Published in final edited form as:

Anal Chem. 2007 April 15; 79(8): 3083–3090. doi:10.1021/ac062154o.

Quantification of cysteine oxidation in human estrogen receptor by mass spectrometry

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Abstract

Redox dependent modifications of sulfhydryl groups within the two Cys₄ zinc fingers of the estrogen receptor DNA-binding domain (ER-DBD) result in structural damage and loss of ER DNA-binding function, which parallels the situation observed in many ER-positive breast cancers. Quantitation of the redox status of cysteinyl thiols within ER-DBD employed cysteine specific oxidants to induce varying degrees of oxidation in recombinant ER, followed by differential alkylation with the stable isotopic labeling reagents [¹²C₂]-iodoacetic acid and [¹³C₂]-bromoacetic acid. Subsequent proteolysis with LysC/Asp-N generated diagnostic peptides of which the C-terminal peptide of the second zinc finger is most strongly detected by mass spectrometry (MS) and serves as a suitable marker of ER-DBD redox status. Data was collected from two different MALDI-MS instruments; a time-of-flight and a linear ion trap (vMALDI-LIT). An analogous but larger synthetic peptide treated with three isotopic variants of the alkylating reagent modeled isotopic overlaps that might complicate the relative quantitation of cysteine oxidation. Despite the isotopic overlaps, excellent relative quantitation was achieved from MS data obtained from both instruments. This was also true of tandem MS/MS data from the vMALDI-LIT, which should facilitate selected reaction monitoring. Relative quantitation by MS also closely matched data from immunochemical methods.

Introduction

Because of its high sensitivity and specificity and its application in automated high-throughput analysis, mass spectrometry (MS) has become the technique of choice for identification of proteins and characterization of modified variants in the study of complex proteomes ^{1, 2}. To monitor any change in a proteome resulting from biologic perturbation, such as during cell growth or following environmental exposure, a means of quantitative recording is essential. Because mass spectrometry can distinguish between different isotopic molecular variants, quantitative techniques have been developed for differentiating protein groups based on the use of chemical derivatives containing stable isotope labels ^{3–8}. Typical of these are the isotope-coded affinity tag (ICAT) reagents, initially developed by the Aebersold group, that contain sulfhydryl reactive groups, enabling a reduction in protein sample complexity by means of selective biotin tagging of cysteines followed by proteolytic digestion and avidin affinity enrichment of cysteine-containing peptides ³. These isotopic mass differences provide relative quantitative information and can distinguish the proteome components of two biological samples. Since ICAT-type reagents will not react with disulfide-linked cysteines, digestion and derivatization is preceded by treatment with an appropriate reducing agent (such as dithiothreitol (DTT)). In studies where one wants to gain information on the oxidative status of specific cysteine residues, sample pre-reduction is not appropriate, thus the application of

an ICAT-type technique to quantify biologic effects of oxidative stress on cysteine disulfide formation requires modification. Nevertheless, an ICAT procedure was used to monitor oxidation induced in proteins in a rabbit heart membrane fraction exposed to hydrogen peroxide, thereby demonstrating that only certain cysteine residues are susceptible to oxidation⁹.

Our long-term studies have focused on oxidative stress effects that alter the structure and function of human estrogen receptor- α isoform (ER- α), a 67 kDa zinc finger transcription factor known to play a pivotal role in the development of most breast cancers^{10, 11}. MS studies have probed the use of isotopic labeling to monitor cysteine oxidation within the two Cys₄-type zinc fingers of the ER DNA binding domain (ER-DBD)^{12–14}. These studies employed differential cysteine alkylation by isotopic reagents before and after sample reduction, followed by ER digestion using two endoproteinases that yielded four diagnostic zinc finger peptides (ZnF1A, ZnF1B, ZnF2A, ZnF2B; Fig. 1). Each oxidized ZnF peptide is separated from the originally reduceable cysteine pairs by 4 Da, representing twice the mass difference of isotopically labeled [¹²C₂]- and [¹³C₂]-carboxymethyl cysteine adducts. However, disulfide formation is not necessarily confined to adjacent pairs of cysteines within each ZnF peptide (Fig. 2) and the detection of other ZnF peptide masses is possible, including a peptide bearing one ¹²C₂-label and one ¹³C₂-label, showing only a 2-Da mass shift. Despite these relatively small mass differences between diagnostic peptides, it is possible to deconvolute these overlapping peptide ions using correction factors determined by the natural abundance of heavy isotopes. A similar strategy using 2 deuterium atoms per modifying group, i.e. a 2 Da mass difference, was successfully employed to probe the active site residues in fructose 1,6-bisphosphate aldolase by aminoethylation of solvent accessible cysteine residues using a 1:1 mixture of d₀ and d₂ reagents¹⁵.

