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Redox proteomics of protein-bound methionine oxidation

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Running title: System-wide analysis of methionine oxidation

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Abbreviations

COFRADIC: combined fractional diagonal chromatography

MetO: methionine-sulfoxide

Msr: methionine-sulfoxide reductase

ROS: reactive oxygen species

Summary

We here present a new method to measure the degree of protein-bound methionine-sulfoxide formation at a proteome-wide scale. In human Jurkat cells that were stressed with hydrogen peroxide, over 2,000 oxidation-sensitive methionines in more than 1,600 different proteins were mapped and their extent of oxidation was quantified. Meta-analysis of the sequences surrounding the oxidized methionine residues revealed a high preference for neighboring polar residues. Using synthetic methionine-sulfoxide containing peptides designed according to the observed sequence preferences in the oxidized Jurkat proteome, we discovered that the substrate specificity of the cellular methionine sulfoxide reductases is a major determinant for the steady-state of methionine oxidation. This was supported by a structural modeling of the MsrA catalytic centre. Finally, we applied our method onto a serum proteome from a mouse sepsis model and identified 35 *in vivo* methionine oxidation events in 27 different proteins.

Introduction

Reactive oxygen species (ROS) are involved in a broad range of processes including signal transduction and gene expression (1), receptor activation (2), antimicrobial and cytotoxic actions of immune cells (3), and aging and age-related degenerative diseases (4). Cellular oxidative stress is associated with increased levels of reactive oxygen species and the molecular damages they cause (5). Of interest here is that some reactive oxygen species specifically modify targeted biomolecules, while others cause non-specific damage. Peroxides for instance are generally more selective compared to hydroxyl radicals (6). A major ROS target are proteins, with oxidation occurring both at the peptide backbone and at amino acid side-chains (6). The major oxidation product of protein-bound methionine is methionine-sulfoxide, further oxidation of which can lead to methionine-sulfone, albeit to a much lesser extent (7). The (patho)physiological importance of this modification is reflected by the methionine sulfoxide reductases (Msr) that are present in nearly all organisms (8, 9): decreased activity of these enzymes was associated with aging and Alzheimer disease (10), and abnormal dopamine signaling was recently found in the methionine sulfoxide reductase A knockout mouse (11). Oxidation of methionine can lead to loss of enzyme activity as shown for a brain voltage-dependent potassium channel (12). Other studies suggest that methionine oxidation prevents methylation (13) or has an effect on phosphorylation on serines and threonines proximate to the oxidized site (14). In this respect, protein kinases are also targeted by methionine oxidation affecting their activity (e.g., (15)). Further, oxidation of surface methionines increases the protein surface hydrophobicity (16) and may perturb native protein folding, and such oxidized proteins further often become targets for degradation by the proteasome (17).

Although methionines are utmost susceptible to oxidation by several types of ROS (18), no adequate proteomic methodologies exist to characterize the exact sites of oxidation and quantify the degree of oxidation. Only very recently, Oien *et al*. generated polyclonal antibodies against

oxidized methionines (19) and although these antibodies identified oxidized proteins, they were unable to identify the exact site of oxidation. By considering the 16 Da mass increase upon oxidation, Rosen *et al*. (20) used spectral counting of both oxidized and non-oxidized peptide species to calculate the general degree of methionine oxidation. However, since methionine-sulfoxide containing peptides were not enriched prior to analysis, it may be expected that many such peptides were overlooked given the complex background of the analyte mixture, and further no attempt was made to distinguish artificial methionine oxidation occurring during sample handling from *in vivo* oxidation.

We here present a COFRADIC (combined fractional diagonal chromatography) proteomics technology to map *in vivo* oxidized methionines and quantify their degree of oxidation. COFRADIC generally isolates a specific set of peptides by altering a peptide functional group or the side-chain of targeted amino acids in between consecutive and identical RP-HPLC peptide separations (21). We here took advantage of an enzymatic reduction of methionine-sulfoxides using a mixture of MsrA and MsrB3. The hydrophobic shift introduced in this way allowed sorting of methionine-sulfoxide containing peptides. Cellular methionine oxidation was studied in human Jurkat T-cells under hydrogen peroxide stress. In total, 2,626 methionine-sulfoxide containing peptides in 1,655 proteins were identified and their degree of oxidation was quantified. Bioinformatic analysis of the data pointed to a sequence motif favoring cellular methionine oxidation. Peptide studies further revealed that the rates of both MsrA methionine-sulfoxide reduction and unexpectedly, also methionine oxidation are influenced by the primary sequence surrounding the methionine. Structural modeling studies on MsrA further confirmed our results. Finally, we performed a differential analysis on serum from a female C57BL6/J mouse in which septic shock was induced by intravenous *Salmonella* infection, and identified 35 *in vivo* oxidized methionine sites in 27 different proteins.

Experimental procedures

Reduction of a methionine-sulfoxide peptide using MsrA and MsrB

The peptide NH_2.IPMYSIITPNVLR.COOH was in-house synthesized using Fmoc-based chemistry. 2 nmol of this peptide was dissolved in 100 µL 1% acetic acid and treated with 0.5% (w/v) of hydrogen peroxide (Sigma-Aldrich, Steinheim, Germany) during 30 min at 30°C followed by immediate injection onto a RP-HPLC column (2.1 mm internal diameter x 150 mm (length) 300SB-C18 column, Zorbax®, Agilent, Waldbronn, Germany) using an Agilent 1100 Series HPLC system. Following a 10 min wash with HPLC solvent A (10 mM ammonium acetate in water/acetonitrile, 98/2 (v/v), water (LC-MS grade, Biosolve, Valkenswaard, The Netherlands) and acetonitrile (HPLC grade, Baker, Deventer, The Netherlands)), a linear gradient to 100% solvent B (10 mM ammonium acetate in water/acetonitrile, 30/70 (v/v)) was applied over 100 min. Using Agilent's electronic flow controller, a constant flow of 80 µl/min was used. The oxidized peptide was collected and split into aliquots of 500 pmol that were vacuum dried. These aliquots were then used for reduction with MsrA (Jena Bioscience, Jena, Germany), MsrB (Jena Bioscience) or a mix of both reductases. The oxidized peptides were re-dissolved in 95 µL 25 mM Tris.HCl pH 7.6 containing 10 mM DTT. To this mixture, 2.5 µL of the stock solution of MsrA and/or MsrB was added and reduction was allowed for 2 hours at 37 °C. The enzymes were then removed using a Ni-NTA (Probond™ Resin from Invitrogen, Paisley, UK). In practice, 20 µL of 50% slurry (in ethanol) was placed on a Pierce® Spin Cups-Paper Filter (Thermo Scientific, Waltham, MA, USA) and washed twice with 150 µL 25 mM Tris.HCl pH 7.6. Next, the reaction mixture was put onto the spin column and binding of the His-tagged MsrA and MsrB enzymes to the Ni-NTA beads was allowed for 20 min at room temperature, followed by a centrifugation step for 4 min at 200 x g. The mixture was acidified with 5 µL 20% acetic acid and analyzed by RP-HPLC as described above.

Oxidation of SILAC labeled Jurkat cells with hydrogen peroxide

Heavy SILAC medium was prepared by adding $^{13}C_5$-methionine (Cambridge Isotope Laboratories, MA, US) to a final concentration of 15 mg/ml (101 µM) together with 20 IU/ml penicillin, 20 µg/ml streptomycine and 10 % (v/v) dialyzed foetal calf serum (devoid of all substances less than about 10 kDa, Invitrogen) to methionine-free RPMI-1640 medium (Invitrogen). Light SILAC-medium was RPMI-1640 medium containing 10% (v/v) dialyzed foetal calf serum, 20 IU/ml penicillin and 20 µg/ml streptomycine. To ensure complete metabolic labeling, Jurkat cells (ATCC, Teddington, UK) were grown for 7 days prior to H_2O_2 treatment. For each proteomics experiment, 100 ml cell suspension (300 x 10^3 cells/ml) was centrifuged for 5 min at 1500 x g, the pellet was washed twice with ice-cold PBS and re-dissolved in 50 ml PBS (37°C). For the first experiment, 5.5 µl of 30% (w/v) H_2O_2 (Sigma) (a final H_2O_2 concentration of about 1 mM) was added to heavy labeled cells and these cells were incubated for one hour at 37°C after which a second bolus of 11 µl 30% H_2O_2 was added and cells were incubated for an additional hour. Following incubation, cells were centrifuged for 5 min at 1500 x g and cell pellets were washed three times with ice-cold PBS to remove all H_2O_2. An analogous proteomics experiment but with swapped labeling was carried out.

