A Twist on Quantification

Measuring the Site Occupancy of S-Nitrosylation

Christopher I. Murray, Jennifer E. Van Eyk

The use of mass spectrometry (MS) as a tool to study proteins and their posttranslational modifications (PTMs) is gaining wider acceptance by the cardiovascular research community. A unique advantage of MS is the potential for large-scale quantitative analysis of protein abundance between biological samples. Several techniques have been developed for this purpose (Online Table I), some of which have been adapted for PTMs. The MS analysis of any PTM can be significantly enhanced if it is preceded by a chemical or immunoaffinity enrichment strategy targeting a PTM's unique physical property. For example, phosphorylation can be enriched by exploiting its negative charge, classically using a positively charged metal affinity column, whereas acetylation can be enriched using antibodies against its acetyl group. Several strategies have also been developed to examine Cys modifications, including S-nitrosylation (SNO, also known as S-nitrosation).[1–7] The most widely used of these techniques is the biotin switch assay.[7] This assay was initially presented as a method for the enrichment and identification of SNO-modified proteins but has evolved into a powerful and flexible technique to identify and quantify many different oxidative PTMs. Advances in the configuration of this assay have allowed researchers to progress from straightforward protein-level identifications to the individual modified amino acid and then to site-specific quantification (Figure).[8–12] A key factor in the improvement of this approach has been the introduction of isotope-labeled thiol-reactive affinity tags (Online Table I). These reagents have permitted the simultaneous MS analysis of ≥2 samples to measure differences in Cys modification at individual residues.

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In the study by Murphy et al,[13] this group combined the classic biotin switch assay with the isotopic cysteine-reactive tandem mass tag (CysTMT) reagent to not only identify specific Cys residues that were SNO-modified in the heart but also determine the occupancy of SNO modification for each site. The use of thiol-reactive isotopic affinity (mass) tags is not new. The earliest isotope-labeled affinity tags were developed by Aebersold's group[14] and reacted with the free thiol groups; however, these were conceived for general protein quantification rather than the targeted analysis of Cys PTMs. Cohen et al[15] first pursued a Cys-specific application of isotope-coded affinity tags (ICAT) in cardiac muscle to investigate the extent of oxidative Cys modifications upon peroxide treatment. CysTMT tags are the most recent development in thiol-targeted isotopic mass tags and can be multiplexed with up to 6 different samples in a single MS analysis.[12] More recently, our group, in collaboration with Thermo Fisher, was the first to combine CysTMT (6' plex) with the biotin switch approach and successfully quantify individual thiol reactivity to SNO modifications.[12] Measurement of thiol reactivity to SNO modification provided a unique look into the hierarchy of which Cys becomes modified under increasing oxidative stimuli. What is novel in the current article is the Murphy et al[13] use of the CysTMT reagent (a light and heavy isotopic version) to determine the extent of SNO modification at each Cys. This exciting twist allowed them to determine each

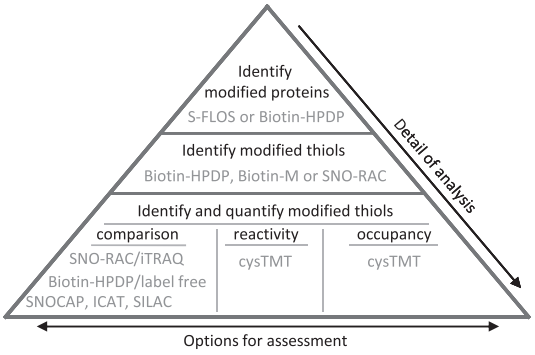

Figure. Options for the analysis of oxidative posttranslational modifications using variations of the biotin switch assay. The current state of redox proteomics has an array of options to evaluate a biological sample's redox state. Basic analysis can determine the modified proteins[8,32] and more refined approaches provide the modified proteins and the position of each affected amino acid residue,[9,33] whereas the most advanced techniques determine the modified residue and provide relative quantification.[10–13,23,34] For S-nitrosylation (SNO)-modified proteins, quantification protocols have been applied to compare the redox state of residues between biological samples, determine individual thiol reactivity, and now individual thiol occupancy. The development of these tools, and others, is invaluable to understanding the mechanisms of cellular redox regulation. Biotin-HPDP indicates N-[6-(Biotinamido)heyl]-3'(2'-pyridyldithio)propionamide; S-FLOS, selective fluorescent labeling of S-nitrosothiols; SNO-RAC, S-nitrosylation-resin assisted capture; iTRAQ, isobaric tags for relative and absolute quantification; ICAT, isotope-coded affinity tag; SILAC, stable isotope labeling by amino acids in cell culture; cysTMT, cysteine-reactive tandem mass tag.

