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Improved Identification and Relative Quantification of Sites of Peptide and Protein Oxidation for Hydroxyl Radical Footprinting

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Abstract

Protein oxidation is typically associated with oxidative stress and aging and affects protein function in normal and pathological processes. Additionally, deliberate oxidative labeling is used to probe protein structure and protein-ligand interactions in hydroxyl radical protein footprinting (HRPF). Oxidation often occurs at multiple sites, leading to mixtures of oxidation isomers that differ only by the site of modification. We utilized sets of synthetic, isomeric “oxidized” peptides to test and compare the ability of electron-transfer dissociation (ETD) and collision-induced dissociation (CID), as well as nano-ultra high performance liquid chromatography (nanoUPLC) separation, to quantitate oxidation isomers with one oxidation at multiple adjacent sites in mixtures of peptides. Tandem mass spectrometry by ETD generates fragment ion ratios that accurately report on relative oxidative modification extent on specific sites, regardless of the charge state of the precursor ion. Conversely, CID was found to generate quantitative MS/MS product ions only at the higher precursor charge state. Oxidized isomers having multiple sites of oxidation in each of two peptide sequences in HRPF product of protein Robo-1 Ig1-2, a protein involved in nervous system axon guidance, were also identified and the oxidation extent at each residue was quantified by ETD without prior liquid chromatography (LC) separation. ETD has proven to be a reliable technique for simultaneous identification and relative quantification of a variety of functionally different oxidation isomers, and is a valuable tool for the study of oxidative stress, as well as for improving spatial resolution for HRPF studies.

Introduction

Oxidative modification of proteins occurs *in vivo* during aging and in certain disease conditions. It can also be induced experimentally by a wide array of oxidizing agents [1, 2]. *In vivo* oxidative modification to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity. Quantitatively profiling changes in modification levels and characterizing the exact amino acid sites of modification have become essential parts of trying to elucidate the mechanisms of a variety of important biological process [3-5]. Apart from this biological role, oxidation of proteins and peptides often occurs during sample processing and preparation. It is important to monitor the degree of oxidative modification of therapeutic proteins manufactured for commercial use. Oxidative protein modification occurring during both manufacture and storage of the protein can affect drug efficacy and potential side effects [6-8]. The most commonly used marker of protein oxidative modification in manufactured proteins is methionine sulfoxide [1, 9]. In some cases, the effect of oxidative modification on bioactivity of the protein is site-dependent, as is seen following oxidation of *Escherichia*

coli-derived stem-cell factor [9]. It would be helpful to have an accurate and reliable analytical method to determine oxidation in proteins in a residue-specific manner in order to assess their biological significance.

Furthermore, oxidative labeling of proteins and peptides is used to probe protein structure and protein-ligand interactions in hydroxyl radical protein footprinting (HRPF) coupled with mass spectrometry (MS) analysis [10, 11], in which the protein of interest is exposed to diffusing hydroxyl radicals and the amounts of oxidation of solvent-exposed residues are monitored by tandem mass spectrometry. HRPF takes advantage of the fact that the rate of oxidation of various amino acid side chains varies directly with their accessibility to solvent [12, 13], allowing the researcher to monitor conformational changes and ligand binding surfaces. The level of accuracy and structural detail generated from the HRPF data relies on improved methods for detecting and quantifying at the residue level adjacent sites of oxidation in the protein sequence. MS is a powerful tool for characterization of oxidative protein modification because of its sensitivity, speed, and specificity of detection. It is routine to differentiate oxidized peptides from their unoxidized counterparts either on the basis of their mass difference and/or liquid chromatographic separation, then the liquid chromatography-mass spectrometry (LC-MS) peak intensity or selected reaction monitoring (SRM)-based methods will be utilized for relative quantification [12, 14-19].

The likelihood of multiple significant oxidation sites existing on a single peptide increases significantly with the development of sub-microsecond HRPF techniques. Earlier methods of HRPF including water radiolysis by gamma (γ) rays [20] and X-rays [13, 21, 22], worked on the millisecond-to-minutes timescales, and required careful control of the extent of oxidation to ensure that the protein maintained the native structure during the HRPF process [23, 24]. In 2005, Hambly and Gross reported Fast Photochemical Oxidation of Proteins (FPOP), an HRPF method that allows extensive labeling of proteins (and therefore more sites of oxidation) during a short time scale of oxidation (sub-microsecond) while retaining their native structure [15-19, 25, 26]. In 2009, Watson *et al* developed a novel method for hydroxyl radical protein footprinting using a Van de Graaf electron accelerator-based method that achieved HRPF by water radiolysis on a similar sub-microsecond timescale [27]. The FPOP method enjoys the advantage of using a much more readily available radiation source and offering more flexibility in the photoactivated chemistry, while the electron accelerator has the advantage of not requiring the addition of any exogenous radical precursors, forming the hydroxyl radicals directly from water. Both methods complete labeling of the protein in a time scale that is faster than the protein can unfold. Therefore, unlike the previous slower methods, these methods are unrestrained by the need to dose-control the radical exposure to maintain native structure. Dose-control of the radical exposure for these sub-microsecond methods is required only to maintain the covalent integrity of the protein in order to yield identifiable fragments, which allows for much higher radical dosages. At these higher radical doses, it is common to label amino acids that are insufficiently reactive to label when native structure must be maintained for longer periods of time. This more extensive labeling results in a higher number of isomeric oxidation products that are co-localized to one tryptic peptide, resulting in an analytical challenge to quantify how much oxidation occurs at each site.