Preparative steps for the COFRADIC isolation of methionine-sulfoxide containing peptides

Cell pellets were lysed in 500 µl 300 mM Tris.HCl (pH 8.7) containing 150 mM NaCl, 1 mM EDTA (Sigma-Aldrich), 0.8% CHAPS (Sigma-Aldrich), 1 M guanidinium hydrochloride and the appropriate amount of the Complete protease inhibitor cocktail (Roche, Basel, Switzerland). Cell lysis was performed for 10 min on ice followed by sonication and cellular debris was removed by centrifugation for 30 min at 16.000 x g and 4 °C. Alkylation of cysteines was carried out by adding iodoacetamide (Sigma-Aldrich) and triscarboxyethylphosphine (Pierce, Rockford, IL

(US)) to final concentrations of 60 mM and 20 mM respectively, for 30 min at 37°C. Both samples were desalted on a NAP™-5 column (Amersham Biosciences, Uppsala, Sweden) in 1 ml of 50 mM ammonium bicarbonate (pH 8.0). Prior to digestion, protein concentrations were measured using the Biorad Protein Assay. Samples were heated for 10 min at 95°C and immediately put on ice for another 15 min. Sequencing-grade modified trypsin (Promega, WI, USA) was added in a 1/100 (w/w) enzyme-to-substrate ratio and following overnight digestion at 37°C, the volume was reduced to 500 µL by vacuum drying.

100 µL (corresponding to approximately 150 µg of protein material) of the digest of the control setup was acidified with 10 µL 10% acetic acid and oxidation of methionines was carried out using 0.5% (w/v) H_2O_2 during 30 min at 30°C. Following oxidation, the pH was adjusted to 8.0 by adding 30 µL of a 1 M stock solution of tri-ethylammoniumbicarbonate (TEAB) (Sigma-Aldrich). 50 µL of 50% catalase-agarose slurry was washed twice with 50 mM TEAB on a spin-cup paper filter (Thermo Scientific), the reaction mixture was then added to the catalase-agarose to neutralize H_2O_2 during 5 min at 30°C. 100 µL (approximately 150 µg of protein material) of the proteome digest of H_2O_2-treated cells was treated analogously except that, instead of hydrogen peroxide, an equal volume of water was added. Finally, samples were mixed in a 1/1 (w/w) ratio and the volume was reduced to 100 µL by vacuum drying.

COFRADIC isolation of methionine-sulfoxide peptides

The peptide mixture was acidified to a final concentration of 1% acetic acid for RP-HPLC separation onto a 2.1 mm internal diameter x 150 mm 300SB-C18 column (Zorbax®, Agilent) with an Agilent 1100 Series HPLC system using the conditions described above. Peptides eluting between 20 and 80 min were collected in 60 fractions of 1 min each (80 µL) in a 96 well plate. Primary fractions that were separated by 15 min were pooled and vacuum dried. Prior to a series of secondary RP-HPLC separations under identical conditions as the primary one, each

pooled fraction was re-dissolved in 90 µL 25 mM Tris.HCl pH 7.6 containing 10 mM DTT, 4 µL of each stock solution of MsrA and MsrB was added and reduction of methionine-sulfoxides took place for 3 hours at 37°C. The reductases were then removed as described above after which the peptide mixture was acidified with 10 µL of a 10 % acetic acid solution and separated by RP-HPLC. Reduced methionyl peptides were collected in an interval ranging from 2 to 10 min following the collection interval of each primary fraction. Such peptides were collected in 6 fractions and, since per secondary run 4 primary fractions were pooled, 24 secondary fractions were collected per run. A total of 360 secondary fractions containing methionyl peptides were in this way collected and to reduce analysis time on the mass spectrometer, secondary fractions that were separated by 15 min were further pooled and vacuum dried. Prior to LC-MS/MS analysis, methionyl peptides were converted into their sulfone form by performic acid oxidation (22). In practice, 900 µL of formic acid (Sigma-Aldrich) was added to 100 µL of 30% hydrogen peroxide (Sigma-Aldrich) and the mixture was kept for 60 min at 25°C. 8 µL of this solution was added to the peptides and the oxidation took place for 45 min on ice followed by the addition of 500 µL water (Baker B.V.) after which the peptide samples were vacuum dried.

LC-MS/MS analysis

The dried peptide fractions were re-dissolved in 15 µL 2% acetonitrile and 8 µL was used for LC-MS/MS analysis using an Ultimate 3000 nano-HPLC system (Dionex, Amsterdam, The Netherlands) in-line connected to a LTQ Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition for the six most abundant ion peaks per MS spectrum. Full scan MS spectra were acquired at a target value of 1E6 with a resolution of 30,000. The six most intense ions were then isolated for fragmentation in the linear ion trap. In the LTQ, MS/MS scans were recorded in profile mode at a target value of 5,000. Peptides were

fragmented after filling the ion trap with a maximum ion time of 10 ms and a maximum of 1E4 ion counts. From the MS/MS data in each LC-run, Mascot generic files (mgf) were created using the Mascot Distiller software (version 2.2.1.0, Matrix Science). When generating these peak lists, grouping of spectra was performed with a maximum intermediate retention time of 30 seconds and maximum intermediate scan count of 5, used where possible. Grouping was done with 0.1 Da tolerance on the precursor ion. A peak list was only generated when the MS/MS spectrum contained more than 10 peaks, no de-isotoping was performed and the relative S/N limit was set at 2. MS/MS peak lists were searched with Mascot using the Mascot Daemon interface (version 2.2.0, Matrix Science). Spectra were searched against the Swiss-Prot database (version 56.4) and taxonomy was set to *Homo sapiens* (20,328 entries). Variable modifications were set to pyro-glutamate formation of N-terminal glutamine and acetylation of the protein's N-terminus. Fixed modifications were methionine sulfone and carbamidomethylated cysteine sulfon. Mass tolerance of the precursor ions was set to ±10 ppm and of fragment ions to ±0.5 Da. The peptide charge was set to 1+, 2+ or 3+ and one missed tryptic cleavage site was allowed. Also, Mascot's C13 setting was to 1. Further, the different SILAC labels (here, $^{13}C_5$-methionine or $^{13}C_5$-methionine) are, during database searching, automatically accounted for by Mascot by selecting these labels in the Mascot Distiller environment. Of note here is that in this context, such labels are considered as so-called "exclusive modifications" that allow any given peptide to hold any of the selected isotopic labels, and, in this way, Mascot tries to optimize the number of significant spectrum-to-peptide matches. Only peptides that were ranked one and scored above the identity threshold score set at 99% confidence were withheld. We calculated the FDR based on the method of Elias and Gygi, 2007 (23) using the following formula: FDR = 2*(#false positives)/(#all identifications).Therefore, we created a shuffled database of the Swiss-Prot *Homo sapiens* database and concatenated this database to the "forward" Swiss-Prot *Homo sapiens* database using DBToolkit (24). For the quantitation protocol, we calculated that 0.20% and 0.27% false

positive sequences were present in respectively the forward and reverse labeling proteome analysis. For the validation protocol, we estimated the FDR at 0.29% and 0.28% for the forward and reverse proteome analysis respectively.

