The Use of Methionine Sulfoxide Reductases to Reverse Oxidized Methionine for Mass Spectrometry Applications

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OVERVIEW

Purpose
To synthesize two active forms of recombinant methionine sulfoxide reductase (MetSR) enzymes and perform proof of principle studies for mass spectrometry applications in intact protein, targeted quantitative peptides, and shotgun proteomics.

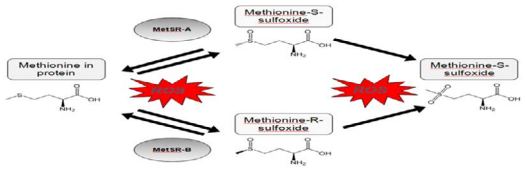
Methods
Two recombinant MetSR and a methionine-rich protein were expressed, purified, and tested for gel mobility shifts prior to use in MS applications. For intact and shotgun proteomics experiments ,hydrogen peroxide was added to induce mild methionine oxidation and compared to non-oxidized controls before and after treatment with MetSR. Reduction was carried out using DTT as a cofactor and samples were analyzed by a Thermo Scientific™ Orbitrap XL™ Hybrid Ion Trap-Orbitrap MS . Targeted quantitative analysis of methionine containing peptide and phosphopeptides were done on a Thermo Scientific™ Q Exactive™ HF MS using Parallel Reaction Monitoring.

Results:
We successfully produced and purified two active MetSR enzymes, as observed by gel shift of an oxidized methionine rich protein, reduction of oxidized methionines in an intact protein, and in shotgun and targeted proteomics experiments at the peptide level. Parallel Reaction Monitoring was used to demonstrate 90-99% reduction in oxidized methionine in MetSR treated peptide and phosphopeptides.

INTRODUCTION

Methionine (Met) is a sulfur-containing essential amino acid that is highly susceptible to oxidation by reactive oxygen species during stress, aging, and disease in cells, and *in vitro*[1]. Met oxidation is the most common modification of purified therapeutic antibodies and other biologics, can affect protein function, and is observed by mass spectrometry (MS) in intact proteins and peptide samples as splitting the MS signal, which reduces sensitivity and increases complexity of analysis. Methionine sulfoxides can be returned to methionine by methionine sulfoxide reductases. The product of Met oxidation is Met sulfoxide (MetO), which exists in two diastereomers that are reduced by two methionine sulfoxide reductases, MetSR-A and MetSR-B (Figure 1).

Figure 1: Schematic of Methionine Oxidation and Reduction: Methionine S-sulfoxide and methioni



Methionine oxidation can also occur during sample preparation, purification , storage , and LC-MS ionization[2,3]. Variable methionine oxidation of purified proteins leads to lower and variable product quality. It also complicates liquid chromatography-mass spectrometry (LC-MS) analysis in 4 ways: **1)** Shifted and split peaks during liquid chromatography[4], **2)** Increased complexity and poorer depth of analysis of intact proteins due to multiple oxidized forms, **3)** Increased complexity & poorer depth of analysis of peptide digests due to multiple oxidized forms, and; **4)** Impaired quantitation of methionine-containing peptides due to the stochastic nature of oxidation of methionine in peptides.

METHODS

Expression of Recombinant Methionine Sulfoxide Reductases

Over a dozen different recombinant methionine reductase vectors were constructed for target protein expression and optimized for E.coli ER2566. 0.1 g of biomass was lysed with cell lysis buffer consisting of 50mM Tris – HCl (pH 8); 100mM NaCl; 0.1% Triton X-100; 1mM EDTA (pH8) and disrupted by sonication. The soluble fraction was diluted with wash buffer consisting of 250mM NaCl, 20mM imidazole, 50mM Tris-HCl, pH=8 and applied to 0.2 ml Thermo Scientific™ HisPur Ni-NTA and washed extensively. Bound proteins were eluted with 50mM Tris, pH8, 250mM NaCl, 500mM imidazole and dialyzed against 50mM Tris-HCl pH 7.6, 4ºC, overnight. Three MetSR genes expressed from three different clones each were tested by gel shift of an oxidized methionine rich protein 6 (Figure 2) and two MetSR/Trx trifunctional enzymes from *Neisseria gonorrhoeae* or *Neisseria meningitidis* were selected for MS applications (Figure 2).

Figure 2: Gel Shift Assay to Reduce MRP6: Three different MetSR genes expressed in three different vectors were tested for their ability to reduce MRP6 after being oxidized by H_2O_2.