Traditionally, HRPF quantification was performed on the peptide level. Slower radical generation techniques that required lower oxidation amounts would often only oxidize a single amino acid in a given tryptic peptide to an appreciable extent, making this peptide-level quantification of oxidation acceptable. However, as the sub-microsecond oxidation techniques have been developed, and as FPOP in particular has been more widely used for HRPF experiments, the more heavily oxidized analytes have resulted in a much more complex array of isomeric oxidized peptides. While traditional oxidation quantification

based on measurements of levels of oxidized peptides allows for structural information, the spatial resolution of such information is strictly limited by the size of the peptide, limiting the applicability of these data to high resolution structural analysis, including constrained modeling approaches. Improved methods for detecting and quantifying oxidation at the residue level, even for adjacent sites of oxidative modification in the protein sequence, will greatly increase the accuracy and level of structural detail of any model generated from the data.

The quantification of the apparent rate of oxidation on any specific residue requires specific quantification of oxidation at that amino acid residue from within a pool of multiple isomers that differ only by the site of oxidation. However, it is still a very challenging task to distinguish and accurately quantify peptide isomers with oxidation at multiple adjacent sites in a single sequence that results in an identical mass shift and cannot be easily separated. Previous research by Woods and co-workers [12] relied upon LC separation of oxidation isomers, combined with semiautomated MS/MS identification of the major oxidation product in each MS/MS spectrum. The spectral intensities from the MS/MS spectrum would be assigned to the major oxidation product for quantification. Similar work with superior chromatography has been reported by Gross and coworkers, using the signal intensities from the separated oxidation isomer for quantification [17-19]. Spectral counting in the MS/MS mode for partially separated oxidation isomers has also been used for quantitative analysis of peptide isomers [16]. However, due to a lack of rigorously known oxidation levels for any of these systems, the accuracy of these methods of analysis remains unknown.

Recently, Vachet and coworkers have reported the success of using electron transfer dissociation (ETD) fragment ion intensity to accurately quantify the residue oxidation level in isomeric mixtures of methionine sulfoxide-containing peptides [28] and peptides that contain both oxidized histidine residues and other oxidized residues [29]. Similarly, Jumper *et al* recently reported use of ETD in quantitating mixtures of carbene footprinting products [30]. In contrast, collision-induced dissociation (CID) MS/MS spectra of these oxidized products rarely reflect the accurate oxidation extent at each given residue. This occurs because the dissociation chemistry of each oxidized isomer can be very different during CID, which gives rise to complicated spectra that are easy to misinterpret [28, 31, 32]. However, insufficient work has been done in this area to demonstrate the accuracy of ETD-based methods for quantifying multiple adjacent sites of oxidation with the various classes of oxidation products, as well as the general applicability of ETD-based dissociation to quantify oxidized peptide isomers having more than one site of oxidation on a single peptide generated in real HRPF samples.

Here we utilized sets of synthetic, isomeric “oxidized” peptides from a model tryptic peptide with a sequence RPMFAIWK to test and compare the ability of different fragmentation methods (both ETD and CID), as well as nano-ultra high performance liquid chromatography (nanoUPLC) separation, to quantify isomers with one oxidation at multiple adjacent sites.. Oxidized isomers having multiple sites of oxidation in each of two peptide sequences in the HRPF product of the protein Robo-1 Ig1-2, a protein involved in nervous system axon guidance, were also identified, and the oxidation extent at each residue was also quantified by ETD without prior LC separation.