Differential analysis using the Mascot Distiller Toolbox

Mascot Distiller Quantitation Toolbox was used in the 'precursor' mode for quantification of the identified peptides. Mascot Distiller detects peaks by trying to fit an ideal isotopic distribution on experimental data. This distribution is predicted using average amino acid compositions for a peptide. This is followed by extraction of the XIC signal of both peptide components (light and heavy) from the raw data. Ratios are then calculated from the area below the light and heavy isotopic envelope of the corresponding peptides (integration method 'trapezium' and integration source 'survey'). To calculate this ratio value, a least squares fit to the component intensities from the scans in the XIC peak was created. MS scans used for this ratio calculation are situated in the elution peak of the precursor determined by the Distiller software (XIC threshold 0.3, XIC smooth 1, max. XIC width 250). To validate the calculated ratio, the standard error on the least square fit has to be below 0.16 and correlation of the isotopic envelope should be above 0.97.

Influence of the primary peptide sequence on methionine-sulfoxide reduction

Peptides (**Supplementary Figs. 7, 10** and **11**) were in-house synthesized using Fmoc-based chemistry. 4 nmol of each peptide was oxidized into its methionine-sulfoxide form with 0.5% (w/v) H_2O_2 in 100 µL of 0.5% TFA for 30 min at 30°C, after which the peptide was immediately analyzed by RP-HPLC (see above). Peptide-sulfoxide forms were collected and split into 1 nmol fractions in 100 µL of 25 mM Tris.HCl pH 7.6 with 10 mM of DTT. 3 of these fractions were treated with 2 µg of MsrA and 2 µg of MsrB3 for 30 min at 37°C, followed by acidification to 1% TFA (final concentration). The remaining peptide fraction served as a negative control. Reaction

mixtures were separated by RP-HPLC (see above) and the degree of reduction was measured by comparing the integrated peak surfaces of the oxidized and the reduced peptides. Data from 3 independent experiments for each peptide were compiled.

Influence of the primary peptide sequence on methionine oxidation

1 nmol of each peptide was dissolved in 85 µL 25 mM Tris.HCl pH 7.6. Methionine oxidation was carried out with 0.1% (w/v) of hydrogen peroxide during 10 min at 25°C followed by immediate separation of the peptide mixture over a RP-HPLC column (see above). The degree of oxidation was measured by comparing the integrated peak surfaces of the oxidized and the non-oxidized peptides. Data from 3 independent experiments for each peptide were compiled.

Structural analysis of substrate binding in the methionine sulfoxide reductase A

The structure of bovine MsrA with PDB code 1FVA was used as template for building the homology model of human MsrA using the FoldX force field (25). The structure of 1FVA was first energy minimized with FoldX to get the most accurate energy predictions.

The crystal contact between the neighbouring molecules in the structure of the MrsA homolog of *M. tuberculosis* with PDB code 1NWA was reconstructed by using the *Crystallize* command in YASARA (26). This command applies crystal symmetry and unit cell data to reconstruct crystal packing of the molecules. Structural superposition of the human model with the bacterial homolog allowed us to position the crystal packing hexapeptide in the human model to obtain a human MsrA-peptide complex model. Superposition and RMSD calculation of the bacterial structure with the bovine structure was carried out in YASARA using the MUSTANG (27) superposition algorithm. After mutating all but the methionine residue in the peptide, FoldX was run to build *in silico* mutations on each position to every amino acid and calculate the energy

difference with alanine as reference. All molecular graphics were created with YASARA (http://www.yasara.org) and PovRay (http://www.povray.org).

Methionine oxidation during Salmonella induced sepsis in mouse serum proteins

Female C57BL6/J mice were from Janvier (Le Genest-St Isle, France) and used at the age of 8 to 10 weeks. The mice were maintained in a temperature-controlled, air-conditioned SPF animal facility with 14h/10h light/dark cycles and received food and water *ad libitum*. All experiments were approved by the animal ethics committee of the Faculty of Sciences of Ghent University (Belgium) and performed according to its guidelines.

Pathogenic *Salmonella enteritidis* (serovar typhimurium) were from ATCC. Bacteria were diluted in lipopolysaccharide (LPS) free PBS to a concentration of 4.10^7 colony-forming units per ml and 0.25 ml was intravenously injected per mouse in the right tail vein. Mice were bled daily at 9 am at the retro-orbital plexus. Blood was allowed to clot for 60 min at 37ºC and further overnight at 4ºC. The clot was removed and cells were pelleted by centrifugation at 14.000 x rpm for 15 min. Serum was separated and stored at -20 ºC until further use. Serum samples collected at day 0, 1 and 4 were each pooled to obtain a total volume of 150 µl of crude serum per condition, followed by depletion of the top three most abundant proteins: albumin, immunoglobulin G and transferrin. A commercially available affinity system was used for this purpose (Multiple Affinity Removal System, Agilent Technologies). 2 ml of the depleted serum was reduced to 900 µl by vacuum drying. The pH of the solution was adjusted to 8.7 by addition of 100 µl of 1 M Tris.HCl pH 8.7. Alkylation of cysteines was carried out using final concentrations of 60 mM of iodoacetamide and 30 mM of TCEP for 30 min at 37°C. Excess reagents were removed by desalting over a NAP-10 (Amersham) desalting column in 1.5 ml of 50 mM triethylammonium bicarbonate buffer pH 7.8. The protein concentration was determined using the Bradford assay. Proteins were heated prior to digestion for 10 min at 95°C and after cooling down on ice,

endoLys C (endoproteinase Lys C, Roche Diagnostics Gmbh, Mannheim, Germany) was added in a 1/200 ratio (w/w) followed by digestion overnight at 37 °C.

After digestion, peptides were propionylated with either the light ($^{12}C_3$) (day 0) or heavy ($^{13}C_3$) (day 1 and day 4) sulfo-NHS-propionyl. Practically, we added 6 mg of this reagent to the digested proteins. Based on the protein concentration measured prior to digestion, we mixed equal amounts of peptide mixtures prior to COFRADIC analysis (about 300 µg of protein material was used per analysis).

COFRADIC analysis of methionine sulfoxides formation during sepsis

For the identification of methionine oxidation of serum proteins in mice we mixed serum proteins from day 0 (propionyl $^{12}C_3$) versus day 1 (propionyl $^{13}C_3$) and from day 0 (propionyl $^{12}C_3$) versus day 4 (propionyl $^{13}C_3$). We only applied the COFRADIC validation protocol onto these samples as described above. Following the COFRADIC sorting procedure we analyzed the obtained fractions onto an Ultimate 3000 nano-HPLC system in-line connected to a LTQ Orbitrap XL mass. Mass spectrometric analysis were performed as described (28).

MS/MS peak lists were searched with Mascot using the Mascot Daemon interface (version 2.2.0, Matrix Science). Spectra were searched against the Swiss-Prot database (version 56.4) and taxonomy was set to *Mus musculus* (15,988 entries). Enzyme was set to Endoproteinase LysC. Variable modifications were set to pyro-glutamate formation of N-terminal glutamine and acetylation of the protein's N-terminus. Fixed modifications were methionine sulfone and carbamidomethylated cysteine sulfon. Mass tolerance of the precursor ions was set to ±10 ppm and of fragment ions to ±0.5 Da. The peptide charge was set to 1+, 2+ or 3+ and one missed cleavage site was allowed. Also, Mascot's C13 setting was to 1, and note that now $^{12}C_3$- or $^{13}C_3$-N-propionylation were selected as the isotope labels in the Mascot Distiller environment (see

above). Only peptides that were ranked one and scored above the identity threshold score set at 99% confidence were withheld.

Mascot Distiller quantitation was performed as described above. For the false discovery rate of both analyses we applied the same procedure as described above and this resulted in a false discovery rate of 1.7% for the d0 versus d4 analysis and 3.5% for the d0 versus d1 sample.