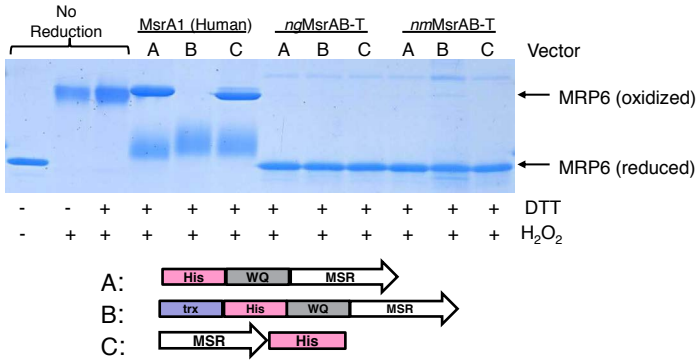
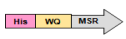


Figure 3: Amino acid sequences of two recombinant MetSR enzymes: Amino acid sequence of *ng*MsrAB-T and *nm*MsrAB-T prior to treatment with WELQut Protease to cleave at the WELQ site.

*ng*MsrAB-T



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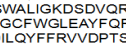
*nm*MsrAB-T



Sequence:*HHHHHHSSGLVPRGSHMWEL*QLALGACSPKIVDAGAATVPHTLSTLKTADNRPA
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Oxidation by Hydrogen Peroxide
For intact analysis, recombinant turboluciferase (TurboLuc, ~16kDa) was selected to perform an initial optimization of methionine oxidation and reduction reactions due to observed sensitivity to oxidation, knowledge of the sequence, and ability to detect with an Orbitrap XL mass spectrometer. All experiments for intact analysis used18μM TurboLuc. TurboLuc has three moles of methionine per mole of protein and thus a 1:1 molar ratio of methionine to H_2O_2 would be 54μM of H_2O_2 for 18μM of TurboLuc. A variety of oxidation conditions were assessed at the following ratios of methionine: H_2O_2 which were 10:1, 1:1, 1:10, 1:25, 1:75, 1:100 and 1:500 (Figure 5 shows 1:10 and 1:75, the rest of the data are not shown).

Reduction of Intact Proteins by ngMsrAB and nmMsrAB
Methionine sufloxide reduction was performed by adding either *ng*MsrAB-T or *nm*MsrAB-T to the TurboLuc control or oxidized sample at an enzyme:sample ratio of 1:4 and DTT to a final concentration of 5mM. Both enzymes were used in separate experiments. Samples were incubated at 37^{0}C for 2 hours & then desalted using 3K MWCO concentrators.

Shotgun Proteomics Coupled with Methionine Oxidation Reduction
Thermo Scientific™ Pierce™ 6 Protein Digest, equimolar, LC-MS grade which was stored in liquid for over a year was used for the oxidation experiments. Control and oxidized samples were reduced using the same procedure as intact protein analysis described above, except for C18 peptide clean up.

LC-MS analysis
The protein sample was injected onto a Thermo Scientific™ ProSwift™ RP-4H Analytical 100 µm x 25 cm monolithic column at a flow rate of 800 nL/min over a gradient of water and acetonitrile in 0.1% formic acid. The shotgun proteomics sample was injected onto a reversed-phase column (15cm x 75 µm I.D., Prepmap C18) and separated using a gradient of water and acetonitrile in 0.1% formic acid at a flow rate of 300 nL/min. All samples used an Eksigent™ nanoLC Ultra 2D plus HPLC coupled to a Thermo Scientific™ LTQ Orbitrap™ XL ETD mass spectrometer.