In the current study, we explored the extent to which our differential alkylation approach yields accurate quantitative information on the degree of cysteine oxidation following ER exposure to selected oxidizing agents using matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS). Two different types of MALDI mass spectrometers are compared: a conventional reflectron time-of-flight (MALDI-TOF) instrument and a novel linear ion trap (vMALDI-LIT) instrument capable of multi-stage MS (MSⁿ) experiments. Because ER is an extremely low abundance protein even when overexpressed in tumors, the use of product ion ratios derived from selected reaction monitoring in MS/MS mode is potentially advantageous to overcome otherwise poor signal-to-noise ratio observed by MS.

Materials and methods

Materials

Recombinant human ER (rER) with a functional receptor concentration of 2.8 pmol/ μ l was obtained from Pan Vera (Madison, WI). The synthetic ZnF peptide, KNRRKSCQACRLRK was custom synthesized by Anaspec Inc. (San Jose, CA). Sulfhydryl-specific derivatizing reagents were obtained as follows: non-isotopically labeled iodoacetic acid [¹²C₂]-IAA, hereafter referred to as IAA, from Sigma-Aldrich (St Louis, MO); singly labeled iodoacetic acid [¹³C₁, ¹²C₁]-IAA (IAA*) and doubly labeled bromoacetic acid [¹³C₂]-BAA (hereafter designated BAA**) from Cambridge Isotope Labs (Andover, MA). The cysteine specific oxidant diamide (azodicarboxylic acid bis[dimethylamide]) was purchased from Sigma-Aldrich (St. Louis, MO) and the thiol reducing agent tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) was from Pierce (Rockford, IL).

Titration of rER with diamide and isotopic labeling for MS analysis

Partial to complete oxidation of the cysteine residues of rER to form disulfides was carried out according to previously published protocols, with minor modifications¹³. Aliquots (4 μ L, 11 pMol) of the protein contained in microcentrifuge tubes made from low binding polymer technology (PGC Scientifics [item # 505-195] MD, USA) were denatured in 4 M guanidine hydrochloride followed by the addition of diamide, a sulfhydryl-specific oxidant, at various concentrations (0 – 180 μ M) and incubation at room temperature for 60 min. This was immediately followed by addition of IAA (4 mM) and incubation in the dark at 37°C for 40 min to label any remaining free sulfhydryls. An aliquot of each resulting solution/mixture containing about 1.2 pmol of rER was saved and stored at –20°C for subsequent western blot analysis. Excess IAA was quenched by the addition of a freshly prepared solution of cysteine (5 mM), followed by the reduction of disulfide bonds with TCEP (10 mM). A second alkylation step was then effected by incubation with BAA** (10 mM) in the dark at 37°C for 90 min to incorporate the “heavy” isotope. The mixture was then diluted with sufficient ammonium bicarbonate solution (25 mM, pH 8) to drop the concentration of reducing and alkylating reagents to less than 1 mM, compatible with effective enzyme digestion. Two stages of proteolysis were performed by incubation with endoproteinase Lys-C (360 ng) from *Lysobacter enzymogenes* (Roche Diagnostics, Indianapolis), followed by endoproteinase Asp-N (140 ng) from *Pseudomonas fragi* (Roche), each for 3 h at 37°C. The resulting peptide mixtures were desalted and concentrated using a C-18 ZipTip (0.6 μ L bed volume, Millipore, MA) and eluted with 25 μ L acetonitrile/trifluoroacetic acid (TFA) in water (0.1% v/v) (60:40 v/v). The eluted peptides were lyophilized to near dryness and reconstituted in 10 μ L 0.1% TFA for subsequent analysis by MALDI-MS.