Results

A COFRADIC method for isolating peptides containing methionine-sulfoxide. Our method both identifies protein-bound methionine-sulfoxides (MetO) and measures the degree of oxidation following isolation of MetO peptides using a diagonal chromatography (COFRADIC) approach (21). In essence, COFRADIC consists of three consecutive steps: 1) a first fractionation of peptides based on their hydrophobicity (RP-HPLC), 2) a chemical or enzymatic reaction that specifically modifies a subset of peptides in each primary fraction, and 3) a series of identical RP-HPLC fractionations of individual, or pooled, modified primary fractions aimed at isolating specific peptides. Here, the primary peptide fractions were treated with methionine sulfoxide reductases A and B3 which reduce MetO peptides into their more hydrophobic Met counterparts. Thus, when subjected to the series of secondary RP-HPLC separations, the so-generated Met peptides shifted to later elution times, while non-MetO peptides, including peptides containing methionines that were not oxidized by ROS, did not shift (**Fig. 1**). Only peptides with altered column retention – thus the reduced MetO peptides – were collected for LC-MS/MS analysis. As expected, both MsrA and MsrB3 were needed to reduce the population of R- and S-sulfoxide diastereoisomers (**Fig. 2**).

Quantification of the degree of methionine oxidation. Given that methionine is an essential amino acid for mammals, quantitative information on the degree of methionine oxidation was readily obtained by coupling the COFRADIC procedure to SILAC labeling (29), using either $^{12}C_5$ or $^{13}C_5$ methionine. One full MetO proteome analysis consisted of two different COFRADIC analyses (**Fig. 3**). For the first COFRADIC analysis ("quantification", **Fig. 3a**), all methionines in the proteome of control cells were converted to methionine-sulfoxide by controlled H_2O_2 oxidation. The so-generated pool of MetO peptides ("reference set") thus represented the maximum set of MetO peptides that could be recovered following proteome analysis of cells

under oxidative stress. This reference set was then mixed with a similar peptide mixture derived from the proteome of cells under oxidative stress, and such a peptide set will contain both Met and MetO peptides ("oxidized set"). COFRADIC was then used to isolate MetO peptides, and subsequent LC-MS/MS analysis led to the identification of isolated peptides, and their MS spectra were used to determine the degree of oxidation of identified Met residues by comparing the ion signals from "reference" peptides with those from "oxidized" peptides (**Fig. 3a**).

To further scan for spontaneous (artificial) and thus unwanted methionine oxidation during proteome handling and peptide preparation, we used a second COFRADIC method ("validation", **Fig 3b**) in which no reference set was created (peptides from the control setup were thus not oxidized). Proteins from both setups were mixed, trypsin digested and MetO peptides were isolated using COFRADIC. We now expect that if spontaneous methionine oxidation took place during preparative steps, it happened in both samples to the same or a highly similar extent and thus corresponding MetO peptides were seen as peptide couples with a ratio close to one in MS spectra (**Fig. 3b**). By combining the results of both COFRADIC analyses, we excluded all doubtful MetO peptides (totaling to about 4% of all identified peptides) and measured the degree of oxidation at the remaining individual sites.

In total, we performed 4 proteome analyses consisting out of two "quantification" COFRADIC analyses (two datasets with swapped labeling) and two "validation" COFRADIC analyses (also with swapped labeling). We manually checked results from the first quantification COFRADIC analysis and decided to use the other three datasets to further validate results from this analysis.

Of particular note here is that prior to LC-MS/MS analysis we converted methionine to methionine-sulfone by performic acid oxidation (30). This stabilizes methionine residues and avoids their spontaneous oxidation to sulfoxides prior to or during MS analysis, which else can interfere with downstream data analysis (e.g., quantification). Note that this performic acid oxidation also converts alkylated cysteines into a S-carboxamidomethyl-cysteine sulfones.

H_2O_2-sensitive methionines in human Jurkat cells. In the first proteome analysis 2,626 MetO peptides in 1,655 proteins were identified, and their degree of oxidation was quantified in human Jurkat cells under H_2O_2 stress (**Supplementary Table 1**[1]). Further validation of this dataset was performed by applying the validation COFRADIC approaches (also with swapped labeling) where true *in vivo* methionine oxidation events were identified as singletons in cells treated with hydrogen peroxide. Of note is that about 56% of the identified sites were also found in a biological replicate (an experiment with a label swap), hinting to the fact cellular methionine oxidation is not a fully random event. Next to this qualitative reproducibility, we also noticed quantitative consistency of the oxidation process (**Supplementary Fig. 1**). The overlap of unique sequences in the four experiments (quantification and validation COFRADIC with label swapping) is shown in **Supplementary Fig. 2**.

An illustration of the obtained data is shown for a MetO peptide from the translational endoplasmic ATPase protein. This peptide contained two possibly H_2O_2-sensitive methionines, and the peptide was found to be oxidized to 50-60% (**Figs. 4a** and **4c**), whereas the corresponding control analyses did not reveal any artificial oxidation at these sites (**Figs. 4b** and **4d**). Our list of identified oxidation sites contains known sites such as methionines 44, 47 and 190 from cytoplasmic actin (P60709). Met-44 and 47 are the first sites that are oxidized by hydrogen peroxide, and further oxidation of Met-190 causes depolymerisation of actin filaments (31). Of note is that we here noticed that Met-44 and 47 were oxidized to about 20% and Met-190 only to 10%. Another example is Met-297 (7% oxidized) from the signal recognition particle 54 kDa protein (P61011). This methionine is located near the G-domain (position 295) that binds GTP. Its *E. coli* homologue was shown to be an important target for hydrogen peroxide oxidation

[1]These data were made available via the PRIDE database (http://www.ebi.ac.uk/pride/); accession numbers 16645 and 16646, username: review69524, password: Q79!f*7e. PRIDE Inspector, available either directly via the link in the PRIDE experiment, or via http://code.google.com/p/pride-toolsuite/wiki/PRIDEInspector is recommended to browse the stored spectra and peptide identifications.

and can be repaired by methionine sulfoxide reductases (32). The oxidized form of the bacterial protein shows reduced interaction with 4.5 S RNA, and upon aligning the sequences of the human and bacterial proteins, Met-297 was found to reside in a short conserved sequence pattern (**Supplementary Fig. 3**), possibly suggesting an alike biological effect of oxidative stress in human cells. We also conducted a DAVID gene ontology search (33) to investigate biological pathways or protein classes that were highly affected by methionine oxidationhowever, in general no particular GO terms stood out from this analysis, suggesting that H_2O_2 oxidation acted throughout all protein complexes and cellular compartments.

Reduction of protein-bound methionine-sulfoxides is sequence-dependent. The possibility of quantifying the degree of oxidation at each identified methionine site in a proteome-wide context is a unique feature of our approach and allowed us to study in detail the degree by which different protein-bound methionines were affected by H_2O_2 stress. In fact, in H_2O_2-stressed Jurkat cells, the majority of the identified methionine oxidation sites (about 81.5%) was weakly or moderately oxidized (arbitrary set to maximum levels of 30% oxidation), whereas only a minor number of sites (18.5%) were oxidized to higher degrees (between 30%-100%) (**Supplementary Fig. 4**). An iceLogo (34) heat map on the dataset of peptides containing only one MetO residue showed that acidic amino acids closely surrounded the oxidized Met, whereas Trp, Tyr, Cys and His were underrepresented in this region (p-value at 0.05) (**Fig. 5** and **Supplementary Fig. 5**). This suggests that methionines within a polar environment, and thus most likely surface-oriented, were the main oxidation targets. Of interest is the underrepresentation of neighboring basic residues, strongly contrasting with the overrepresented acid residues. This is further corroborated by the poor presence of lysine just in front of the oxidized methionine (**Fig. 5**). To some extent this could be due to the use of trypsin in generating peptides for LC-MS/MS. The occurrence of multiple basic residues around MetO could for

instance have generated too short peptides, escaping LC-MS/MS identification. It is also possible that the presence of a basic residue before MetO generated a tryptic peptide with an N-terminal MetO that is not or only poorly reduced during COFRADIC. However, a more feasible explanation is that when interpreting the iceLogo data, it is important to consider the fact that the cells were still viable after H_2O_2 removal (90% viability compared to 95% viability in control cells after two hours of H_2O_2 treatment). Hence, the observed methionine-sulfoxide proteome represents a steady-state situation in which oxidation, a chemical event, is balanced by reduction, an enzymatic process.