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.112.278721/-/DC1.

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(*Circ Res*. 2012;111:1253-1255.)

***Circulation Research* is available at http://circres.ahajournals.org**
DOI: 10.1161/CIRCRESAHA.112.278721

site's SNO occupancy: the ratio (or percentage) of the SNO-modified and unmodified form. Site occupancy is an extremely important aspect of quantification that is often overlooked, in part because there are few tools available that allow this type of information to be gathered. Yet, knowing the occupancy of SNO for each modifiable Cys residue is, in our opinion, key to understanding the role of these modifications and should be part of all future studies involving SNO. In fact, site occupancy should be a goal for all PTM studies, not just for SNO.

Why is site occupancy so critical? It is clear that many amino acid residues can undergo PTM—100s to 1000s of sites being modified at any given time (eg, phosphorylation[16–18] and acetylation[19–21]). The extent of site occupancy will vary at each modified residue, and many will be present in a sample at a very low percentage. In these cases, when investigating changes in PTM status after a stimulation or treatment, the current method of quantifying fold changes can be misleading. For example, a low occupancy site (<1%) may increase 5-fold upon ischemia reperfusion to <5% occupancy, whereas the same 5-fold increase to a site with moderate occupancy (20%) would result in 100% occupancy. Conversely, a site already 50% occupied cannot increase by >2-fold. Fold change analysis without assessing site occupancy may not reveal the most biologically important modification amino acid residues.

Determination of site occupancy is also advantageous when multiple PTMs occur in the same amino acid residue. Thus, the extent of site occupancy for each PTM is critical for determining the contribution of the various competing PTMs at a given Cys residue. It is already clear from the literature that there are specific Cys residues that can have a plural modification status.[22,23] To date, this type of analysis has not yet been performed, but quantitative information dissecting the extent of each Cys PTM will be key, particularly to support the emerging concept that SNO is protective against other more damaging Cys oxidative PTMs. One of the current limitations in the field is that relative quantification does not allow to compare the levels of PTM at multiple Cys residues in different proteins. The different ionization efficiency of peptides requires absolute quantification of the unmodified and modified residue (mole:mole) to compare the extent of modification at different sites. Absolute quantification using MS can be achieved using multiple reaction monitoring (also termed selective reaction monitoring), where a known quantity of an isotope-labeled peptide is spiked into a sample and quantified based on a standard curve.[24–27] This method has already been applied to quantify phosphorylated proteins (including mitochondria[28] and cardiac troponin I),[29] but its application to Cys-PTMs has been limited to semiquantitative analysis rather than absolute quantification.[30,31] We believe that with time and further development, this approach will ultimately be the future of PTM quantitative analysis.

Until that time, the application of the CysTMT reagent to determine site occupancy for Cys PTMs is a very significant step in the right direction. Although there are still many technical and feasibility issues remaining (as outlined in their article), the work by Murphy et al[13] demonstrates that this approach is possible and has opened the door to a host of interesting insights into the biochemical effects of NO production, ischemia reperfusion injury in the heart, and beyond.

Sources of Funding

This work was funded by the American Heart Association Predoctoral Fellowship 0815145E (to C.I.M.) and National Heart, Lung, and Blood Institute's Proteomic Initiative Contract NHLBI-HV-10-05(2) (to J.E.V.E.), and PO1HL77189-01 (to J.E.V.E.).

Disclosures

None.

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KEY WORDS: mass spectrometry ■ proteomics ■ quantification ■ S-nitrosation ■ S-nitrosylation

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Circ Res. 2012;111:1253-1255
doi: 10.1161/CIRCRESAHA.112.278721
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571