Experimental

Materials

Hydrogen peroxide (30%) was obtained from J.T. Baker (Phillipsburg, NJ). Dithiothreitol (DTT), HPLC-grade acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). Methionine amide was purchased from Bachem (Torrance, CA, USA). Catalase, formic acid

and L-glutamine were obtained from the Sigma-Aldrich Corporation (St. Louis, MO). Sequencing-grade modified Lys-C was purchased from Promega Corporation (Madison, WI, USA). All reagents were used as provided. Purified water (18 M Ω) was obtained from an in-house Milli-Q Synthesis system (Millipore, Billerica, MA). The peptide RPMFAIWK, and its oxidized isomers: RP*MFAIWK (Pro2 hydroxyproline), RPF*AIWK (Phe4 tyrosine), and RPMFA*IWK (Ala5 serine) were synthesized by GenScript USA Inc. (Piscataway, NJ). Peptide RPM*FAIWK (Met3 methionine sulfoxide) was prepared in house by adding 90 μ M unmodified peptide RPMFAIWK to a solution of hydrogen peroxide (3%) and incubated in the dark for 1 h, followed by C₁₈ reverse phase HPLC purification of the methionine sulfoxide-containing product. The amount of these synthetic peptides was individually quantified by amino acid analysis and was nominally in 50 μ M range. Working stocks of each peptide were prepared by diluting solutions down to a concentration of 2 μ M in acetonitrile, water, and formic acid (50:50:0.1, v/v/v).

Construction, Expression and Purification of Robo 1 Ig1-2

The protein sequence of the translation of the human ROBO1 gene was analyzed for domain boundaries using the UniProt database and a truncated protein sequence comprised of Ig domains 1 and 2 (plus flanking regions) was chosen for gene synthesis. Optimized codon utilization for human protein expression was employed in the synthesis of the corresponding coding region and the resulting DNA fragment was cloned into our mammalian expression vector (pGen2) using restriction digestion and ligation into corresponding restriction sites in the vector. Large scale DNA preparations were prepared and transiently transfected into HEK293S GnTI- suspension culture cells and recombinant protein was harvested after 6 days of transfection. The recombinant protein was purified from the culture supernatant using Ni²⁺-NTA chromatography and concentrated to ~1mg/mL. The resulting protein preparation was digested with recombinant TEV-GFP and EndoF1-GFP and then further purified by Ni²⁺-NTA chromatography and gel filtration.

Sample Preparation

The working stock solutions of the four peptides were mixed in five different volume ratios (1:1:1:1, 1:2:3:4, 4:3:2:1, 1:1:9:9, 9:9:1:1) in triplicate at a final total molar concentration of 2 μ M to prepare the mimetic oxidative isomeric mixtures generated under different conditions.

Protein Robo1 Ig1-2 was oxidized using laser flash photolysis of hydrogen peroxide as described previously [12, 18]. Briefly, a 248 nm GAM EX100 KrF excimer laser operating at 50 mJ per pulse output was used to photolyze H₂O₂. Protein Robo1 Ig1-2 was reconstituted in an ammonium bicarbonate buffered solution (50 mM, pH=7.8) at a concentration of 30 μ M. Each 20 μ L reaction buffer (50 mM ammonium bicarbonate) was prepared with a final concentration of 6 μ M Robo1 Ig1-2, 3% hydrogen peroxide (freshly prepared and added right prior to irradiation) and 20 mM glutamine. The solution was then loaded into a 100 μ L syringe and introduced via a syringe pump coupled to the 100 μ m i.d. fused silica tubing. The flow rate was set to 12.19 μ L/min, with the laser pulse repetition rate of 5 Hz to illuminate each volume of sample with a single focused laser pulse as it passed through the laser path, with 10% of the total volume remaining unirradiated to correct for diffusion and laminar flow effects. The capillary outflow from a sufficient number of laser shots was collected in a microcentrifuge tube, to which was added immediately 20 μ L of water-washed agarose-immobilized catalase (0.5 mg/mL) and methionine amide (0.5 mg/mL) to remove any remaining H₂O₂ and other reactive oxygen species. Following irradiation, the sample (40 μ L) was mixed with ammonium bicarbonate buffer (40 μ L, 50 mM) and DTT (20 μ L, 50 mM) and then incubated at 65°C for 30 min to denature and reduce the protein. After cooling to room temperature, a 1:100 weight ratio of

Lys-C:protein was added to the protein sample and incubated at 37 °C overnight while rotating. The digested samples were stored at –20°C for LC-MS/MS analysis.

Instrumentation

Mass spectral analyses were performed on the Waters Synapt G2 Q-TOF mass spectrometer configured with both ETD and CID capabilities, and with a nano-ESI source that was coupled to either a nanoACQUITY (Waters) UPLC system, or operated in infusion mode. For direct injection experiments, the sample was delivered at 0.4 µL/min using a syringe pump. For nanoUPLC-MS/MS analysis, the samples were separated on a Symmetry C₁₈ 180 µm × 20 mm, 5 µm trapping column and a BEH130 C₁₈ 75 µm × 150 mm, 1.7 µm analytical column. The gradient elution was performed from 2–40% acetonitrile in 0.1% formic acid over 50 min at a flow rate of 0.4 µL/min, and then increased to 80% acetonitrile in 0.1% formic acid for 5 min followed by a 5 min re-equilibration step. Nitrosobenzene was used as ETD reagent. Trap wave height was set at 0.2 V to allow the ETD reagent to mix with the sample and react. Supplemental transfer CE of around 5 V was used to aid the ETD of doubly-charged ions. For CID, the collision energy was set at 25 V for doubly-charged ion and 15 V for triply-charged ion, respectively. All mass spectra were acquired in positive ion mode, with spray voltage of 3 kV and source temperature 100°C.