Therefore, we studied the kinetics of methionine-sulfoxide reduction by MsrA and MsrB3 using a set of synthetic peptides in which the MetO resided in a region of acidic residues but in which the amino acid N-terminal to MetO was substituted. We observed a strong effect of the nature of this residue, with slower reduction when this position was occupied by Asp, Glu or Pro, and faster reduction with basic residues (**Supplementary Fig. 6**). Further explanation and support for these differential reductase activities came from our modeling studies. Interaction of substrates with MetO reductases was modeled using structural data available for MsrA (no structural data for MsrB3 are currently available), for which the human protein structure was modeled from its bovine homolog (35) that shares 89% sequence identity with structurally solved residues. Unfortunately the bovine structure does not contain a peptide substrate for specificity analysis, but a more remote MsrA homolog of *M. tuberculosis* was solved where a fortuitous crystal-packing contact was observed in which a methionine of one MsrA molecule binds to the active site pocket of a neighboring MsrA molecule (36). The sequence identity with the human homolog is only 36%, but structural superposition with the bovine structure shows a similar fold with an RMSD of 0.967Å over 151 structurally aligned residues. Since the methionine is located at the extreme N-terminus, structural data is available only for the hexapeptide sequence XXMXXX. To this extent the observed interaction can serve as a suitable model to analyze the interaction of

MsrA with peptide substrates. Here, the free energy changes upon mutation of the substrate peptide using the FoldX algorithm were predicted using Ala as reference (**Supplementary Fig. 7**). The influence of every amino acid was checked at different positions and, in agreement with our peptide studies (**Supplementary Fig. 6**), we found that Pro, Asp and Glu proximate to the oxidized Met are less well preferred and tolerated by MsrA. On the other hand, proximate Arg and Lys are preferred (also agreeing with our peptide study, **Supplementary Fig. 6**), and the formation of extra hydrogen bonds when these basic amino acids bind within the catalytic centre explains this (**Fig. 6**). Only the modeling results for proximate Ile and Val did not match the peptide reduction data; the former predicted that such peptides would not be reduced efficiently due to steric hindrances in the catalytic centre (**Supplementary Fig. 7**), however we noticed that such peptides were not reduced at significantly slower rates (**Supplementary Fig. 6**). This discrepancy could indicate the existence of different possible conformations of the peptide-receptor complex. Our modeling data also revealed that Pro at position -1 and +2 away from the oxidized methionine negatively influences substrate binding, a feature that is also apparent in the iceLogo of sites that were strongly (over 30%) oxidized in cells (**Supplementary Fig. 8**). We further confirmed that such positioned prolines also lead to slower reduction rates using synthetic peptides (**Supplementary Fig. 9**).

We thus conclude that the sequence surrounding of oxidation-sensitive methionines is a determining factor for the reduction efficiency of methionine-sulfoxides by methionine-sulfoxide reductases, and thus such sequence features will finally influence the degree of methionine oxidation at individual sites. It is thus tempting to believe that probably all surface-accessible methionines are targets for oxidation, but that the reverse process (reduction) will be sequence-dependent. In this respect, we also checked if the rate of methionine oxidation was sequence-dependent and quite to our surprise we could indeed detect oxidation rate differences, with

peptides having Lys-Met, Arg-Met, His-Met and Pro-Met motifs getting oxidized at slower rates (**Supplementary Fig. 10**).

Methionine oxidation upon *Salmonella* induced septic shock. Intravenous administration of *Salmonella* into mice causes a severe septic shock and leads to oxidative burst (37) during which oxygen and nitrogen-derived radicals are produced and released to create a toxic environment for invading pathogens. Excess radicals are scavenged by serum proteins, leading to protein modifications such as tyrosine nitration (28). Using our COFRADIC approach we next investigated whether this oxidative burst led to oxidation of methionine in serum proteins of mice infected with *Salmonella*. Prior to COFRADIC analysis, we depleted mouse serum of its three most abundant proteins (albumin, IgG and serotransferrin). We first compared methionine oxidation in serum proteins from control mice (day 0) and from *Salmonella* infected mice following one day of infection (day 1). Because the mice were not metabolically labeled we needed to use a post-metabolic labeling strategy, and instead of using trypsin as the protease, proteins were now digested with endoproteinase LysC and the resulting peptides were post-metabolically labeled using *N*-hydroxysuccinimide (NHS) esters of $^{13}C_3$ (day 1) or $^{12}C_3$ (day 0) propionic acid. Here, the majority of the generated peptides start with a free alpha-amino group and end on a lysine, carrying an epsilon-amino group. Both amino groups are modified with the isotopic variants of the propionyl group and thus a mass difference of 6 Da is introduced between peptides from control mice (day 0) and from the sepsis mouse model (day 1). Following peptide labeling, the two peptide pools were mixed and MetO peptides were isolated and identified. In a second analysis, we analyzed methionine oxidation in serum proteins from mice at day 0 and day 4 post *Salmonella* infection. Here, peptides from the day 0 sample were labeled by $^{13}C_3$-propionylation and those from the day 4 sample were labeled by $^{12}C_3$-propionylation.

Following LC-MS/MS analysis, a first selection of candidate methionine-sulfoxide peptides was based on the Mascot Distiller quantification quality control. Mascot Distiller allows automatic quantification of the light and heavy isotopic labeled peptides, and its intrinsic quality control checks several parameters such as the presence of overlapping isotopes. When a quantified peptide meets all of the required quality controls, it receives the status 'TRUE', on the other hand if one of the quality checks failed, the quantified peptide receives the status 'FALSE'. We decided to discard all peptides of which the quantification status was indicated 'FALSE' and this reduced the number of unique peptides from 287 to 87 in the d0 versus d1 comparison, and from 417 to 133 in the d0 versus d4 comparison. Next, we removed all the peptides carrying artificially (spontaneous) oxidized methionine, which reduced the number of unique peptides further to 5 (d0 versus d1 analysis) and to 29 (d0 versus d4 analysis, (**Supplementary Table 2** [2])). Compared to the Jurkat studies, here we observed a high degree of spontaneous methionine oxidation, the reason probably related to the more intensive preparative steps (e.g., post-metabolic labeling) prior to the actual COFRADIC analysis.

Taken together, these data indicate that our COFRADIC method is sufficiently sensitive to identify *in vivo* methionine oxidation in very complex samples. At first sight however, both lists seem to contain rather irrelevant oxidation sites in intracellular proteins that are not expected in serum. We believe that the presence of these proteins in serum actually represents a series of necrotic events taking place during the onset of sepsis. For instance, we found that oxidation of Met-132 from myoglobin and Met-30 from creatine kinase-M were early oxidation events. Rhabdomyolysis (38), or the disruption and necrosis of skeletal muscle leading to the leakage of intracellular muscle constituents into the serum, is an early sepsis event which might explain that

[2]These data were made available via the PRIDE database (http://www.ebi.ac.uk/pride/); accession number 16647, username: review69524, password: Q79!f*7e. PRIDE Inspector, available either directly via the link in the PRIDE experiment, or via http://code.google.com/p/pride-toolsuite/wiki/PRIDEInspector is recommended to browse the stored spectra and peptide identifications.

these proteins were here identified. Further given that necrosis is associated with excess production of free radicals, this might further explain why these muscle proteins were found to hold methionine-sulfoxides. Myoglobin is quickly removed via the kidneys, possibly explaining why we did not identify myoglobin as an oxidation target.