Western blot and densitometric analysis of oxidized rER

Aliquots of diamide-titrated rER described were resolved by non-denaturing 1-D SDS-PAGE in duplicate on Bis-Tris gels (4–12%) (Invitrogen, Carlsbad, CA). The separated ER proteins were subsequently transferred onto polyvinylidene dihydrofluoride (PVDF) membranes by electroblotting. The resulting duplicate blots were probed either with a novel anti-oxER antibody produced against the cyclized/disulfide form of the synthetic ZnF2B peptide (Scott et al, in preparation), or to assess total ER, with the commercial monoclonal anti-ER antibody F-10 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) that detects a C-terminal epitope in human ER α . Densitometric quantitation of bands from scanned immunoblots was carried out with Labworks™ Analysis software (version 3.0.02), UVP Inc. (Upland, CA). The relative amount of oxidized (disulfide linked) versus free cysteine was determined from the ratio of the densitometric signal measured from the oxER band to that of total ER concentration.

Isotopic labeling of a synthetic peptide to model MS quantitation of cysteine disulfides

An aliquot (10 μ L) containing 100 pM/ μ L of synthetic peptide stock solution (KNRRKSCQACRLRK) was treated with TCEP (10 mM, 40°C, 45 min) in distilled water for complete reduction of any disulfide bonds. The resulting solution was diluted with water to yield a peptide concentration of 25 pM/ μ L then divided into aliquots of 5 μ L. To each aliquot, 5 μ L of a 20 mM reagent solution was added, either IAA, IAA* or BAA**, and diluted with ammonium bicarbonate (pH 8, 25 mM) to a final concentration of 3 mM. The reaction mixtures were incubated in the dark at 37°C and monitored by MALDI-TOF to ensure complete alkylation of all cysteine residues in each reaction mixture. The reaction with BAA** was carried out for twice the time necessary for reaction with either IAA or IAA* to compensate for the lower sulfhydryl reactivity noted previously¹⁴. The reactions were terminated by addition of 0.1% TFA in water to yield a final peptide concentration of 2 pmol/ μ L. Three-component peptide mixtures of the alkylated peptides were prepared by mixing fractions of these solutions in the following molar ratios: IAA:IAA*:BAA**; 1:1:1, 1:1:2, 1:1:4, 1:1:0.5,

1:1:0.25. The quantities of each component were adjusted to give a constant total peptide concentration of 50 fmol/ μ L in each mixture. Similarly, binary peptide mixtures were also prepared in molar ratios: 1:1, 1:2, 1:4, 1:0.5, 1:0.25, for IAA:BAA^{**}. In all cases, alkylated peptide mixtures were prepared in triplicate from common stock solutions for separate analyses. All peptide mixtures were stored at -20°C for subsequent analysis by MALDI-MS.

MALDI-MS analysis of isotope labeled synthetic peptides and rER digests

Analysis of peptides by MALDI-MS was carried out using two different instruments. For MALDI-TOF analysis, a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) equipped with a N_2 laser (337 nm) was used, and equal volumes (1:1 v/v) of the peptide mixture and α -cyano-4-hydroxycinnamic acid (CHCA) solution (5 mg/ml in 50% v/v acetonitrile/water 0.1% v/v TFA) were mixed and 1 μ L was manually spotted onto the MALDI target plate by the dried droplet method. Mass spectra were recorded in positive ion reflector mode with manual selection of the crystals to be irradiated, 100 shots per spot being accumulated for each spectrum. The laser power was adjusted to give adequate signal strength without peak overloading. After applying internal mass calibration based on the masses of known peptides, each spectrum was smoothed with a Gaussian function and the baseline was corrected using the manufacturer's software. Quantitation of peptides was achieved using the Data ExplorerTM software for integration of the monoisotopic (mi) peaks for profile data format 16.