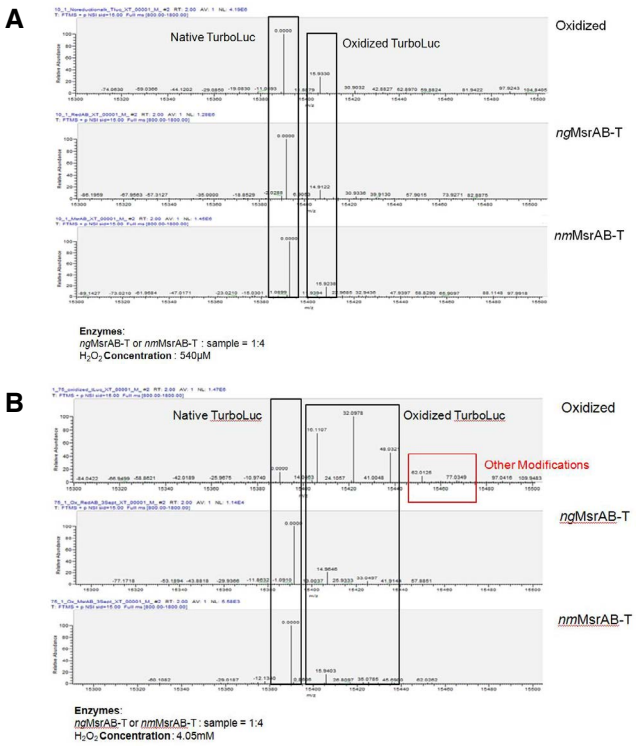
Targeted Quantitative Peptide Analysis
Three heavy peptide standards (two phosphopeptides) containing an oxidized methionine (100 fmol/µL) were mixed with the Pierce 6 protein digest, equimolar, LC-MS grade (40 ng/µL). The mixed sample was then reduced by either *ng*MsrAB-T or *nm*MsrAB-T as described earlier. The resulting peptide mixture was then injected onto a C18 column (Easy-Spray PepMap C18, 3μm, 75μm x 150 cm) and separated by a gradient of water and acetonitrile in 0.1% formic acid using a Thermo Scientific™ Dionex™ Ultimate™ 3000 RSLCnano system. The sample was infused into the Q Exactive HF mass spectrometer via electrospray ionization utilizing parallel reaction monitoring (Figure 4) for collection of targeted spectra. The resulting raw data were processed and visualized using Skyline 3.1.0.7382 analysis software.

RESULTS

Intact Protein Analysis

The ability for both MetSR enzymes to reduce an intact protein was demonstrated using Thermo Scientific™ TurboLuc™ . Figure 5 demonstrates how oxidation is reversed by either MetSR. Figure 5A shows that 1:10 moles of methionine to moles of H_2O_2 can decrease the forms of oxidized TurboLuc mildly. When looking at highly oxidized samples shown in Figure 5B (1:75 ratio) significant oxidation was able to be reversed, but some oxidation events occurred which could either be caused from other amino acids being oxidized or creation of the sulfone that are not reversible by MetSR. Remaining protein oxidation may be due to sulfone formation, oxidation of other amino acids, or re-oxidation during electrospray ionization.

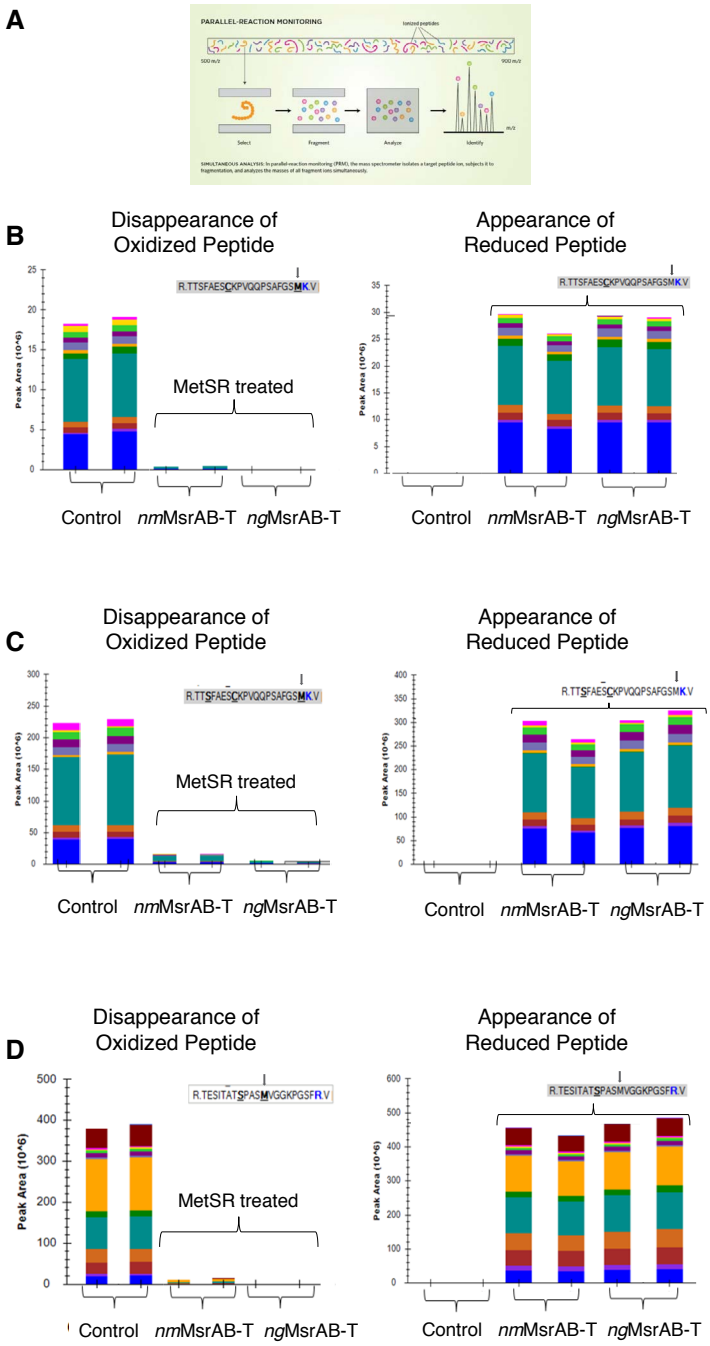
Figure 4: Intact Protein MS Results: Spectra compared after Thermo Scientific™ Xcalibur™ Xtract Raw file for oxidized and reduced samples using *ng*MsrAB-T or *nm*MsrAB-T. Figure 5A shows data from the 1:10 moles of methionine to H_2O_2, whereas Figure 5B is for 1:75 moles of methionine to H_2O_2. The peaks assigned as 0.0000 is the TurboLuc protein without any methionine oxidation, and shifts by 16 Daltons are indicative of oxidation (15 Daltons are sometimes observed due to data processing).