Interesting late targets include the histones H3 and H4 (respectively oxidized at Met-121 and Met-85). Elevated levels of nucleosomes in serum resulting from cell necrosis during sepsis have been described and strongly correlate with the severity of sepsis (39). Finally, oxidation of Met-361 in the NADP-dependent malic enzyme was the only oxidation event that was identified in both experiments. This protein also is an intracellular protein and could also be indicative for the necrotic events taking place during sepsis.

Discussion

Central in our technology is the reduction of MetO peptides by combined action of MsrA and MsrB3 thereby covering both MetO diastereoisomers. This conversion to methionine produces a chromatographic shift, specific for the MetO peptides only such that these shift out of a complex background into less crowded chromatographic zones where they are isolated. Combining this sorting step with methionine-SILAC labelling, we were further able to measure the extent of oxidation at each individual site. Using H_2O_2-treated human Jurkat T-lymphocytes as a cellular inflammation model, we identified and quantified 2,626 MetO sites in 1,655 proteins, thus establishing the largest pool of *in cellulo* methionine oxidation events hitherto reported.

The unique feature of being able to discriminate between heavily and moderately oxidized residues, further allowed us to compare properties of each class. For instance, we noticed that in general MetO peptides were preferably surrounded by acidic amino acids, with a noticeable enrichment for proline in heavily oxidized peptides. These *in cellulo* observations were further supported by analyzing the reduction rates of synthetic MetO-containing peptides and by structural modelling of the MsrA active site docked with various MetO-containing peptides, demonstrating favorable docking when methionine was preceded by Lys or Arg. Taken together this strongly supports the concept that the steady-state methionine oxidation status of a cell is primarily determined by the substrate specificities of the reductases, which repair the oxidized residue faster when it is located in a local basic environment. On the other hand, we noticed a trend towards inhibition of reduction when proline residues were present in the critical zone.

Although for the first time a global picture of protein-bound methionine oxidation is presented in cells exposed to hydrogen peroxide, the different steps leading to this picture are far from explained. Indeed, next to different reduction rates, methionine oxidation rates, which are not enzyme-driven and therefore expected to be more random, should be taken into account as well. Our results hint to the fact that oxidation targets a same set of residues. These H_2O_2-sensitive

methionines are located in areas enriched for charged and depleted for hydrophobic residues and are most probably surface-exposed methionines stabilized by surrounding polar residues, and are better targets for oxidation than methionines embedded in a hydrophobic environment which are more stable in the protein inner core. Even when methionines are favorably exposed, we still have to take into account that they can be oxidized at different rates depending on the nature of their neighboring residues as suggested by our studies on synthetic peptides. Although we do not know to which extent our peptide experiments reflect the real situation in a cellular environment where catalases and peroxidases are active, these *in vitro* results should be kept in mind when interpreting the peroxide proteome.

In conclusion, for the first time ever we have been able to generate a global and quantitative view on methionine oxidation of an entire cellular proteome after being exposed to an oxidizing agent. This revealed unexpected features in which the cellular methionine sulfoxide reductases play a pivotal role. Our method was initially applied to a semi-artificial cell system where oxidation was expected to be quite prominent. However, we have here also demonstrated that our method is equally powerful when applied to *in vivo* samples such as the sepsis samples, opening opportunities to use our method to search for specific markers for oxidative stress. It should however be noted that the sepsis serum proteome analysis was performed on serum depleted from three of its major protein fractions (albumin, IgG and serotransferrin). Their removal will have blocked identification of their methionine that are targeted during oxidation since these abundant serum proteins are known to scavenge reactive oxygen species (40). However, our interest was to identify methionine oxidation on less abundant proteins (e.g., intracellular proteins), and without removing abundant serum proteins, such oxidation events would have been difficult, if not impossible, to detect.

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Figure legends

Figure 1: Isolation of methionine-sulfoxide containing peptides from a complex mixture using COFRADIC. Peptides are separated during a primary RP-HPLC run in several primary fractions (n, n+1, …). Next, peptides from these primary fractions are treated with methionine sulfoxide reductases A and B3, reducing only methionine-sulfoxide (■) and not affecting non-methionine peptides ( ) and non-oxidized methionine peptides (,). When such treated primary fractions are re-separated during an identical RP-HPLC run, column retention of the reduced methionine-sulfoxide containing peptides altered (Δt), resulting in a hydrophobic shift out of the primary interval, while non-affected peptides eluted within the same time interval as during the primary run. The shifted peptides are collected for further LC-MS/MS analysis.

Figure 2: Using MsrA and MsrB3 as sorting tools for the COFRADIC based isolation of methionine sulfoxide containing peptides. Oxidation of IPMoxYSIITPNVLR with H2O2 (●) results in a hydrophilic shift compared to the unoxidized form (*). Treating the oxidized methionyl peptide with MsrA (b) or MsrB (c) results in incomplete reduction due to the stereospecificity of the reductases. Only when a combination of MsrA and MsrB is applied, full reduction is achieved (d). Reduction resulted in a hydrophobic shift of about 4 min which is sufficiently large for our diagonal chromatographic setup. Indeed, oxidation of methionines gives rise to two diastereoisomers; R- and S-sulfoxide, and MsrA reduces the S-sulfoxide whereas MsrB specifically targets the R-sulfoxide. This implies that both reductases are needed to achieve full sorting of methionine-sulfoxide containing peptides.

Figure 3: COFRADIC setup for quantifying the degree of methionine oxidation. Cells are SILAC labeled with different isotopic variants of methionine. (**a**) Following cell lysis, proteins are

digested with trypsin and methionyl peptides from the control setup ($^{12}C_5$-Met labeled peptides) are completely converted into their sulfoxides (red circles) by controlled oxidation. Methioninie-sulfoxide peptides in this pool of reference peptides thus represent the maximum degree to which a certain methionine residue can be oxidized (Mx). Next, peptides from both setups are mixed and COFRADIC is performed to isolate methionine-sulfoxide containing peptides using a mixture of MsrA and MsrB3 in the actual sorting step. These peptides are analyzed using LC-MS/MS, and following inspection of the peptide MS spectra, the ratio of the heavy versus the light peptide represents the degree of oxidation. (**b**) In order to point out artificial methionine oxidation, proteins obtained from both setups are mixed prior to digestion. If during sample preparation methionines oxidize spontaneously (green circles), one expect that both isotopic peptide variants oxidize to similar degrees, while *in cellulo* or *in vivo* oxidized methionines (red circles) only originate from cells under oxidative stress. Hence, in peptide MS spectra, COFRADIC-siolated methionyl peptides caused by spontaneous oxidation are present as a couple with the isotopic variants being present in very similar amounts, whereas *in cellulo* or *in vivo* oxidation will give rise to peptide singletons.

Figure 4: Measuring methionine oxidation in the translational endoplasmic ATPase: an illustration. VINQILTEMDGMSTK was identified in four biological replicates to contain (a) methionine(s) that are oxidized by hydrogen peroxide. Panels A and C show MS-spectra from two experiments representing a swapped labeling experiment in which the degree of methionine oxidation was calculated (quantification protocol), in panel A the heavy labeled sample was *in vivo* treated with hydrogen peroxide, and in panel C the light labeled sample was *in vivo* treated with hydrogen peroxide. Panels B and D show MS-spectra from two validation proteomic experiments (also swapped labeling) during which peptides from the control cells were not oxidized. In the latter experiments, the peptide was present as a singleton in the proteome

derived from hydrogen peroxide stressed Jurkat cells, meaning that it was only oxidized in the set-up that was treated with H_2O_2 and also that it didn't oxidize spontaneously during sample preparation.

Figure 5: Heat map of oxidized methionines in H_2O_2-stressed Jurkat cells. When compared to the theoretical human proteome at 95% significance, a clear preference (green color) of charged amino acids close to oxidized methionines (position M) appears, while aromatic amino acids are not preferred (red color). Immediately N-terminal to the targeted methionine (position -1), a clear absence for the basic amino acid lysine is apparent (only five N- and C-terminal residues to the oxidized methionine are shown).