A second MALDI-MS analysis employed a vacuum MALDI linear ion trap mass spectrometer (vMALDI-LTQ, ThermoElectron, San Jose, CA, USA), hereafter referred to as MALDI-LIT. The ionization region in this instrument is maintained at 170 mTorr and also employs a N_2 -laser at 337 nm (20 Hz, 250 μ Joules/pulse). The MALDI-LIT operating parameters included a crystal-positioning system (CPS) and auto spectrum filter (ASF), the ASF threshold being 1000 counts for an MS scan and 250 counts for MS² scans. Automatic gain control (AGC) was employed to allow the instrument software to automatically adjust the number of laser shots to maintain spectral quality by effectively controlling the number of ions admitted into the trap. For quantitation of isotopic peptide ion pairs, the instrument was operated in zoom scan mode with a mass isolation window of ± 8 Da centered around the parent mass. Ten microscans were acquired in profile data mode for each experiment. Corresponding MS² scans were performed with a normalized collision energy value of 40, activation Q value of 0.25, activation time of 30 ms, and 5 microscans for each experiment. Data analysis was performed with the Xcalibur software package supplied by the manufacturer.

Results and Discussion

The use of MALDI-MS for quantitation of isotope labeled peptides

A potential problem in quantitative studies by MALDI-MS is variability in sample preparation, leading to crystal heterogeneity, discrimination and suppression of some signals. Consequently this ionization technique has not been employed widely for peptide quantification compared to electrospray ionization. Various approaches to minimize MALDI discrimination in the quantitation of peptides have included the production of thin films by rapid drying of volatile solvents¹⁷, the use of electrospray to produce thin homogeneous films¹⁸ and the use of ionic liquid matrices that do not involve crystal production¹⁹. A study of quantitation of a number of analyte classes including peptides using ionic liquid matrices demonstrated that although high linearity and reproducibility could be achieved, careful production of dried crystals led to better standard deviations²⁰. In the current work particular care was taken to maintain uniform protocols for sample preparation and MALDI data collection, all work being carried out by a single individual. Furthermore, since compounds differing only in the isotopic content of small derivatizing groups should behave almost identically, comparison of *relative* peptide

abundances in MALDI-MS spectra should be largely unaffected by changes in overall ion yield, as has been shown previously for ICAT labeling ^{4, 21}

In the current work, oxidation of rER *in vitro* with diamide or H₂O₂, followed by differential alkylation with the stable isotope reagents IAA and BAA** and double enzyme digestion with Lys-C and Asp-N as described previously ¹³, gave MALDI-TOF spectra of the mixed peptides. A region of one such spectrum is shown in Fig. 3A(i) for three overlapping peaks occurring at *m/z*'s 1180.6, 1182.6 and 1184.6. This constitutes a mass signature that characterizes the different redox states of cysteine residues within the C-terminus of the second ER zinc finger (ZnF2B) as illustrated in Fig. 2: the lowest mass arising from peptides originally having two free sulfhydryls (or coordinated to the zinc atom), each alkylated with the "light" reagent; the intermediate mass arising from one free sulfhydryl plus a cysteine residue disulfide-linked with a distal sulfhydryl outside the C-terminal region of ZnF2, giving both "light" and "heavy" adducts; and the highest mass representing peptides containing a disulfide bond between the two cysteine residues of ZnF2B, giving two "heavy" adducts.

The choice of isotopic reagents employed for quantitation

In previous studies ^{14, 22} we have demonstrated that to circumvent any adventitious disulfide formation or scrambling in the target protein during sample work-up, it is possible to rapidly alkylate all free sulfhydryls, then to reduce and alkylate with the alternative isotopic variant of the reagent, *prior* to digestion. Earlier data ¹⁴ suggest that, perhaps because the initial derivatization is carried out on the protein rather than peptides, the presence of a bulky biotin tag as in the standard ICAT reagents could cause steric hindrance that might impede reaction at adjacent or inaccessible cysteine residues within the targeted protein. To avoid this effect that could lead to a loss of critical redox information, we opted to use smaller reagents that do not contain the biotin group, although an affinity chromatography step is precluded. Standard ICAT reagents created for labeling sulfhydryls in a protein were designed such that the mass difference between the "heavy" and "light" labeled peptides was sufficient to minimize the overlap of their isotopic envelopes. This was achieved by incorporating eight H/D or nine ¹²C/¹³C differences to give a separation of 8 or 9 Da between the two peptides. Carbon isotopes are preferred over H/D substituted groups as the former do not experience separation in reversed phase chromatography, but it is not possible to achieve such large mass differences based on carbon isotopes with small derivatizing agents such as IAA, possessing only two carbon atoms, i.e. a 2 Da difference between "light" and "heavy" versions. Therefore we applied theoretical isotopic profiles based on the known abundances of naturally occurring heavy isotopes to correct for isotopic overlaps. Peptides containing two originally oxidized cysteines will exhibit less isotopic overlap as the mass difference should be 4 Da per peptide compared with the same peptide containing two free cysteines.