Quantitative Analysis

For direct injection and co-eluted samples, the fragment ion intensities from ETD and/or CID are used for the calculation of oxidation rate at specific residue site using a similar approach reported by Jumper *et al* [30]. Briefly, peptide isomers with oxidation distributed throughout the sequence can generate both oxidized and unoxidized sequence ions in its MS/MS spectrum. The actual fractional oxidation of a given sequence ion is defined as the ratio between the oxidized sequence ion intensity to the sum of the intensity of the corresponding oxidized and unoxidized sequence ion. For example, for ETD spectra generating a c-ion series, the actual fractional oxidation is defined as Equation 1:

$$f(c_i)_{actual} = \frac{I(c_i)_{oxidized}}{I(c_i)_{oxidized} + I(c_i)_{unoxidized}} \quad (1)$$

where $f(c_i)_{actual}$ denotes the fractional oxidation of c-ion i. $I(c_i)$ denotes the intensity of the c ion i, whether the oxidized and unoxidized form. The sum of the molar fraction made up of peptides that contain an oxidized residue within sequence ion i is defined as the theoretical value of $f(c_i)$. This is shown in Equation 2:

$$f(c_i)_{theoretical} = \sum_{i=1}^i f(i_{oxidized}) \quad (2)$$

Where the $f(i_{oxidized})$ is the molar fraction of the peptide containing an oxidation at residue i.

The relative oxidation rate for a specific residue i is calculated as the difference between the fractional oxidation of adjacent residues. This is shown in Equation 3:

$$oxidation \ rate/residue_i = f(c_i)_{actual} - f(c_{i-1})_{actual} \quad (3)$$

A similar approach is followed for CID spectra generation b-ions. For UPLC peaks of separated isomers, the selected ion monitoring (SIM) peak area is used for relative quantification.

Results and Discussion

Quantification of Synthetic Peptide Isomers using CID and ETD without Prior LC Separation

It has been demonstrated that side chain oxidation of the methionine residue often drastically affects the fragmentation pattern during CID, preventing correct peptide identification, and as a result, relative quantification [28, 33]. To investigate the possible fragmentation pattern effect caused by CID on the quantification of oxidized peptide isomers, the tandem mass spectra of both CID and ETD for each of the four oxidation isomers of the model peptide RPMFAIWK was obtained by direct infusion of individual isomer. The sequence RPMFAIWK was chosen in order to meet a variety of criteria. First, we wanted to cover each of the biophysical categories of major oxidation products that we could cover with synthetic peptides: large aliphatics (Pro → hydroxyproline), sulfur-containing residues (Met → methionine sulfoxide), aromatics (Phe → tyrosine), and small aliphatics (Ala → serine). We were unable to cover the generally less reactive ionic amino acids, as their major oxidation products are not readily available for peptide synthesis, and oxidation of ionic amino acids can easily change their ionization efficiencies in dramatic ways [34]. Second, we wanted to be able to probe multiple charge states by both CID and ETD to determine the effect of charge state on quantification. This required an additional basic site to ensure the presence of the +3 charge state. Third, we wanted to ensure that the peptide had a reasonable retention on a UPLC column to test quantification by UPLC. This caused us to include the hydrophobic Ile and Trp residues near the C-terminus. Finally, we wanted our peptide to be a possible tryptic peptide, as most HRP experiments are performed from tryptic digests of the protein. This caused us to place the extra basic site N-terminal to a Pro, which prevents tryptic cleavage. Having the basic site at the N-terminus also made the presence of abundant +2 and +3 charge states more likely due to the charging of the N-terminus and the N-terminal Arg side chain interfering with one another, preventing the peptide from existing entirely as a +3 ion. The sequence RPMFAIWK was analyzed by a protein BLAST search against the non-redundant protein sequences database [35] and found to be strongly homologous to a large number of sequences present in biology, indicating that this peptide is not a wholly artificial and unreasonable model sequence for testing analytical methods (data not shown).

Sequence isomers were confirmed straightforwardly on the basis of their sequence-specific fragment ions. For example, the CID spectrum of RP*MFAIWK shows oxidized b2-b8 ions, indicating Pro2 is oxidized, while the CID spectrum of RPM*FAIWK shows an unoxidized b2 ion and oxidized forms of the b3-b8 ions, indicating that Met3 is oxidized. Both ETD and CID gave confident sequence information of the site of oxidative modification on each individual isomer even for the labile side chain modification methionine sulfoxide (data not shown).

