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Review

Methodologies for the characterization, identification and quantification of S-nitrosylated proteins ☆

Matthew W. Foster *

Division of Pulmonary, Allergy and Critical Care Medicine, Duke University Medical Center, Durham, NC 27710, USA

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ABSTRACT

Background: Protein S-nitrosylation plays a central role in signal transduction by nitric oxide (NO), and aberrant S-nitrosylation of specific proteins is increasingly implicated in disease.
Scope of Review: Here, methodologies for the characterization, identification and quantification of SNO-proteins are reviewed, focusing on techniques suitable for the structural characterization and absolute quantification of isolated SNO-proteins, the identification and relative quantification of SNO-proteins from complex mixtures as well as the mass spectrometry-based identification and relative quantification of sites of S-nitrosylation (SNO-sites) in proteins.
Major Conclusions: Structural characterization of SNO-proteins by X-ray crystallography is increasingly being utilized to understand both the relationships between protein structure and Cys thiol reactivity as well as the consequences of S-nitrosylation on protein structure and function. New methods for the proteomic identification and quantification of SNO-proteins and SNO-sites have greatly impacted the ability to study protein S-nitrosylation in complex biological systems.
General Significance: The ability to identify and quantify SNO-proteins has long been rate-determining for scientific advances in the field of protein S-nitrosylation. Therefore, it is critical that investigators in the field have a good understand the utility and limitations of modern analytical techniques for SNO-protein analysis.
This article is part of a Special Issue entitled: Regulation of cellular processes by S-nitrosylation.

1. Introduction

It is increasingly recognized that protein S-nitrosylation, the post-translational modification of Cys thiol by nitric oxide (NO) to generate S-nitrosothiol (SNO), transduces many of the biological effects of NO [1–3]. Aberrant S-nitrosylation is implicated in numerous cardiopulmonary, skeletomuscular and neurodegenerative diseases [4,5]. Largely driven by methodological limitations, early studies in the field largely focused on: abundant and readily detectable endogenous species, namely SNO-hemoglobin (SNO-Hb) and SNO-albumin (SNO-Alb) [4]; proteins that could be easily obtained in purified form for in vitro analyses (e.g. SNO-GAPDH and SNO-caspase); and the physiological effects of NO that could often be only indirectly ascribed to S-nitrosylation [6]. The development of new techniques for the enrichment and identification of endogenous SNO-proteins, and mapping of sites of S-nitrosylation (SNO-sites) have prompted most of the major discoveries in the field over the last 10 years, many of which are highlighted elsewhere in this Special Issue.

There are now perhaps hundreds of published permutations of assays for SNO-protein characterization, identification and quantification. However, assays generally fall into one of three classes (Table 1). They involve: 1) direct detection of a NO-modified thiol; 2) chemical reduction or photolytic breakdown of the SNO to a more readily identifiable NO-based species; or 3) tagging of the S-nitrosylated Cys thiol for subsequent enrichment and identification of SNO-proteins as well as facile mapping of SNO-sites. In general, methodologies in class 1 are mostly biophysical techniques—perhaps the most powerful being X-ray crystallography—that are best suited for characterization of single, isolated SNO-proteins. Techniques specific to class 2 detect SNO-derived NO and nitrite and are amenable to absolute quantification of total amounts of protein S-nitrosylation (but not specific SNO-proteins) in biological mixtures. The third class of methodologies is particularly suited for identification of SNO-proteins and SNO-sites from complex mixtures and relative (but not absolute) quantification of these species across multiple samples. Here we present an overview of both "tried and true" and promising new methodologies for SNO-protein characterization, identification and quantification.

2. Characterization of intact S-nitrosoproteins and protein-derived S-nitrosopeptides

Purified SNO-proteins are amenable to characterization by a number of biophysical techniques, including mass spectrometry (MS) and X-ray crystallography (see below), as well as ultraviolet/

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* Tel.: +1 919 225 4552.
E-mail address: mwfoster@duke.edu.

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Table 1
Overview of methodologies for detection of SNO-proteins.

Class 1: Detection of intact SNO[a]
X-ray crystallography
UV–Vis spectroscopy
NMR spectroscopy
Mass spectrometry
SNO-specific antibodies
Class 2: Detection of SNO-derived nitrite and NO[b]
Saville assay
DAF-2 assay
GC–MS
Photolysis chemiluminescence
Reductive chemiluminescence
NO electrode
Class 3: Labeling of SNO-derived Cys thiol[c]
Biotin switch technique (BST)
SNO-site identification (SNO-SID)
S-nitrosothiol capture (SNOCAP)
Resin-assisted capture of SNO-Proteins (SNO-RAC)
Spin trapping after UV photolysis
Organomercurial-binding
Phosphine-based ligation

[a] These methods, except for SNO-based antibodies, are mostly suitable for the characterization of purified SNO-proteins and are generally of low-sensitivity.
[b] These methods are suitable for quantifying total levels of endogenous SNO-proteins but have limited utility for the analysis of specific SNO-proteins from complex mixtures.
[c] These methods are useful for the enrichment, identification and relative quantification of SNO-proteins from complex mixtures and for the facile identification of SNO-sites.

visible spectroscopy (UV/Vis) [7,8] and ^{15}N nuclear magnetic resonance spectroscopy (NMR) [7,9]. The techniques are mostly applicable to the characterization of isolated SNO-proteins. UV/Vis can be used for quantification of low-mass SNOs [10], but SNO-proteins are not generally produced in sufficient quantities to be easily detected, while NMR has little demonstrated utility. SNO-specific antibodies, raised against an S-nitrosocysteine epitope, have also been used for the enrichment and identification of SNO-proteins in situ (see below).