The MS-Isotope program in Protein Prospector (University of California San Francisco. <http://prospector.ucsf.edu/>) was used to simulate the theoretical isotopic abundances of overlapping peaks for the derivatized peptides predicted to be generated by our double digestion protocol. Assuming that monoisotopic peaks at *m/z* 1182.6 and 1184.6 give rise to isotope distribution patterns similar to that at *m/z* 1180.6, the overlapping peaks shown in Fig. 3A(i) were corrected as follows: Observed peak intensity (*Obs Pi*) at *m/z* 1182.6 is contributed by 29.6% of the peak intensity at *m/z* 1180.6 (Fig. 3A(ii)) and therefore corrected peak intensity (*corr Pi*) at *m/z* 1182.6 = *Obs Pi* at *m/z* 1182.6 – 29.6% *Obs Pi* at *m/z* 1180.6]; For the peak at 1184.6 the *corr. Pi* at *m/z* 1184.6 = *Obs Pi* at *m/z* 1184.6 – 3.2% *Obs Pi* at *m/z* 1180.6 – 29.6% *corr Pi* at *m/z* 1182.6. Figure 3A(iii) depicts a histogram of the resultant corrected overlapping peak ratios as: IAA: IAA/BAA**:BAA**, 1.00: 1.06: 1.03; indicating nearly equal proportions of the three major redox states of the cysteine residues within ZnF2B, as illustrated in Fig. 2.

To confirm the validity of this relative quantification of overlapping peaks, we employed a model synthetic peptide KNRRKSCQACRLRK spanning the ZnF2B region of ER (Fig. 1). The spectrum shown in Fig. 3B(i) with peaks at m/z 1863, 1865 and 1867 was derived from an equimolar ternary mixture of differentially alkylated synthetic peptides (using 2IAA, 2IAA* and 2BAA** as isotope labels). Computation of a correction factor for overlapping peaks was performed in a similar manner to that described above: $corr\ Pi\ at\ m/z\ 1867 = obs\ Pi\ at\ m/z\ 1867 - 60\% corr\ Pi\ at\ m/z\ 1865 - 10.3\% obs\ Pi\ at\ m/z\ 1863$. After correction, the peak intensities for the equimolar mixture of three differently labeled species gave the experimental ratio for IAA:IAA*:BAA** equal to 1.00:1.02:1.13 (Fig. 3B(iii)). Furthermore, this confirmed that under the reaction conditions selected for derivatization, all reactions went to completion, even though BAA is known to be less reactive than IAA.

Comparison of different MALDI-MS instrument platforms

If a sensitive and accurate assay could be developed using MALDI ionization, this might have some advantages over electrospray ionization (ESI) for routine clinical analysis as the former method is more robust, even though ESI has been more widely used in quantitative studies. In the past most MALDI instruments were based on time-of-flight but alternatives are now becoming available, including the MALDI-LIT and Fourier transform (FT) instruments. As both TOF and LIT instrument types were available for this study we evaluated both platforms here. FT instruments are capable of higher performance in terms of mass range, peak resolution and mass accuracy but are less widely available and were not considered in this study. Furthermore, our digestion strategy was to develop a digest that produces peptides of a size suite to the TOF and LIT instruments. Theoretically our assay would be based on a comparison of the peak intensities of only two differentially labeled species, the monoisotopic peaks being at m/z 1180.6 and 1184.6, i.e. separated by 4 Da. But an intermediate species was also prevalent (mixed derivatives) so this was included in our model studies based on the somewhat larger synthetic peptide analog. Mixtures of the synthetic peptide modified by the 3 stable isotope reagents IAA, IAA* and BAA** were prepared in molar ratios 1:1:1, 1:1:2, 1:1:4, 1:1:0.5 and 1:1:0.25 (IAA: IAA*: BAA). Each experiment was repeated independently three times and in every case MALDI mass spectra were obtained from both instruments. Manual correction of overlapping peak intensities was performed on individual spectra as described earlier to resolve the monoisotopic species at m/z 1862.9, 1864.9 and 1866.9. Regression analysis of scatter plots of the calculated and experimentally derived ratios (IAA/BAA) gave rectilinear relationships between detector response and the relative peptide intensities (Fig. 4); with both MALDI-TOF and MALDI-LIT producing virtually identical correlation coefficient (r^2) values of 0.9980 and 0.9982 respectively. These results provide a direct comparison of relative peptide quantification in MS mode on two instruments having totally different mass analysis methods. In the MALDI-TOF instrument, delayed ion extraction coupled with reflectron operation compensates for kinetic energy differences of ions and thus good resolution of peaks over a wide mass range is standard. In the case of the linear ion trap mass analyzer, operation in normal scan mode produces only poorly resolved peaks, incompatible with accurate quantification. However, operation in the zoom scan mode over the narrow selected mass range centered at m/z 1865 $\pm$ 8 Da gave Gaussian-shaped peaks at higher resolution that were excellent for peptide quantification.