The mixtures of the four isomers: RP*MFAIWK, RPM*FAIWK, RPMF*AIWK, and RPMFA*IWK, at five varying ratios: 1:1:1:1, 1:2:3:4, 4:3:2:1, 9:9:1:1, and 1:1:9:9, respectively, were then subjected to tandem mass spectrometry analysis by both ETD and CID. The representative ETD spectra of both doubly- and triply-charged ions of mixture at ratio of 1:1:1:1 are shown in Figure 1. The representative CID spectra of both charged-state ions of mixture at ratio 1:1:1:1 are presented in Figure 2. The representative ETD spectra of both charged-state ions of mixtures at other ratios are presented in Figure S1-Figure S2. The representative CID spectra of triply-charged ion of mixtures at other ratios are presented in Figure S3. The mere presence or absence of an oxidized fragment ion is only sufficient to determine, at best, the N-terminal and C-terminal sites of oxidation. In order to determine internal oxidations, as well as to quantify any mixture of isomeric oxidations, the ratio of product ion intensities of the oxidized fragment to the sum of the unoxidized and oxidized

version of that fragment ion must be compared. The measured percentages of the oxidized form based on c-type ions ($f(c_i)_{\text{actual}}$) of ETD and b-type ions ($f(b_i)_{\text{actual}}$) of CID are plotted against the theoretical percentages oxidized ($f(c_i)_{\text{theoretical}}$ and $f(b_i)_{\text{theoretical}}$, Figure 3a, b, c and d), respectively. The reader should note that the experimental and theoretical percentages oxidized are for the fragment ion, not the amino acid residue. For example, a c3 ion's fractional oxidation would represent the sum of all fractional oxidation occurring on the first three amino acids of the peptide. As shown in Figure 3a and b, the molar ratio of each isomer in the mixture calculated based on abundance of the c-type product ion fragmented by ETD at both doubly- and triply-charged states correlates well to the theoretical values. Except a few outliers, the quantification method based on ETD fragmentation ion intensity at doubly- and triply-charged states exhibited an accuracy (relative error) ranging from -18 to 24% based on the average of triplicates. The data suggest that oxidized residues present in a mixture at 5% relative abundance can be accurately quantitated by ETD fragmentation.

As shown in Figure 3c and d, the precursor charge state plays a major role in CID quantification; only spectra (Figure 3d) of the triply-charged ions reveals a good correlation between the abundance of the oxidized versus unoxidized b-type product ion and the abundance of the appropriate oxidation isomers. The accuracy for quantification based on CID mass spectra of doubly-charged precursor ion ranged from -29 to 1200%. By contrast, the accuracy for quantification based on CID mass spectra of triply-charged precursor ion ranges from -26 to 22% and most of data points (over 85%) present accuracy within $\pm 20\%$. We attempted CID of a three-component peptide mixture excluding the methionine sulfoxide-containing peptide, and found that the +2 charge state still did not yield quantitative fragmentation results (data not shown), indicating that the presence of methionine sulfoxide is not the only confounding issue in CID-based quantification at this charge state. These results lend support to the hypothesis that oxidation-influenced fragmentation is a charge-remote process that can be suppressed in the presence of sufficiently mobile protons. Further work would be required to test this hypothesis. In addition to the successful determination of the N-terminal sequence (b and c ions), several C-terminal fragment ions were also observed in the CID and/or ETD spectra of triply-charged ions (Table S1 and Table S2). The molar ratios of isomers RPMF*AIWK and RPMFA*IWK calculated based on abundance of C-terminal fragment ions (y or z ions) are close to expected, in concert with the ratios calculated based on abundance of N-terminal fragment ions (b and/or c ions). It should be noted that some y ions were also formed in the ETD spectra of triply charged ion, possibly due to in-source dissociation or supplemental energy activation. The mass of observed y ion series is 16.0187 da higher than the z ion series of the same amino acid residue, and the oxidized z ion is 15.9994 da higher than unoxidized, which means there is only 0.0193 da mass differences between the oxidized z ion and y ion of the same amino acid residue. High resolution MS allows these two peaks to be distinguished. For example, the oxidized z5 ion at m/z 664.343 could be resolved from the y ion at m/z 664.363 (Figure S4), which makes the quantification of oxidized isomers based on z ion intensity reliable by excluding the interference from y ions. However, in mass spectrometers with lower resolution, the interference between unoxidized y-ions and oxidized z-ions prevents accurate quantification based on C-terminal ions in ETD.

Given the observed fact that reliability of CID-based quantification of oxidation isomers depends heavily on charge state, while ETD-based quantification is robust across both +2 and +3 charge states, it stands to reason that ETD-based quantification will be more reliable in cases where the isomeric state is unknown and the charge state sufficient for quantitative CID fragmentation is not determined beforehand.

