Top-Down Quantitation of Methionine Oxidation

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Introduction

Methionine oxidation plays a critical role in many processes of biological and biomedical importance, including cellular redox responses and stability of protein pharmaceuticals. Bottom-up methods for analysis of methionine oxidation can suffer from incomplete sequence coverage, as well as an inability to readily detect correlated oxidation between two or more methionines. Similarly, the study of protein structure and interactions by hydroxyl radical protein foot printing (HRPF) is a rapidly growing field. However, the methodology for quantifying HRPF modifications in top-down analyses are lacking. Using apomyoglobin and calmodulin as a model proteins we partially converted methionine's (Met) into methionine sulfoxide by incubation in H_20_2. Using top-down ETD and ECD based fragmentation, we quantified the amount of oxidation at each methionine and compared the quantified values to those from traditional bottom-up fragmentation. We find that quantification of apomyoglobin methionine oxidation by top-down MS/MS is accurate in good agreement with traditional, bottom-up methods. However ETD fragmentation of calmodulin gave variable results compared to ECD. It could be due to miss assignment of peaks due to low intensity compared to ECD. ECD is giving more promising results. It could be due to high resolution and intensity.

Top-down ETD analysis

1mM of apomyoglobin and colmadulin was incubated for approximately one hour with 100mM H_20_2, shielded from UV light. The reaction was halted with acetone precipitation and dissolved in 500ul of 50% ACN, 0.1%formic acid and 2% propylene carbonate [1] for top-down analysis. Prepared sample was injected to Thermo Orbitrap Fusion Tribrid in direct infusion mode in positive mode with spray voltage 3200, capillary temperature 300ºC at 120000 Orbitrap resolution for full scan and for ETD fragmentation isolation window of ten *m/z* units. ETD reaction times 25 milleseconds. ProSight Lite was used for initial sequence coverage analysis [2] but all data-were ultimately analyzed and annotated manually. For ECD analysis data was acquired on Bruker solariX XR12Telsa FTMS. With infusing 80ul per hr. With 3s accumulation time, ECD pulse length 0.03sec, ECD bias 1.5V, and ECD lens 40V.

Bottom-up analysis

Bottom sample was prepared as standard protocol and one µL of the digested sample was injected onto a PepMap RSLC 75µm×15cm C18 analytical column (2 µm particle size,100Å pore size) using an Ultimate 3000 Nano LC system coupled to a Thermo Orbitrap Fusion Tribrid in positive mode with spray voltage 2300, capillary temperature 200ºC at 60000 Orbitrap resolution. Mobile phase A was LC-MS grade water,0.1% formic acid, and mobile phase B was ACN with 0.1% formic acid. A gradient elution was performed by equilibrating with 2% B for 6min, increasing to 35% B over 23min, further increasing to 95% over 5 min, holding at 95% B for 3min, returning to 2% B over 10min

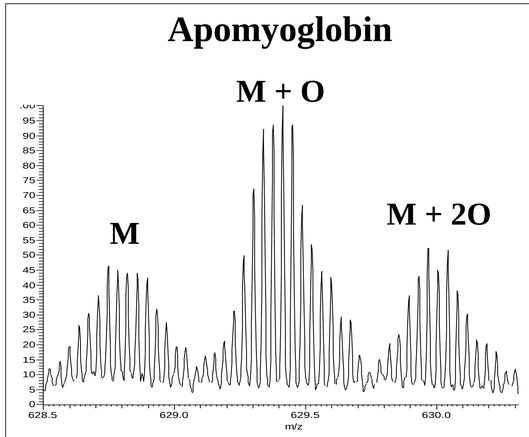

Figure 1. Representative spectra of +27 charge state of apomyoglobin incubated for 1 hr. with 1M H_20_2.

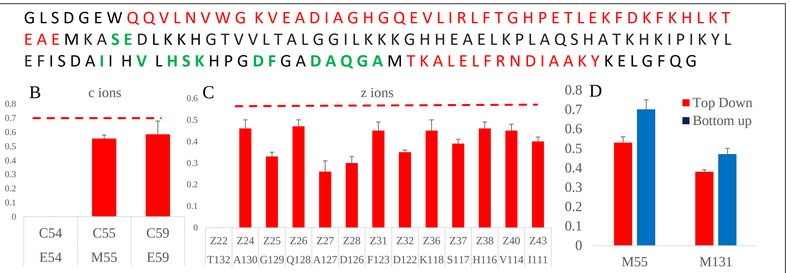

Figure 2. ETD-based top-down analysis of all oxidized and unoxidized forms of the +27 charge state of apomyoglobin after peroxide oxidation. (**A**) Sequence coverage of apomyoglobin. **Red:** only detected as unoxidized. **Green:** detected in both oxidized and unoxidized forms, and used for oxidation quantification. **Black:** insufficient intensity for quantification (**B**) Oxidation of c-ions. All data points represent two technical replicates. Hashed line represents the average oxidation of M55 based on bottom-up data; error bars indicate one standard deviation. (**C**) Oxidation of z-ions Hashed line represents the average oxidation of M131 based on bottom-up data. (**D**) Average of all values of oxidation taken for M55 and M131 by **(Red)** top-down analysis and **(Blue)** bottom up analysis.