For effective quantitation it was important to establish that the zoom scan technique did not induce any non-linearity or other discrimination. Naturally occurring isotopic ratios for non-labeled peptides were found to match with the expected abundances and there was good agreement between ratios determined by TOF and LIT. This suggests that there is no significant discrimination over the $\pm$ 8 Da mass window we selected in the zoom scan. Interestingly, most labeling strategies such as ICAT have sought to expand the mass separation between the light and heavy variants but a method that gives relatively small mass differences may have an

advantage in minimizing any discrimination caused by a non-linear response in the zoom scan. To our knowledge, this is one of the first studies that compares peptide quantitation in the MS mode with the above mentioned MALDI platforms that employ completely different mass analysis methods, and further extends the comparison to include MSMS mode with the linear ion trap (see below).

Monitoring oxidation of ER by MALDI-MS: a comparison with immunochemistry

Having established linear association of experimental and expected/calculated ratios by MALDI-MS for isotopically labeled peptide mixtures, it was important to confirm the oxidation status of these cysteine residues in ER by an alternative assay technique. Titrating rER with increasing concentrations of the specific cysteine oxidant diamide or the more general reagent hydrogen peroxide, produces progressive cysteine disulfide formation. For MS analysis after oxidant treatment, rER was treated with the “light” reagent IAA, reduced, treated with “heavy” BAA**, digested and the peptide mixture then analyzed by MALDI-TOF. Representative spectra generated from in-solution digests of diamide-treated rER are shown in Fig. 5A. The spectra showed systematic increments in the relative amount of oxidized ZnF2B, represented by the peak at m/z 1184.6, with a concomitant decrease in the non-oxidized species corresponding to the peak at m/z 1180.6. For immunoassay, aliquots of the same set of diamide-treated rER samples, separated by 1-D SDS-PAGE, were immunoblotted using a novel anti-oxER antibody that specifically detects cysteine disulfides within ZnF2B²³, normalized by total ER immunoblot and detected using the F10 commercial ER monoclonal antibody that is reactive with the C-terminus of human ER- α , as shown in fig 5B. The densitometric data from the blot parallels the MS generated data, as illustrated graphically by the histogram in Fig. 5C, confirming that MALDI-TOF-MS and immunochemistry are similarly capable of quantifying cysteine oxidation in ER ZnF2B.

Comparison of MS and MSMS

Direct MS analysis of peptide mixtures generated by digestion of a recombinant protein yield relatively clean spectra that are largely devoid of overlapping signals that would introduce major errors in quantitation. However, it is anticipated that the equivalent analysis of ER peptides in clinical samples will be subject to extensive chemical noise that may well invalidate such results. Tandem mass spectrometric methods such as selected reaction monitoring provide well-established protocols for detecting low-level species in complex mixtures. A number of instrument platforms have been employed to carry out MALDI-MS/MS experiments, including the TOF/TOF^{24, 25} and Q-TOF^{26, 27} and more recently LIT²⁸.