Relative Quantification of Synthetic Peptide Isomers by nanoUPLC-MS in Selected Ion Monitoring Mode

Due to similar properties, separation of isomers by liquid chromatography (LC) requires intensive screening of various columns and mobile phase conditions. In cases of hydrophilic oxidized peptides, this task becomes even more challenging due to limited retention on traditional reversed-phase HPLC columns. During the process of the optimization of nanoUPLC conditions, two different columns: BEH130 C₁₈ column (75 μm $\times$ 100 mm, 1.7 μm) and porous shell Halo C₁₈ column were tested. With an optimized LC gradient, 3 of the 4 peptide isomers were separated from each other, while RP*MFAIWK and RPMFA*IWK were co-eluted (data not shown). As shown in Figure S5, a longer BEH130 C₁₈ column (75 μm $\times$ 150 mm, 1.7 μm) is able to produce excellent peak shapes for all four oxidation isomers of the model peptide RPMFAIWK. Baseline separation within 60 minutes was achieved with linear gradient elution. It should be noted that gradient separation conditions are necessary for complete baseline separation of the four isomers on BEH130 C₁₈ column. Different isocratic separation conditions were tested on the columns mentioned above, under the best of circumstance, only three peaks could be observed and peaks for RP*MFAIWK and RPMFA*IWK were co-eluted.

The four isomeric peptides analyzed were of synthetic origin, so that their individual assignment in the UPLC-MS/MS runs was straightforward. The retention time for the four isomers: RP*MFAIWK, RPM*FAIWK and RPMF*AIWK and RPMFA*IWK, is 32.8 min, 30.5 min, 29.5 min, and 33.4 min, respectively. The molar ratio of each isomer was determined by integration of the SIM peak. The peak area of each isomer correlated well with the defined amount of the isomer in solution when the data was calculated with methionine sulfoxide-containing isomer (RPM*FAIWK) excluded (Figure 4). The amounts of methionine sulfoxide-containing isomer are overestimated by peak area in all tested samples. This variation was not observed when the isomers were individually injected and eluted under a step-gradient to strong elution conditions using a mobile phase of 90% acetonitrile (UPLC peak areas are used for quantification, data not shown). Under these step-gradient conditions, no separation of the methionine amide diastereomers can occur, eliminating peak shape irregularities at the cost of any UPLC resolving power. These data, combined with the successful quantification by ETD fragmentation, suggests that the discrepancy between the UPLC peak areas and the theoretical abundance of each isomer is not due to differences in ionization efficiency between the isomers. Based on the results above, UPLC-based quantification may be unreliable, even in cases where the oxidation isomers can be cleanly separated to baseline. This may be due to the fact that, in our sample, the MetSO-containing peptide is the only peptide that is present as a mixture of two stereoisomers, while the other oxidation isomers are stereochemically pure. Analysis of the MetSO peak in Figure S5 indicates peak broadening, shouldering or splitting, which may be due to partial separation of these stereoisomers. This peak asymmetry may be causing the overestimation of MetSO quantity by UPLC. Unfortunately, in HRPF and most oxidative stress situations, oxidative modification of peptides will not result in a stereochemically pure sample. Therefore, this peak asymmetry may occur rampantly in any optimized UPLC protocol, further complicating quantification by nanoUPLC analysis.

Relative Quantification of Mixtures of Oxidized Isomers having Multiple Sites of Oxidation in HRPF Product of Protein Robo-1 Ig1-2 by ETD

To demonstrate the ability of ETD-based dissociation to quantify oxidized peptide isomers having more than one site of oxidation on a single peptide generated in real HRPF samples, we performed a HRPF study of the protein Robo-1 Ig1-2 and identified a series of oxidized products with multiple oxidation sites on a single peptide sequence. An example is the

identification and quantification of sites of oxidation of an identified peptide LMITYTRK by ETD.

Protein Robo-1 Ig1-2 was subjected to hydroxyl radical footprinting and Lys-C digestion, and the peptide LMITYTRK produced by Lys-C digestion was sequenced based on the tandem mass spectra by ETD (Figure S6a). A singly-oxidized product of this peptide LMITYTRK generated by HRPf was also identified and sequenced as LM*ITYTRK based on its ETD spectrum (Figure S6b). The ETD spectrum of LM*ITYTRK, $[M+O+3H]^{3+}$ contains unoxidized c1 ion, oxidized c2-c7 ions, and unoxidized z2-z3 ions, which indicate that Met2 is the sole site of oxidation.

