Abstract

This review discusses proteomic methods to detect and identify S-nitrosated proteins. Protein S-nitrosation, the post-translational modification of thiol residues to form S-nitrosothiols, has been suggested to be a mechanism of cellular redox signaling by which nitric oxide can alter cellular function through modification of protein thiol residues. It has become apparent that methods that will detect and identify low levels of S-nitrosated protein in complex protein mixtures are required in order to fully appreciate the range, extent and selectivity of this modification in both physiological and pathological conditions. While many advances have been made in the detection of either total cellular S-nitrosation or individual S-nitrosothiols, proteomic methods for the detection of S-nitrosation are in relative infancy. This review will discuss the major methods that have been used for the proteomic analysis of protein S-nitrosation and discuss the pros and cons of this methodology.

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1. Introduction

Of all the potential post-translational thiol modifications that have been suggested to be involved in the transmission of intra-
intracellular signal through its interaction with thiols is at first a rather unlikely one. Metal centers (e.g. ferrous hemes) and free radicals (e.g. superoxide) are kinetically preferable targets, and NO does not react with thiols at any biologically meaningful rate [2,3]. The direct reaction between NO and thiols is a redox reaction generating nitrous oxide, sulfenic acid and/or disulfide, but not RSNO [4,5]. However, in the presence of oxygen and thiols, NO generates colored RSNO. These compounds were shown to have some synthetic chemical use, for instance as intermediates in the formation of mixed disulfides [6], and had antibacterial properties [7]. It was not until S-nitroso-N-acetyl-penicillamine (SNAP) was shown to have vasodilatory properties [8] that the biological potential of RSNO in mammalian systems became apparent. With the discovery of NO as a physiological mediator of vascular responses [9,10], as well as many other processes, the role of endogenously produced RSNO, first measured by Stamler et al. [11], as mediators of a sub-set of NO-dependent responses became, and remains, an area of great interest.

While in vivo mechanisms of S-nitrosation remain the subject of debate, and are clearly more complex than a simple association of NO with a thiol, RSNO can be detected in vivo under basal and pathological conditions [11–14]. Much investigation has focused on examining the role of S-nitrosation in modifying specific cellular pathways. For example, the ability of NO to affect apoptosis has been linked to modification of a catalytically important thiol in caspase-3 [15,16]. Relatively few studies have examined S-nitrosation from a more global perspective by placing individual effects of nitrosative stress in the context of the proteome of S-nitrosated (or otherwise modified) thiols. This article will assess current methods for the detection of global protein S-nitrosation using proteomic techniques.

2. The Chemistry of S-Nitrosothiols

As the mechanism of thiol nitrosation is important in attempting to understand the S-nitrosated proteome, in this section we will briefly describe mechanisms of S-nitrosothiol formation. There are four major mechanisms of S-nitrosation that have the potential to occur in biological systems. (i) S-Nitrosothiols can be formed from the reaction of nitrous acid (HONO) with thiols (Eqs. (1) and (2)).

$$\text{HONO} + \text{H}^+ \rightarrow \text{H}_2\text{ONO}^+ \quad (1)$$

$$\text{H}_2\text{ONO}^+ + \text{RSH} \rightarrow \text{RSNO} + \text{H}_2\text{O} + \text{H}^+ \quad (2)$$

This is major mechanism of RSNO synthesis in the test tube [17,18], and only occurs at pH values significantly below the physiological norm. As the $pK_a$ of nitrous acid is 3.37, only vanishingly low levels of acid are present at physiological pH, and it is thought that HONO itself requires protonation before it can S-nitrosate thiols [19]. While this reaction may play some role in the gastro-intestinal tract, it is not clear that tissue pH could ever drop low enough to make significant levels of RSNO. (ii) It is possible for RSNO to be formed by the direct addition of nitrosonium (NO$^+$) to a thiol at neutral pH (Eq. (3)).

Peroxidase

$$\text{RSH} + \text{NO}^+ \rightarrow \text{RSNO} + \text{H}^+ \quad (3)$$

complexes I and II [20,21] have been shown to be reduced by NO, presumably generating nitrosonium. However, the major limitation of this mechanism is that nitrosonium is unstable in water at neutral pH, immediately hydrolyzing to nitrite, and so the thiol must be in the immediate vicinity of source of nitrosonium. Myeloperoxidase-dependent N-nitrosation has been reported [22], but so far there are no reports of S-nitrosation by peroxidase-mediated mechanisms. (iii) Direct thiol nitrosation by N$_2$O$_3$ occurs at a relatively fast rate forming an S-nitrosothiol and nitrite (Eq. (4)) [23]. In order

$$\text{RSH} + \text{N}_2\text{O}_3 \rightarrow \text{RSNO} + \text{NO}_2^- + \text{H}^+\quad (4)$$
to generate N$_2$O$_3$ however, NO needs to be oxidized to NO$_2$, which will then combine with NO to form N$_2$O$_3$. While this chemistry clearly happens in the test tube, the oxidation of NO to NO$_2$ by oxygen occurs by a reaction that is second order in NO and first order in oxygen (Eq. (5)) [23–25], and consequently very slow at biological concentrations of NO. It has been suggested that hydrophobic areas in membranes and proteins could increase the local concentration of both NO and oxygen and so accelerate this reaction [26,27], thus increasing the probability of its occurrence in physiological systems. Other mechanisms of NO$_2$ formation are possible such as the

$$2^\ast\text{NO} + \text{O}_2 \rightarrow 2\text{NO}_2\quad (5)$$
oxidation of nitrite by peroxidase complexes I and II [28]. At a level of inflammation, where NO, nitrite and hydrogen peroxide may be generated in the presence of peroxidases, it is possible to envisage the chemical requirements for N$_2$O$_3$ formation. In addition, N$_2$O$_3$ could be formed from the condensation of nitrous acid (Eq. (6)). As discussed above, the level of HONO will be very small at neutral pH, and as this

$$\text{HONO} + \text{HONO} \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O}\quad (6)$$

reaction depends on the square of the concentration of HONO, it is likely to have little impact at neutral pH. However, this mechanism may well be responsible for the formation of S-nitrosothiols that have been reported at around pH 6.0 [29] and may be a relevant reaction during ischemic injury, where tissue pH may drop to such low values. (iv) The addition of NO to a thyl radical (Eq. (7)) will form an S-nitrosothiol by a diffusion

$$\text{RS}^\ast + \cdot\text{NO} \rightarrow \text{RSNO}\quad (7)$$

limited radical–radical combination reaction [30,31]. Consequently any process that can give rise to a thyl radical has the potential to also generate S-nitrosothiols. The fact that glutathione (GSH) is able to repair other free radicals (such as the tyrosyl radical) by hydrogen (or electron) donation suggests that thyl radicals may be a general feature of oxidative free-radical exposure. Another possible mechanism of RSNO formation that deserves further investigation is the direct addition of NO to a thiol to form a putative RSNOH intermediate, followed by reaction with oxygen (or a one-electron acceptor) to