

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.

Article, see p 1308

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 group¹⁴ 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 al¹⁵ 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 al¹³ 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

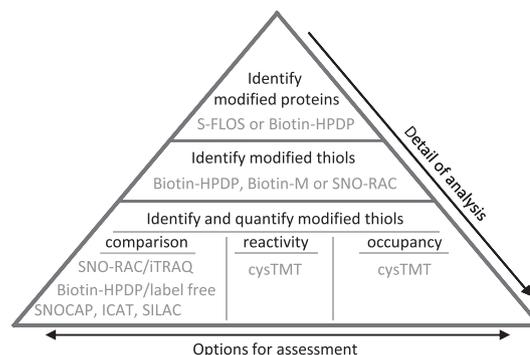


Figure. Options for the analysis of oxidative posttranslational modifications using variations of the biotin switch assay. The current state of redox proteomics has an array of options to evaluate a biological sample's redox state. Basic analysis can determine the modified proteins^{8,32} and more refined 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)hexyl]-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.

From the Departments of Biological Chemistry (C.I.M., J.E.V.E.) and Medicine (J.E.V.E.), Division of Cardiology and Biomedical Engineering (J.E.V.E.), Johns Hopkins University, Baltimore, MD.

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

Correspondence to Jennifer E. Van Eyk, Mason F. Lord Bldg, Center Tower, Room 602, 5200 Eastern Ave, Johns Hopkins University, Baltimore, MD 21224. E-mail jvaneyk1@jhmi.edu

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

© 2012 American Heart Association, Inc.

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 mitochondria²⁸ 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 al¹³ 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.

References

- Murray CI, Van Eyk JE. Chasing cysteine oxidative modifications: proteomic tools for characterizing cysteine redox-status. *Circ Cardiovasc Genet*. 2012; In Press.
- Bechtold E, King SB. Chemical methods for the direct detection and labeling of S-nitrosothiols. *Antioxid Redox Signal*. 2012;17:981–991.
- Jacob C, Battaglia E, Burkholz T, Peng D, Bagrel D, Montenarh M. Control of oxidative posttranslational cysteine modifications: from intricate chemistry to widespread biological and medical applications. *Chem Res Toxicol*. 2012;25:588–604.
- Wang H, Xian M. Chemical methods to detect S-nitrosation. *Curr Opin Chem Biol*. 2011;15:32–37.
- Chouchani ET, James AM, Fearnley IM, Lilley KS, Murphy MP. Proteomic approaches to the characterization of protein thiol modification. *Curr Opin Chem Biol*. 2011;15:120–128.
- Forrester MT, Foster MW, Benhar M, Stamler JS. Detection of protein S-nitrosylation with the biotin-switch technique. *Free Radic Biol Med*. 2009;46:119–126.
- Jaffrey SR, Snyder SH. The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE*. 2001;86:p11.
- Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat Cell Biol*. 2001;3:193–197.
- Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures. *Proc Natl Acad Sci USA*. 2006;103:1012–1017.
- Forrester MT, Thompson JW, Foster MW, Nogueira L, Moseley MA, Stamler JS. Proteomic analysis of S-nitrosylation and denitrosylation by resin-assisted capture. *Nat Biotechnol*. 2009;27:557–559.
- Paige JS, Xu G, Stancevic B, Jaffrey SR. Nitrosothiol reactivity profiling identifies S-nitrosylated proteins with unexpected stability. *Chem Biol*. 2008;15:1307–1316.
- Murray CI, Uhrigshardt H, O'Meally RN, Cole RN, Van Eyk JE. Identification and quantification of S-nitrosylation by cysteine reactive tandem mass tag switch assay. *Mol Cell Proteomics*. 2012;11:M111.013441.
- Kohr MJ, Aponte A, Sun J, Gucek M, Steenbergen C, Murphy E. Measurement of S-Nitrosylation occupancy in the myocardium with cysteine-reactive tandem mass tags. *Circ Res*. 2012;111:1308–1312.
- Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol*. 1999;17:994–999.
- Sethuraman M, McComb ME, Huang H, Huang S, Heibeck T, Costello CE, Cohen RA. Isotope-coded affinity tag (ICAT) approach to redox proteomics: identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J Proteome Res*. 2004;3:1228–1233.
- Lundby A, Secher A, Lage K, Nordsborg NB, Dmytryiev A, Lundby C, Olsen JV. Quantitative maps of protein phosphorylation sites across 14 different rat organs and tissues. *Nat Commun*. 2012;3:876.
- Olsen JV, Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P, Mann M. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell*. 2006;127:635–648.
- Deng N, Zhang J, Zong C, et al. Phosphoproteome analysis reveals regulatory sites in major pathways of cardiac mitochondria. *Mol Cell Proteomics*. 2011;10:M110.000117.
- Choudhary C, Kumar C, Gnäd F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*. 2009;325:834–840.
- Lundby A, Lage K, Weinert BT, Bekker-Jensen DB, Secher A, Skovgaard T, Kelstrup CD, Dmytryiev A, Choudhary C, Lundby C, Olsen JV.

- Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. *Cell Rep.* 2012;2:419–431.
21. Mischerikow N, Heck AJ. Targeted large-scale analysis of protein acetylation. *Proteomics.* 2011;11:571–589.
 22. Wang SB, Murray CI, Chung HS, Van Eyk JE. Redox-regulation of mitochondrial ATP synthase. *Trends Cardiovasc Med.* 2012;16:1323–1367.
 23. Kohr MJ, Sun J, Aponte A, Wang G, Gucek M, Murphy E, Steenbergen C. Simultaneous measurement of protein oxidation and S-nitrosylation during preconditioning and ischemia/reperfusion injury with resin-assisted capture. *Circ Res.* 2011;108:418–426.
 24. Picotti P, Aebersold R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. *Nat Methods.* 2012;9:555–566.
 25. Shi T, Su D, Liu T, Tang K, Camp DG 2nd, Qian WJ, Smith RD. Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. *Proteomics.* 2012;12:1074–1092.
 26. Calvo E, Camafeita E, Fernández-Gutiérrez B, López JA. Applying selected reaction monitoring to targeted proteomics. *Expert Rev Proteomics.* 2011;8:165–173.
 27. Fu Q, Schoenhoff FS, Savage WJ, Zhang P, Van Eyk JE. Multiplex assays for biomarker research and clinical application: translational science coming of age. *Proteomics Clin Appl.* 2010;4:271–284.
 28. Lam MP, Scruggs SB, Kim TY, Zong C, Lau E, Wang D, Ryan CM, Faull KF, Ping P. An MRM-based workflow for quantifying cardiac mitochondrial protein phosphorylation in murine and human tissue. *J Proteomics.* 2012;75:4602–4609.
 29. Zhang P, Kirk JA, Ji W, Dos Remedios CG, Kass DA, Van Eyk JE, Murphy AM. Multiple reaction monitoring to identify site-specific troponin I phosphorylated residues in the failing human heart. *Circulation.* 2012;126:1828–1837.
 30. Chen HJ, Chen YC. Reactive nitrogen oxide species-induced post-translational modifications in human hemoglobin and the association with cigarette smoking. *Anal Chem.* 2012;84:7881–7890.
 31. Held JM, Danielson SR, Behring JB, Atsriku C, Britton DJ, Puckett RL, Schilling B, Campisi J, Benz CC, Gibson BW. Targeted quantitation of site-specific cysteine oxidation in endogenous proteins using a differential alkylation and multiple reaction monitoring mass spectrometry approach. *Mol Cell Proteomics.* 2010;9:1400–1410.
 32. Santhanam L, Gucek M, Brown TR, Mansharamani M, Ryoo S, Lemmon CA, Romer L, Shoukas AA, Berkowitz DE, Cole RN. Selective fluorescent labeling of S-nitrosothiols (S-FLOS): a novel method for studying S-nitrosation. *Nitric Oxide.* 2008;19:295–302.
 33. Huang B, Chen C. Detection of protein S-nitrosation using irreversible biotinylation procedures (IBP). *Free Radic Biol Med.* 2010;49:447–456.
 34. Zhang X, Huang B, Zhou X, Chen C. Quantitative proteomic analysis of S-nitrosated proteins in diabetic mouse liver with ICAT switch method. *Protein Cell.* 2010;1:675–687.

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

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



A Twist on Quantification: Measuring the Site Occupancy of S-Nitrosylation

Christopher I. Murray and Jennifer E. Van Eyk

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

Copyright © 2012 American Heart Association, Inc. All rights reserved.

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/content/111/10/1253>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation Research* is online at:
<http://circres.ahajournals.org/subscriptions/>