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Review

Gel-based methods in redox proteomics ☆

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ABSTRACT

Background: The key to understanding the full significance of oxidants in health and disease is the development of tools and methods that allow the study of proteins that sense and transduce changes in cellular redox. Oxidant-reactive deprotonated thiols commonly operate as redox sensors in proteins and a variety of methods have been developed that allow us to monitor their oxidative modification.
Scope of the review: This outline review specifically focuses on gel-based methods used to detect, quantify and identify protein thiol oxidative modifications. The techniques we discuss fall into one of two broad categories. Firstly, methods that allow oxidation of thiols in specific proteins or the global cellular pool to be monitored are discussed. These typically utilise thiol-labelling reagents that add a reporter moiety (e.g. affinity tag, fluorophore, chromophore), in which loss of labelling signifies oxidation. Secondly, we outline methods that allow specific thiol oxidation states of proteins (e.g. S-sulphenylation, S-nitrosylation, S-thionylation and interprotein disulphide bond formation) to be investigated.
Major conclusions: A variety of different gel-based methods for identifying thiol proteins that are sensitive to oxidative modifications have been developed. These methods can aid the detection and quantification of thiol redox state, as well as identifying the sensor protein.
General significance: By understanding how cellular redox is sensed and transduced to a functional effect by protein thiol redox sensors, this will help us better appreciate the role of oxidants in health and disease. This article is part of a Special Issue entitled Current methods to study reactive oxygen species — Pros and cons.

1. Introduction

Cellular oxidants were once considered primarily harmful entities that damage cellular biomolecules to cause dysfunction. However, accumulating evidence suggests a role of oxidants in the normal functioning of the healthy cell. The transduction of an oxidant signal into a biological response can be mediated in several ways, one of which involves them chemically reacting with protein cysteine residues to induce their oxidation. Cysteine thiol (–SH) groups can undergo a variety of disparate redox reactions depending upon the species and concentrations of oxidants they come into contact with. However, not all thiol groups are susceptible to modification under the redox conditions that can exist in the cellular environment. Low pKa thiols that ionise to the thiolate anion ($-S^-$) state at physiological pH are particularly predisposed to oxidative post-translational modifications. Therefore, the likelihood of a thiol being susceptible to oxidation, which in turn enables the redox-regulation of protein function, will depend upon its specific local environment within the protein (as this determines its pKa), together with its proximity and accessibility to cellular oxidants. A number of different post-translational oxidative modifications can occur such as S-sulphenylation, S-nitrosylation and disulphide-modified proteins, as shown in Fig. 1. Many thiol oxidative modifications are reversible, usually by reducing enzymes such as thioredoxin or glutaredoxin [1,2]. Irreversible modifications, such a sulphinic and sulphonic acids are often considered in terms of damage. However, it has been shown that in at least one case, a sulphinylated form of 2-cysteine peroxiredoxin can be reduced by the ATP-dependent protein sulphiredoxin [3].

Defining how cellular redox changes alter proteins by affecting crucial thiol residues will help us understand the role of cysteine-targeted oxidation in the control of cellular functions. Consequently, a number of different methods have been developed that allow detection, quantification and often identification of protein thiol redox state. Some methods also allow the identification of the cysteine residue where the redox modification occurs. This review focuses on gel-based methods that can be used to investigate protein thiol oxidative modifications.

2. Thiol-tagging methods

Reduced thiols in proteins can be chemically derivatised using a number of readily commercially available compounds, such as N-ethylmaleimide (NEM), iodoacetamide (IAM), iodoacetic acid (IAA) and thiosulphates. These chemicals can have restricted accessibility to some protein thiols, preferentially labelling only those on the surface. However, labelling the protein under denaturing conditions (e.g. in

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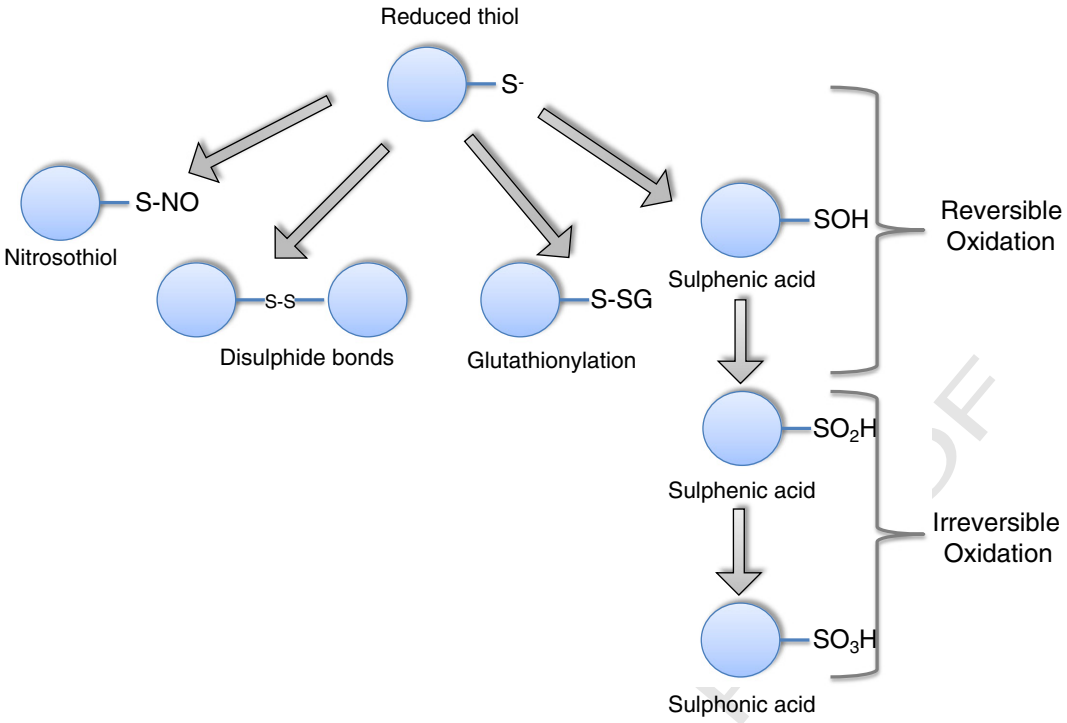