Figure 6: Modeling of different substrates in the catalytic centre of MsrA. Several substrates (pink color) were modeled in the catalytic centre of MsrA. When Arg (**a**) or Lys (**b**) at the N-terminal side of methionine binds in the catalytic centre, an extra hydrogen bond (dotted line) is formed promoting binding of this substrate. When binding of methionyl peptides with Asp (**c**) or Pro (**d**) at the N-terminal side of methionine, no stabilizing bonds can be formed.

Figure 1

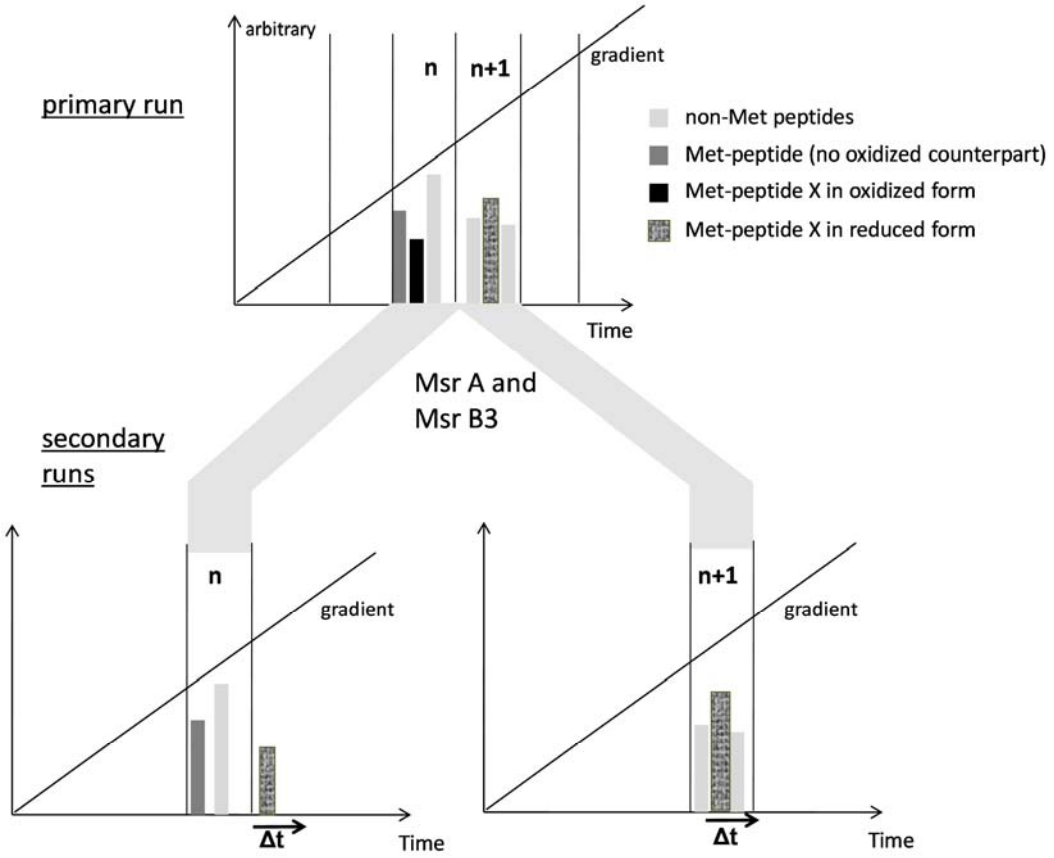

Figure 2

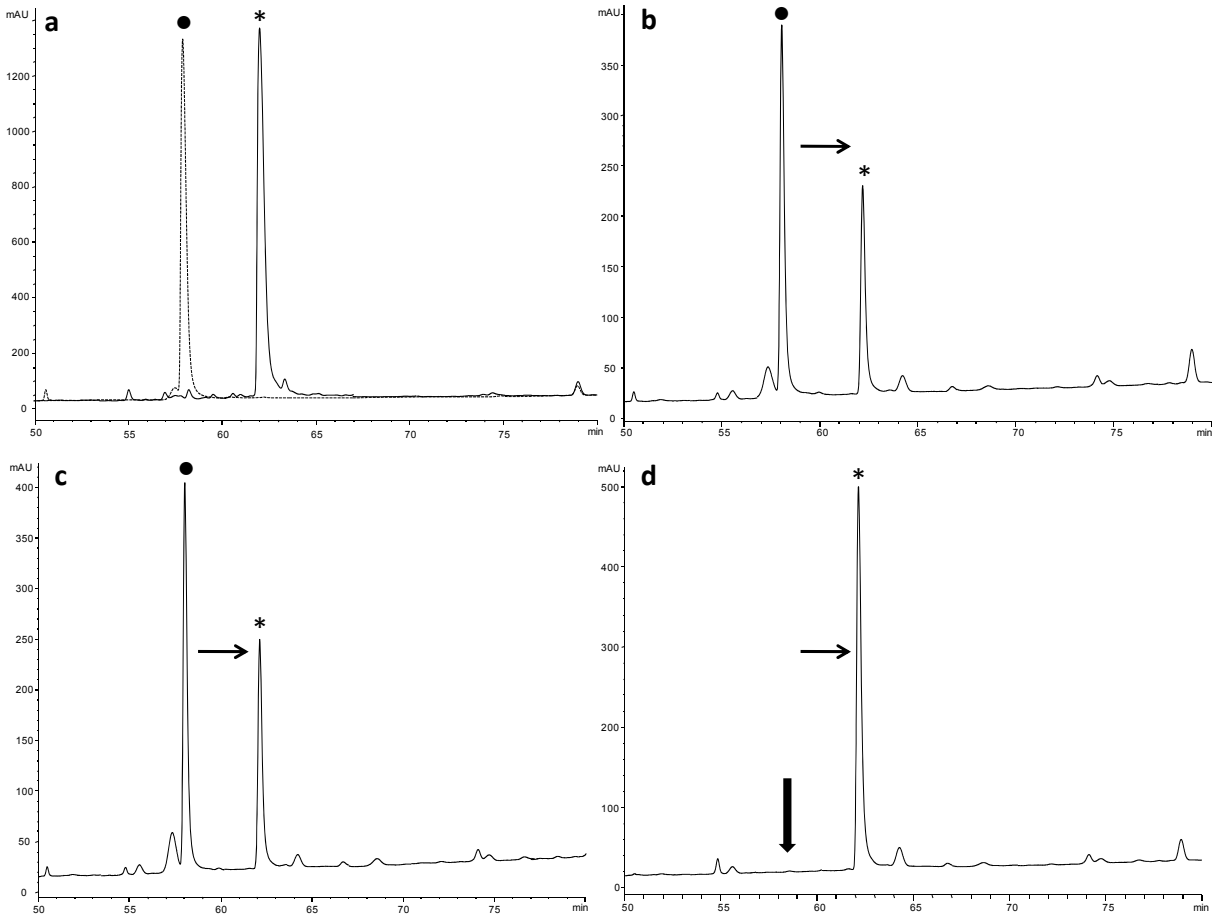

Figure 3

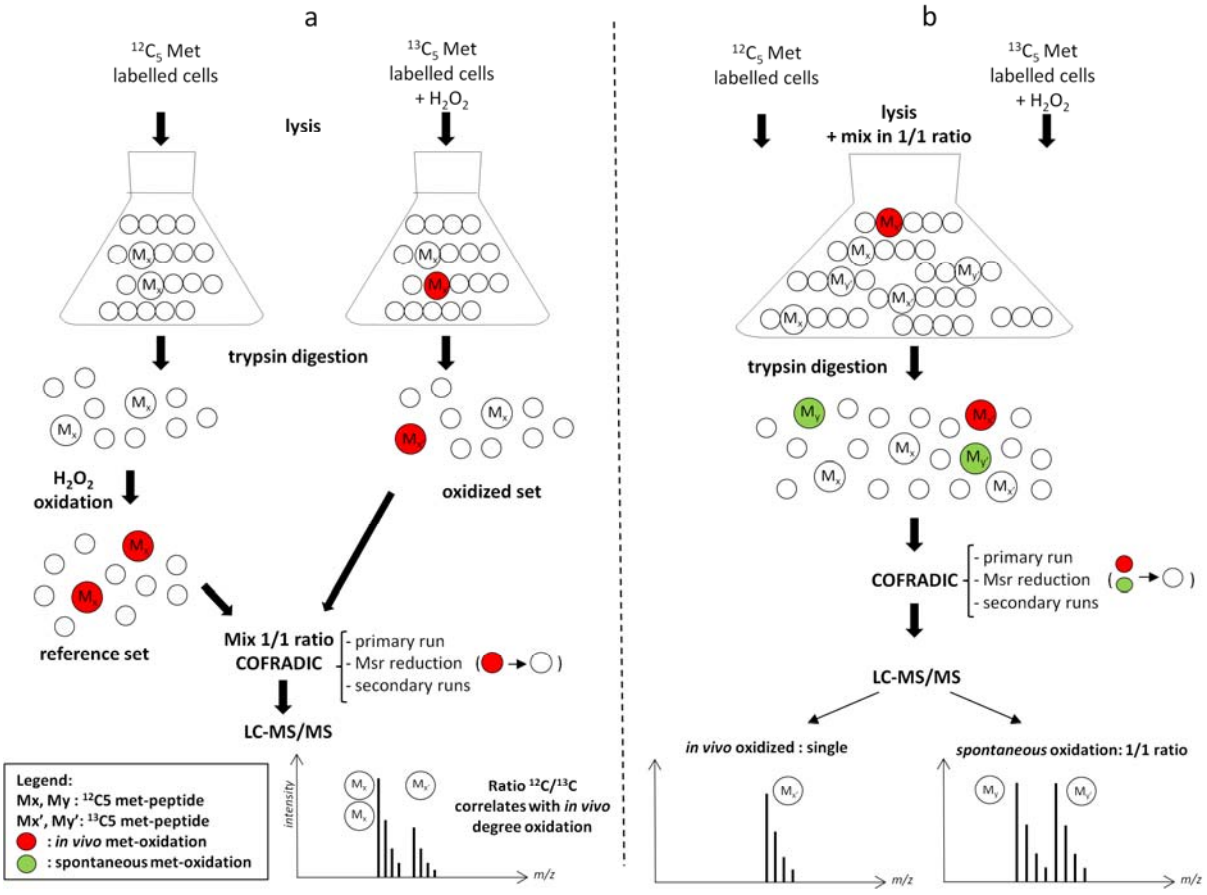

Figure 4

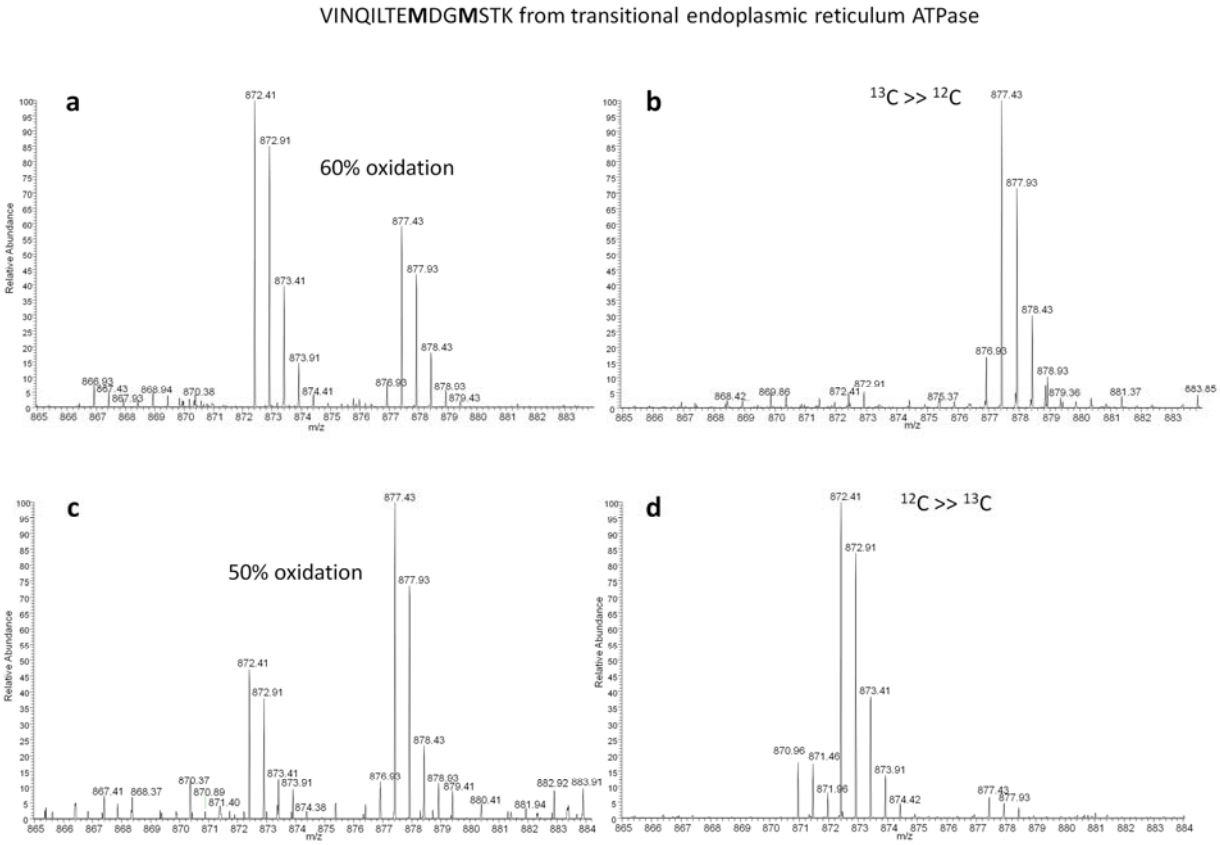

Figure 5

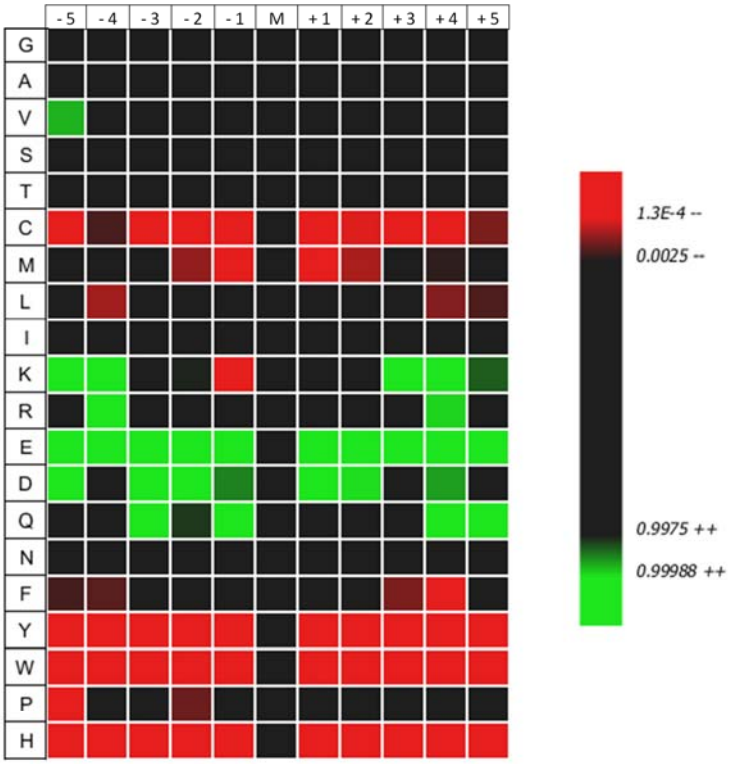

Figure 6