MALDI-LIT instruments allow MS/MS and MSⁿ experiments and therefore should be suited to such analyses. As a preliminary step in the use of MS/MS it was necessary to identify fragment peaks in the MALDI-LIT tandem spectra of isotopically labeled synthetic peptides that retain the isotopic information that identifies the reagent with which they were reacted, i.e. fragment ions that retain one or more derivatized cysteine residues. We prepared and analyzed five binary mixtures of the IAA and BAA** labeled synthetic peptides in the ratios 1:1, 1:2, 1:4, 1:0.5, 1:0.25. Fig 6A and 6B show representative mass spectra acquired in MS mode for equimolar mixtures of the two peptides by MALDI-TOF and vMALDI ion trap respectively. As described earlier, correction for peak overlaps was carried out viz: $\text{corr } P_i \text{ at } m/z \text{ 1184.6} = \text{obs } P_i \text{ at } m/z \text{ 1184.6} - 10.3\% \text{ obs } P_i \text{ at } m/z \text{ 1180.6}$. By selecting a mass window centered at $m/z \text{ 1865} \pm 8 \text{ Da}$ in a double play zoom scan mode, the parent ions were fragmented in the ion trap and relative quantification performed on product ion pairs showing either 2-Da or 4-Da mass differences, i.e. representing fragment ions containing one or two labeled cysteine residues respectively. Fig. 6C shows product ion pairs containing one cysteine residue (m/z : 967.5/969.5; 984.5/986.5; 1147.5/1149.5; 1770.9/1772.9) or two cysteines residues (m/z : 1716.9/1720.9).

The fragment ion sequences were identified from the MS/MS spectra and the elemental compositions were used to generate the theoretical isotopic distributions needed for correction of isotopic overlaps (Table 1), allowing corrected peak ratios to be determined. Based on the five samples with relative concentrations ranging from 1:4 to 4:1, scatterplots were generated for the parent ion and each fragment ion. For each ion a correlation coefficient, $r^2 = 1$ and intercept = 1, would imply a perfectly linear association between experimental and predicted values. The three strongest ion pairs m/z 1770.9/1772.9, 984.5/986.5 and 1147.6/1149.6, gave the best statistical results, i.e. ratios closest to 1.0, lowest standard deviations over three separate experiments and correlation coefficients closest to 1.0, compared with the 2 weakest ion pairs m/z 1716.9/1720.9 and 967.5/969.5. As was pointed out by a reviewer, the (A+1)/A ratios for the two weakest pairs were obviously higher than predicted from natural isotope ratios, unlike the three strongest ion pairs, making any quantitation from the weak peaks unreliable. Possible contributors to background ions are multiple fragmentations of the peptide ions, which is not uncommon in singly charged ions, or fragmentation of matrix clusters. The second of these explanations seems less likely as matrix peaks would have different fractional masses and would appear as separate components or would broaden the peaks, which did not occur. Whatever the cause, it is prudent to exclude data from peaks that have (A+1)/A isotopic ratios that are obviously incorrect. After excluding the weakest peaks the data further confirm that mass spectrometric quantification gives accurate results in both MS and MS/MS modes. However, tandem-MS mode is usually preferred for quantification in complex mixtures as the reduced chemical noise should afford higher accuracy for quantitative analyses.

On a related topic, isotopic distributions can be difficult to distinguish in the presence of deamidation, which can occur during sample preparation or in MS analysis. However, isotope ratios measured for the unlabeled peptides showed no evidence for deamidation, and there is no reason to assume that labeled peptides would behave differently.

Conclusions

We previously established that the C-terminal half of the structurally more labile ER second zinc finger (ZnF2B) appeared not only to be most sensitive to oxidative stress, but also yielded a peptide sequence rich in basic residues (SCQACRLRK) that is readily ionized and detected by MALDI-MS. Here we have employed a synthetic peptide incorporating the ZnF2B sequence, in conjunction with three stable isotope versions of a very common thiol-alkylating agent (IAA), to model isotopic overlaps encountered during relative quantitation of cysteine oxidation in oxidant modified ER. Often the choice of relatively more expensive sulfhydryl reactive stable isotope reagents such as ICAT for quantitative studies has been based on the perceived need to avoid isotopic overlaps of diagnostic ion pairs in the mass spectra; thus simplifying relative peptide quantitation. This study has demonstrated that by application of theoretically generated correction factors, isotopic overlaps do not prevent reliable quantitative information being obtained. Using MALDI-MS we demonstrate that relative quantification of cysteine oxidation in ER can be achieved in both the MS and tandem-MS modes, regardless of the presence of isotopic overlaps. We also employed two different mass spectrometer types, a time-of-flight and linear ion trap, to demonstrate that these findings are independent of instrument platform. Additionally, we establish that results from our MS-based quantitative protocol are consistent with results from immunochemical methods. Thus we have suitable methodology to monitor the effects of oxidative stress on the key protein that is critical to the biology of ER-positive breast cancer at the molecular level.