Targeted Protein Analysis

Figure 6 shows the disappearance of the oxidized peptide and appearance of the corresponding reduced peptide after treatment with *ng*MsrAB or *nm*MsrAB. These data indicate that treatment with *ng*MsrAB or *nm*MsrAB results in a >90% reduction of an oxidized methionine and resulted in a corresponding increase in the peptide with a reduced methionine. In addition, after treatment some of the oxidized peptides were not even detectable (Figure 6 A and C for *ng*MsrAB-T).

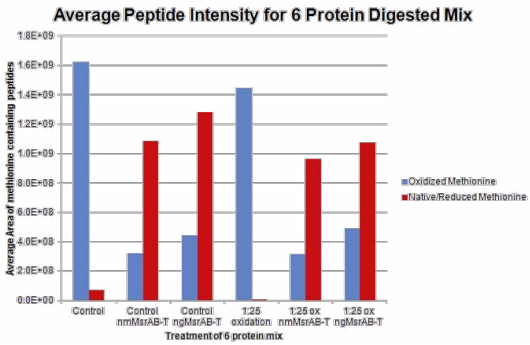
Figure 5: Parallel Reaction Monitoring of three methionine containing peptides: Schematic of parallel reaction monitoring for targeted peptide quantitation [5,6](A). Quantitative reversal of methionine oxidation in a peptide standard (B), a phosphopeptide of the same peptide standard (C), and a second phosphopeptide (D). The bar graph area shows the cumulative area under the curve of individual b-ions and y-ions. Each experiment was performed in duplicate.



Shotgun proteomics

The Pierce 6 protein LC-MS grade digest was used to assess the two MetSR enzymes using both non-oxidized and mildly oxidized samples (Figure 7). Substantial methionine reversal was seen with all reaction ratios less than 1:100 moles of methionine:H_2O_2. At 1:100 sulfones and other amino acids were oxidized which greatly reduced the identification of peptides due to random amino acid oxidation events (data not shown). In addition, DTT alone did not show any reversal of methionine oxidation. Lastly, numerous oxidized peptides were no longer detected after treatment with MetSR. Remaining peptide oxidation may be due to sulfone formation, oxidation of other amino acids, or re-oxidation during electrospray ionization.

Figure 6: Average intensity of 6-protein digested mix: A comparison of the average peptide intensity of detectable peptides before and after treatment with two forms of MetSR.



CONCLUSIONS

Methionine oxidation can be reversed in intact proteins and peptides prior to analysis by SDS-PAGE and intact or peptide LC-MS analysis.

Reversal of methionine oxidation by MetSR has benefits to MS experiments involving intact proteins, targeted quantitative peptide analysis, and shotgun proteomics.

A modified LC-MS ionization source design may alleviate the residual modifications observed at the protein and peptide level[7].

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