Fig. 1. Outline of potential thiol modifications. Protein thiols that are exposed to oxidation can form a variety of intermediates, nitrosothiols, sulphenic acid. These intermediates may be converted into more stable states such as inter- or intra-protein disulphides. These types of modifications are reversible, however longer exposure to oxidation can lead to irreversible modifications, such as sulphinic and sulphonic acid formation.

the presence of SDS) renders interior thiols accessible [4]. The reaction between thiols and maleimide-based compounds, such as NEM, occurs by addition reactions across the double bonds of these commonly used reagents [5]. NEM reacts with cysteine over a wider pH range [6], though it is not fully selective for thiol side-chains as it can also react with tyrosine, histidine, methionine and lysine residues. IAM and IAA have less reactivity with tyrosine, histidine, methionine and lysine and therefore are more thiol-selective [4]. Despite the increased selectivity of IAA and IAM, NEM has been shown to be more effective at alkylating thiols [6]. Similarly, the reaction between NEM and thiols is much faster than the reaction with IAA and IAM. This may be of importance in sample preparation as a longer incubation time for alkylation will result in oxidation or thiol disulphide exchange occurring in samples [6].

Biotin, fluorophores, radiophores or polyethylene glycol (PEG) can be chemically combined with NEM, IAM and IAA to generate thiol-reactive compounds that enable detection and quantification. For example, PEG-maleimide will react with a thiol to increase the mass of the protein. A variety of PEG-maleimide derivatives that add different masses (e.g. 5, 10 or 20 kDa) per thiol it reacts with are commercially available. Proteins can subsequently be resolved by SDS-PAGE and then monitored on Western blots using an antibody to a specific protein of interest. Proteins with oxidised thiols will not incorporate PEG-maleimide and consequently will be detected at a lower molecular weight than the same protein with reduced thiols that will react with the probe. The degree of shift will depend upon the number of modified thiols and the weight of the tag used [5]. Fluorescent or radioactive reporter moieties have also been combined with thiol reactive compounds to try and identify oxidised thiols. Under oxidising conditions when protein thiols may become oxidised, less of these reporter molecules are incorporated and this can be routinely monitored using commonly available detection methods [7].

Biotinylated IAM (BIAM) and biotinylated NEM (Bt-NEM) have both been commonly used to label protein thiols. BIAM-labelling preferentially reacts with protein thiolates, a chemical feature that has been utilised in the development of a method that selectively labels protein cysteines that are oxidant sensitive. This BIAM-labelling method is carried out at pH 6 (or lower) to promote the full protonation of the majority of thiols which typically have a higher pKa [8]. Thus most reduced protein thiols will not label with BIAM as it reacts selectively with those in the reactive thiolate state. However, low pKa oxidant-reactive thiols will still be partially deprotonated at low pH. As oxidants also preferentially react with these low pKa thiols, low pH BIAM-labelling provides a method that allows oxidant-sensitive thiols to be selectively studied. This theoretically Q4 overcomes a generic problem with methods that use loss of thiol labelling to monitor oxidative stress, namely that most thiols are not oxidant sensitive. Thus, practically it is difficult to detect oxidation of the relatively few oxidant-reactive protein thiols against a very high background because of the abundance of non-redox active thiols. The loss of BIAM-labelling, indexed on Western blots using streptavidin-HRP, indicates protein thiol oxidation. The biotin tag also enables the purification of proteins susceptible to oxidation using avidin-based affinity matrices and their identification using LC–MS/MS.

3. Tag-based methods for detecting specific forms of protein thiol oxidation

Most of the tag-based methods described in the previous section detect cysteine oxidation by loss of labelling due to thiol modification. However, identifying an oxidant-induced loss of signal is challenging, particularly against a high background signal (due to protein thiols that are not sensitive to oxidation being labelled). An alternate, potentially better approach would be to identify modified thiols by a gain of signal. In this way, alkylating (or tagging) reagents may be used to block any unmodified thiols prior to reversal of the thiol modification and subsequent labelling of the free thiol. For example, NEM or IAM may be used to block all free thiols in a cell lysate prior to removal of the unincorporated alkylation reagent, before the selective reduction of the oxidative modification to generate a free thiol. This reduced thiol can then be subsequently labelled to aid with identification.

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