We also observed a doubly-oxidized product of the same peptide sequence LMITYTRK by comparing the full mass spectrum of unoxidized Robo-1 Ig1-2 sample with oxidized sample. The SIM chromatogram shows only one single peak corresponding to this doubly-oxidized product with optimized nanoUPLC separation on the same column used for the synthetic peptide isomers. However, the ETD spectrum shows unoxidized c1 and singly-oxidized c2 ions, and singly- and doubly-oxidized forms of c3-c7 product ions (Figure 5). The presence of only singly-oxidized c2 ion and singly-oxidized and doubly-oxidized c3-c7 product ions indicate that 100% Met2 was singly-oxidized. In addition, the increasing percentages of these doubly-oxidized forms of c3-c6 product ions provide clear evidence that Ile3, Thr4, Tyr5, Thr6 and Lys8 are all partially oxidized (Table 1). Therefore, this single SIM chromatographic peak at m/z 353 ($[M+2O+3H]^{3+}$ species of the peptide LMITYTRK actually represents a mixture of five doubly-oxidized isomers differing by the site of second oxidation. Indeed, the percentages of these doubly-oxidized forms of c3-c8 product ions indicate that the relative molar ratio of the five doubly-oxidized isomers with oxidation on Met2/Iso3, Met2/Thr4, Met2/Tyr5, Met2/Thr6 and Met2/Lys8 is approximately 15:32:20:19:14.

Another example in which the ETD spectrum has demonstrated the capability of simultaneously identifying and quantifying the oxidation sites of peptide isomers involves another HRPf product of protein Robo-1 Ig1-2: peptide IVEHPSDLIVSK. The ETD spectrum of the triply-charged oxidized peptide from an unseparated sample not only identifies three oxidation sites but also provides the degree of oxidation at each residue (Figure 6 and Table 2). As shown in Table 2, the percentages of oxidized c1-c3 and c5 ions are consistent, indicating only the N-terminal amino acid is oxidized to a significant extent. Meanwhile, it is clearly shown that c6 ion is significantly oxidized, as the percentage increases substantially at this residue. The analysis of the percentage of the oxidized forms indicates that the degree of oxidation at Ile1, Ser6 and Lys12 is about 40%, 20%, 40%, respectively. Examination of Figure 6 indicates that ETD fragmentation efficiency of this peptide was not particularly robust, which has obvious consequences when examining the increased standard deviations of oxidation measurement shown in Table 2. However, even with the poor signal-to-noise, we are clearly able to identify and quantify the major oxidation isomers of this peptide, which were impossible to resolve by reverse phase UPLC separation.

Conclusion

In this work, we have demonstrated the accuracy of quantification of mixtures of synthetic oxidation isomers based on ETD fragment ion intensities. The amount of oxidation occurring at given residues could be accurately determined from ETD spectra of peptides in both doubly- and triply-charged states. In contrast, CID spectra of doubly-charged precursor ions for such peptides rarely reflect the accurate oxidation extent, though CID spectra of the triply-charged ion allows accurate determination for such peptides. Based on the current

limited data set (only one set of four synthetic peptide sequences was tested), we are unable to predict with certainty what charge state would result in quantitative CID fragmentation for a given peptide sequence, although initial results from our model peptide lead to the hypothesis that each basic site in the peptide must be protonated for quantitative CID.

LC separation is one of major techniques currently used for relative quantification of mixtures of oxidation isomers [12, 17-19]. But carefully optimized LC conditions are needed to fully separate the oxidized isomers. In addition, even though these isomers could often (but not always) be separated successfully after intensive optimization of LC conditions, the relative quantification of the isomers based on individual chromatographic peak area shows significant variation for each individual isomer. The only isomer that gave aberrant readings in our model UPLC separation-based quantification was the methionine sulfoxide-containing peptide. This error cannot be explained by differences in ionization efficiency, as ionization efficiency differences would be revealed in any ESI-MS-based method, be it LC-MS based or fragment ion intensity-based. The methionine sulfoxide peptide is the only model peptide that existed as a mixture of two structures: the two stereoisomers of the sulfoxide. The broader peak shape and shouldering of the oxidized methionine peak shown in Figure S5 (probably due to these diastereomers) compared to the other isomeric oxidation products may result in poor peak area calculations, leading to the errors in quantification. Similarly, when isomers were injected individually and eluted with a strong step-gradient to eliminate peak shape aberrations, accurate quantification of all four isomers could be achieved, although this method sacrifices the resolving power of the UPLC and is impractical for mixtures. Unfortunately, in HRPf experiments, these oxidation products almost always exist as multiple isomers within a single amino acid residue. In our experience, these HRPf-based mixtures often have UPLC peaks with apparent partial separation of these in-residue isomers, leading to peak broadening and poor peak shapes. If these peak shapes lead to errors in peak area calculations, then LC-MS-based quantification of these oxidation isomers is likely to be rife with errors. Further work comparing ETD-based and UPLC-based quantification of complex HRPf-derived samples should be pursued to probe the extent of this potential issue.