Apomyoglobin

Amino acid residue	Bottom up	Top down ETD+27
M55	0.70 ± 0.05	0.53 ± 0.03
M131	0.47 ± 0.03	0.38 ± 0.11

Calmodulin

Amino acid residue	Bottom up	Top down +15
M76	0.45 ± 0.04	ETD 0.30
M144	0.57 ± 0.03	ECD 0.35

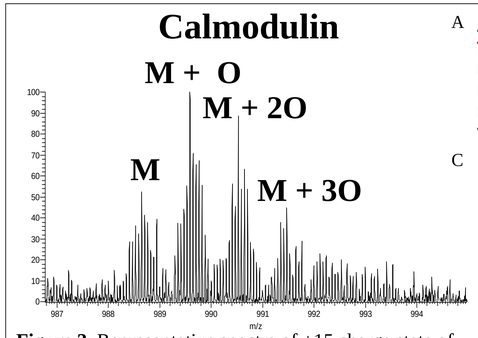

Figure 3. Representative spectra of +15 charge state of calmodulin incubated for 1 hr with 1M H_20_2.

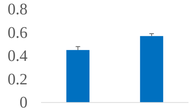

Figure 4. Methionine M76 and M144 oxidation as measured by bottom-up

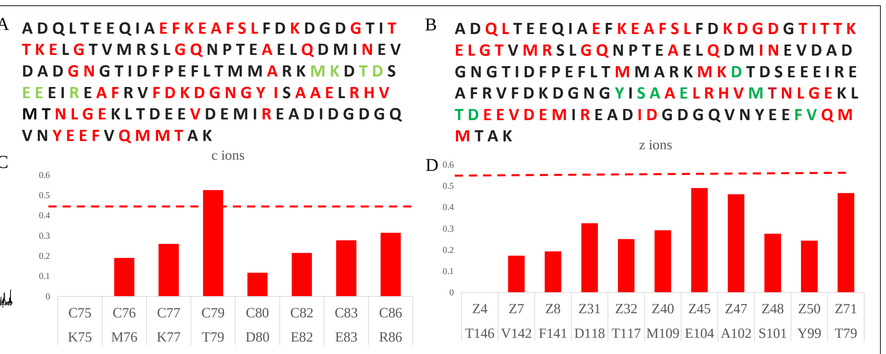

Figure 5. ETD and ECD based top-down analysis of all oxidized and unoxidized forms of the +15 charge state of calmodulin after peroxide oxidation. (**A,B**) Sequence coverage of calmodulin for A: ETD and B: ECD based quantitation. Ions labeled in **Red**: only detected as unoxidized. Ions labeled in **Green:** detected in both oxidized and unoxidized forms, and were used for oxidation quantification. Ions labeled **Black:** insufficient intensity for quantification (**C**) Oxidation of C-ions (ETD), as measured based on oxidized versus unoxidized product ion intensity. Hashed line represents the average oxidation of M76 based on bottom-up data. (**D**) Oxidation of Z-ions (ECD), as measured based on oxidized versus unoxidized product ion. Hashed line represents the average oxidation of M144 based on bottom-up data

Conclusion

➢Top-down ETD-based MS/MS of apomyoglobin are largely consistent with bottom-up measurements, although bottom-up consistently gave higher levels of oxidation. It is unclear if the higher levels of oxidation in bottom-up are due to measurement differences, or oxidation during digestion.

➢Top down ETD-Based MS/MS of calmodulin gave more variable levels of oxidation for the more complex sample. Low intensity of oxidized product ion signal at any amino acid results in a high contribution of background noise to oxidation quantitation.

➢Top down ECD fragmentation gave better intensity and oxidation ions are consistent with bottom up. ECD fragmentation could give better statistical values compared to ETD.

➢Better coverage of oxidized product ions required to detect correlation between sites of oxidation

Acknowledgements

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References

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2. 2. Fellers, R.T., et al., *ProSight Lite: graphical software to analyze top-down mass spectrometry data.* Proteomics, 2015. **15**(7): p. 1235-8.