of deuterated peptides show a loss of 84 Da instead of 81 Da, which is consistent with a loss of ND_3 and SO_2 ; (5) when the N-terminus of these peptides is acetylated, the dissociation pathway is shut down (e.g., Figure 3), and the loss of 81 Da is replaced by a neutral loss of 66 Da (i.e., H_2SO_2). These experimental data allow us to propose a mechanism for this reaction, which is shown in Scheme 1. The intramolecular interaction between sulfinic acid and the N-terminal amine group generates a six-membered cyclic intermediate, which facilitates the neutral losses of NH_3 and SO_2 . This mechanism is analogous to the mechanism invoked to explain the preferential cleavage of peptide bonds adjacent to cysteine sulfinic acid residues [22]. The mechanism is also consistent with all of our observed experimental data.

Peptides with Oxidized Methionine Residues

Like oxidized cysteine residues, oxidized methionine residues can also dramatically limit the amount of sequence information obtained from CID of peptides containing this residue. The CID spectrum of oxidized RRWQWRM*KKLG, [$M + O + 3H$] $^{3+}$, where M* is

Scheme 1. Proposed dissociation mechanism for the loss of 81 Da from doubly oxidized C*LRRASLG.

Figure 4. (a) CID of oxidized RRWQWRM*KKLG [$M + O + 3H$] $^{3+}$ and (b) ETD of oxidized RRWQWRM*KKLG [$M + O + 3H$] $^{3+}$.

methionine sulfoxide, illustrates this effect (Figure 4a). The dominant peak in this spectrum is at m/z 499.6, which corresponds to the loss of methane sulfinic acid (CH_3SOH). Jiang et al. have demonstrated that this is a diagnostic product ion from the side-chain of methionine sulfoxide [24]. The spectrum also shows several product ions of low abundance, including [$y_{10} + O$] $^{3+}$, $y_5 + O$, and $y_6 + O$. The facile loss of CH_3SOH limits other dissociation pathways that might provide more sequence information. In contrast, the ETD spectrum of RRWQWRM*KKLG, [$M + O + 3H$] $^{3+}$ contains unoxidized $c_2 - c_6$ ions, oxidized $c_7 - c_9$ ions, and oxidized $z_6 - z_9$ ions, which provide almost complete sequence coverage and clearly indicate that Met7 is oxidized (Figure 4b).

Another example in which ETD facilitates sequencing of a peptide containing methionine sulfoxide involves the peptide KKS RGDY M* T M* Q I G (Figure 5). The CID spectrum of this peptide actually contains

Figure 5. (a) CID of oxidized KKS RGDY M* T M* Q I G $[M + O + 2H]^{2+}$ and (b) ETD of oxidized KKS RGDY M* T M* Q I G $[M + O + 2H]^{2+}$.

several sequence ions despite the dominant loss of CH_3SOH , but the spectrum does not give definitive data about the oxidation status of Met8 and Met10 (Figure 5a). Indeed, the CID data seems to indicate that Met10, but not Met8, is oxidized despite the fact LC data (not shown) indicate that the dissociated peptide ion is actually a mixture of two isomers differing only by which Met residue is oxidized. Once again, the ETD spectrum gives much clearer sequence information, especially with regard to the oxidation status of Met8 and Met10 (Figure 5b). Indeed, the relative product ion ratios for c_8/c_8+O and c_9/c_9+O indicate that the Met8 isomer of this peptide comprises about 75% of the total precursor ion abundance. Thus, the ETD data provide an accurate indication of the oxidation status of both Met residues, whereas the CID data were ambiguous in this regard.

The facile loss of methane sulfenic acid, which occurs when the number of charges on a peptide ion is equal to or less than the number of basic residues in the peptide, often leads to limited sequence information in CID spectra of methionine sulfoxide-containing peptides. Because of the nature of the ETD mechanism, this

neutral loss is nonexistent in the ETD spectra of these peptides, and so sequence information can be more readily obtained for these peptides. We have obtained results similar to those shown in Figures 4 and 5 for several other peptides containing methionine sulfoxide, including YGGFMKR, TMVVHEKPDDLGRGGNEESTK, and TMVVHEKPDDLGR.

ETD Is Relatively Insensitive to Side-Chain Chemistry

Based on observations described above, ETD provides more sequence information than CID for oxidized cysteine and methionine-containing peptides because ETD seems to be less sensitive to the side-chain chemistry of these oxidized residues. We decided to further investigate this idea by cataloging the relative ion abundances for c and z ions in the ETD spectra of all the unoxidized and oxidized peptides that we have studied. In each case, the spectra were normalized to the most abundant

Figure 6. (a) ETD of unoxidized YGGFMKR $[M + 2H]^{2+}$ and (b) ETD of oxidized YGGFM*KR $[M + O + 2H]^{2+}$.

product ion in the spectrum. Not surprisingly, the relative abundances of most sequence ions are similar in the ETD spectra of unoxidized and oxidized peptides. As an example, the ETD spectra of the unoxidized and oxidized forms of YGGFMKR are shown in Figure 6. The similarities between the relative ion abundances of the observed product ions are clear. The only major differences between the two spectra are that many of the product ions in YGGFMKR are oxidized because Met5 is oxidized. When the ETD spectra of this peptide and other cysteine- and methionine-containing peptides and their oxidized counterparts are compared, the percentage of product ions that have similar relative abundances is 65%. This percentage was obtained by dividing the total number of product ions with similar relative abundances (i.e., within 10%) by the total number of product ions observed in the spectra; this was done for all the unoxidized and oxidized peptides. As an example of this evaluation, Figure 6 shows that the z_3 , z_4 , z_5 , z_6 , and c_6 product ions have relative abundances that are very similar (i.e., within 10% relative abundance) in both spectra, whereas the relative abundances of the z_2 product ion are 14 and 27%, respectively, in the spectra of the unoxidized and oxidized peptides. By this analysis, the z_3 , z_4 , z_5 , z_6 , and c_6 product ions are established as similar in both spectra, but the z_2 product ion is considered dissimilar. For comparison, if the same analysis is done for the CID spectra of all the unoxidized and oxidized peptides, the percentage of product ions that have similar relative abundances is 3%. The higher percentage of similarity in the case of the ETD spectra indicates that this technique is significantly less sensitive to the side-chain chemistry of oxidized cysteine and methionine residues than CID. So, ETD can provide more reliable sequence information and more straightforward spectral interpretations than CID for peptides containing oxidized cysteine and methionine residues.

Conclusions

The results reported here clearly show that ETD can provide more sequence information than CID for peptides with oxidized cysteine and methionine residues, particularly when the peptide's charge state is equal to or less than the number of basic residues. Peptide dissociations after ETD are much less sensitive to side-chain modifications on cysteine and methionine residues. We also report for the first time a unique dissociation pathway for peptides having N-terminal cysteine sulfinic acid residues. The mechanism for this new dissociation pathway involves a six-membered cyclic intermediate that involves the side-chain of cysteine sulfinic acid and the N-terminal amine and leads to the facile loss of NH_3 and SO_2 , which corresponds to a mass loss of 81 Da.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (RO1 GM075092).

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