2.1. X-ray crystallography

High-resolution crystal structures have been recently solved for a number of SNO-proteins, namely S-nitrosylated hemeproteins [11–13], protein tyrosine phosphatase 1B [14] and thioredoxin (SNO-Trx) [8]. Collectively, these structural analyses have not only enabled SNO-site identification, but also have helped to characterize the effects of S-nitrosylation on protein structure, the "solid-state" conformations of protein-bound SNO (e.g. R–S–N–O dihedral) and potential mechanisms of S-nitrosylation (Fig. 1). Treatment with NO or low-mass S-nitrosothiol has been performed both prior to [8,13] and after crystallization [11,12,14]. SNO-proteins can survive lengthy crystallization protocols, as evidenced by the stability of SNO-myoglobin after one month at room temperature (under light- and metal-free conditions) [13]. However, the S-NO moiety appears to be unstable to synchrotron radiation [13,15], which is preferred for solving high-resolution structures.

2.2. Mass spectrometry (MS)

MS has been applied to the analysis of both intact and protease-digested SNO-proteins. SNOs are unstable to analysis by matrix-assisted laser desorption ionization MS (MALDI-MS) but can be detected by electrospray ionization MS (ESI-MS) [16]. Deconvoluted spectra of intact SNO-proteins exhibit a mass shift of +29 amu per bound NO and can suggest SNO:protein stoichiometry [16–19]. Alternatively, SNO-containing peptides have been identified from proteolyzed proteins using a variety of digestion conditions, instrumentation and acquisition methods [20–22]. SNO-Hb was characterized by pepsin digestion (50:1 protein:protease, 5% formic acid, 37 °C, 1.5 h) followed by reverse phase-LC–MS, varying the cone voltage between 20 and 80 V across multiple injections [20]. A voltage-dependent shift of −29 amu was observed for a peptide corresponding to residues 89–105 of β-Hb (Cys93 is the known SNO-site). A SNO-site in argininosuccinate synthetase (AS) was localized by neutral loss scanning using a triple quadrupole mass spectrometer [21]. SNO-AS was digested with trypsin (50:1, pH 8.0, 37 °C, 4 h) and infused into the ESI source. Parent ions that lost 30 or 15 m/z (singly or doubly charged ions, respectively) in the first quadrupole were allowed to pass into the second quadrupole for mass analysis. More recently, a different technique was used to map a SNO-site on *Arabidopsis thaliana* NPR1 after digestion with trypsin (20:1, pH 5.5, 37 °C, 1 h) and analysis by nano-RP-LC–MS^3 on a LTQ-Orbitrap [22].

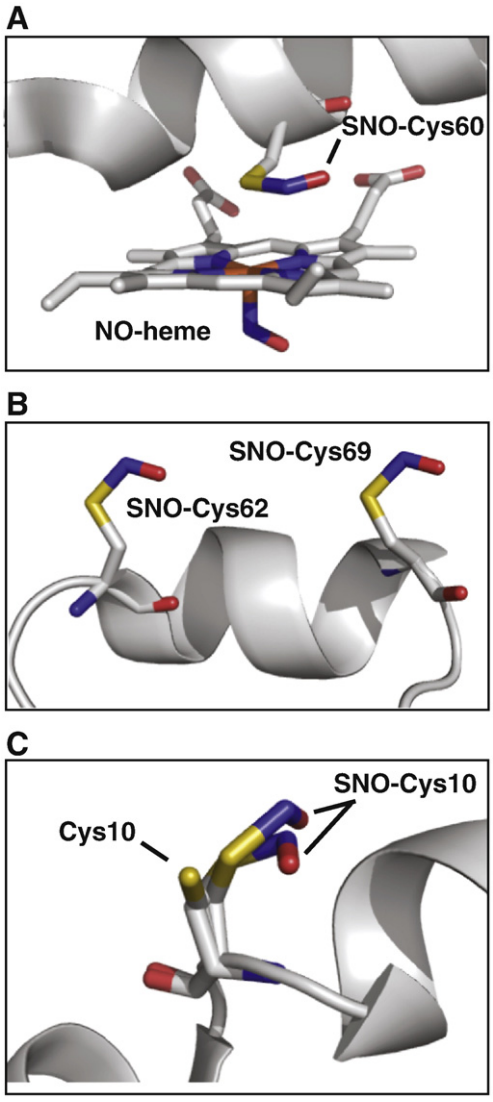

Fig. 1. X-ray crystallography of S-nitrosylated proteins. A) Exposure of *Cimex lectularius* nitrophorin crystal to NO led to distal heme nitrosylation and S-nitrosylation of Cys60 on the proximal side of the heme (PDB ID: 1Y21) [12]. Stopped-flow UV–Vis spectroscopy revealed that heme reduction is coupled to S-nitrosylation of the protein by NO. B) Human thioredoxin was S-nitrosylated at two adjacent sites (Cys62 and Cys69) of an α-helix (PDB ID: 2HXK). The S-nitrosylated Cys residues both adopt a cis-planar configuration. Cys62, which exhibits higher reactivity than Cys69 [8], sits at the helix N-terminus, a newly identified S-nitrosylation motif [55]. Interestingly, SNO-Cys62 is buried and faces the interior of the protein (not shown) [8]. C) The structure of CysNO-treated Blackfin Tuna myoglobin (PDB ID: 2NRM) revealed the presence of both reduced and S-nitrosylated Cys10 [13]. Two conformations of SNO-Cys10, each adopting a cis-planar configuration, were observed. Images were generating using Pymol.