The application of ETD in simultaneously identifying and quantifying the oxidation sites has also been demonstrated through analyzing a series of oxidized products with multiple oxidation sites on two peptide sequences in HRPf product of protein Robo-1 Ig1-2. As discussed above, it is quite challenging to separate these isomers by LC due to their similar properties and high hydrophilic character, even using the nanoUPLC system which has demonstrated the capability to separate four synthetic peptide isomers with adjacent oxidation. In such a situation, ETD-based tandem mass spectrometry provides a fast, convenient and accurate method that allows simultaneous identification and relative quantification of the oxidation of each individual amino acid in proteins. Its advantages in ability to obtain structural details on modified peptides present in complex mixtures, as well as its accuracy for quantitative analysis, make it a powerful tool in the study of oxidative stress, commercial protein oxidative modification, as well as for improving spatial resolution for HRPf studies. The latter capability should further advance the HRPf power for the determination of protein structure and protein-ligand interactions. While the well-known limitations of ETD (the need for higher charge states, inability to cleave N-terminal to proline) may limit its applicability in some cases, in most cases it is an important tool in the quantification of peptide oxidation isomers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative ETD spectrum of mixture of four oxidation isomers of the model peptide RPMFAIWK at (a) doubly-charged state and (b) triply-charged state by direct infusion. The mixture contains RP*MFAIWK, RPM*FAIWK, RPMF*AIWK, and RPMFA*IWK at a molar ratio 1:1:1:1. The asterisks indicate the product ions that are oxidized.

Figure 2.

Representative CID spectrum of mixture of four oxidation isomers of the model peptide RPMFAIWK at (a) doubly-charged state and (b) triply-charged state by direct infusion. The mixture contains RP*MFAIWK, RPM*FAIWK, RPMF*AIWK, and RPMFA*IWK at a molar ratio 1:1:1:1. The asterisks indicate the product ions that are oxidized.

Figure 3.

Comparison of theoretical and experimental quantification of mixtures of four oxidation isomers of the model peptide RPMFAIWK based on fragment c-type ions intensity by ETD at (a) doubly-charged state, and (b) triply-charged state, and on fragment b-type ions intensity by CID at (c) doubly-charged state and (d) triply-charged state, respectively. The mixtures contain: RP*MFAIWK, RPM*FAIWK, RPMF*AIWK, and RPMFA*IWK at five varying molar ratios of 1:1:1:1, 1:2:3:4, 4:3:2:1, 9:9:1:1, and 1:1:9:9 ($n=3$). Each replicate of the triplicate analysis is plotted as an individual data point.

Figure 4.

Comparison of added molar ratio and experimental quantification calculated with RPM*FAIWK included (a) and excluded (b) of mixtures of four oxidation isomers of the model peptide RPMFAIWK based on SIM peak area after UPLC separation (n=3). Each replicate of the triplicate analysis is plotted as an individual data point.

Figure 5.

The ETD spectrum of doubly-oxidized peptide LMITYTRK $[M+2O+3H]^{3+}$. The single asterisks indicate the product ions that are singly oxidized. The double asterisks indicate the product ions that are doubly oxidized.

Figure 6.

The ETD spectrum of singly oxidized peptide isomers IVEHPSDLIVSK $[M+O+3H]^{3+}$. The asterisks indicate the product ions that are oxidized.

Table 1

Percentage (Mean $\pm$ SD, n = 3) of oxidized product ions observed in the ETD spectrum of the doubly oxidized peptide LMITYTRK

Product Ion	m/z _{unoxidized}	m/z _{singly oxidized}	m/z _{doubly oxidized}	Ratio of singly oxidized to unoxidized product ion	Percentage of doubly oxidized product ion
c1	131	147	163	0	0.0
c2	262	278	294	100	0.0
c3	375	391	407	100	15.1 $\pm$ 2.5
c4	476	492	508	100	46.8 $\pm$ 2.0
c5	639	655	671	100	66.7 $\pm$ 3.5
c6	740	756	772	100	85.9 $\pm$ 3.2
c7	896	912	928	100	89.6 $\pm$ 6.3
c8	---	---	---	100	100

Table 2

Percentage (Mean $\pm$ SD, n = 3) of oxidized product ions observed in the ETD spectrum of the singly oxidized peptide isomers IVEHPSDLIVSK

Product ion	m/z _{unoxidized}	m/z _{oxidized}	Percentage oxidized
c1	131	147	40.3 $\pm$ 8.0
c2	230	246	33.3 $\pm$ 9.3
c3	359	375	42.9 $\pm$ 3.5
c4	---	---	---
c5	593	609	32.7 $\pm$ 8.6
c6	680	696	60.7 $\pm$ 5.5
c7	795	811	61.1 $\pm$ 12.2
c8	---	---	---
c9	1022	1038	72.6 $\pm$ 5.6
c10	1121	1137	69.3 $\pm$ 6.5
c11	1208	1224	73.6 $\pm$ 15.3
c12	---	---	100
z2	218	234	42.9 $\pm$ 14.6
z3	317	333	39.4 $\pm$ 5.3
z4	430	446	46.3 $\pm$ 3.4
z5	543	559	44.7 $\pm$ 2.5
z9	980	996	53.7 $\pm$ 7.0
z12	---	---	100