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Fig. 1.

Functional domains of ERα showing the amino acid sequence of the DBD; the predicted zinc finger peptides derived by double enzyme digestion with Lys-C and Asp-N as well as the synthetic peptide used in the study.

Fig.2.

Schematic illustration of the possible different forms of cysteine disulfides that may be formed within the C-terminus of zinc finger 2 (ZnF2); the differential alkylation strategy used for monitoring redox status of cysteine within ZnF2B peptides is presented: resulting in free sulfhydryls (m/z 1180.56) or mixed oxidized/reduced species (m/z 1182.56) or disulfides (m/z 1184.56)

Fig 3.

Expanded MALDI-TOF spectra of : A(i) diagnostic ZnF2B peptide derived from isotopic labeling and digestion (Lys-C/Asp-N) of diamide treated rER; B(i) Equimolar ternary mixture of isotopically labeled synthetic peptide: Theoretical isotopic distribution profiles of: A(ii) ZnF2B (m/z 1180.56) and B(ii) synthetic peptide (m/z 1863): Graphical output of corrected peak overlaps of A(iii) ZnF2B and B(iii) equimolar mixture of labeled synthetic peptide

Fig. 4.

Regression analysis of A) MALDI-TOF and B) MALDI-LIT data derived from quantitative analysis of five ternary mixtures of stable isotope conjugates of the synthetic peptide used in study: IAA:IAA*:BAA, 1:1:1, 1:1:2, 1:1:4, 1:1:0.5, 1:1:0.25 as molar ratios.

Figure 5.

Changes in the redox status of cysteine residues within DBD of diamide titrated rER monitored by: A) MALDI-TOF showing representative spectra of oxidized (m/z 1180.56) and unoxidised (m/z 1184.56) ZnF2B peptides at different concentrations of oxidant; B) immunochemical technique (Western blot) by utilizing a novel anti-oxER antibody to detect oxidized ER normalized by total ER content. C) Comparison of techniques used for quantitation of oxidized ER: by densitometry and MALDI/TOF-MS.

Figure 6. MALDI-MS spectra of an equimolar mixture (IAA:BAA**, 1:1) of isotope labeled synthetic peptide (KNRRKSCQACRLRK) analyzed by: A) MALDI/LIT; B) MALDI-TOF; C) MS/MS from MALDI-LIT showing the base peak ion chromatogram of cysteine containing fragment ion pairs (inserts) of the labeled synthetic peptide

Table 1

Peptide sequences of cysteine-containing product ions used to obtain the theoretical isotopic distributions. Gradients and correlation coefficients from scatterplots of experimental versus expected ratios for product ion pairs are presented.

Product ion (m/z)	Sequence	Gradient (mean $\pm$ SD, n =3)	r ² (mean $\pm$ SD, n=3)
967.5	NRRKSCQA-H ₂ O-NH ₃	0.855 $\pm$ 0.005	0.9949 $\pm$ 0.005
984.5	NRRKSCQA-H ₂ O	1.093 $\pm$ 0.001	0.9968 $\pm$ 0.002
1147.5	KNRRKSCQA (C ₉)	1.008 $\pm$ 0.001	0.9996 $\pm$ 0.003
1716.9	KNRRKSCQACRLR (b ₁₃ , y ₁₃ -H ₂ O)	0.824 $\pm$ 0.009	0.9903 $\pm$ 0.01
1770.9	KNRRKSCQACRLRK-HSCH ₂ CO ₂ H	1.038 $\pm$ 0.001	0.9953 $\pm$ 0.009