Contemporary techniques for detecting and identifying proteins susceptible to reversible thiol oxidation

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Abstract
Elevated protein oxidation is a widely reported hallmark of most major diseases. Historically, this ‘oxidative stress’ has been considered causatively detrimental, as the protein oxidation events were interpreted simply as damage. However, recent advances have changed this antiquated view; sensitive methodology for detecting and identifying proteins susceptible to oxidation has revealed a fundamental role for this modification in physiological cell signalling during health. Reversible protein oxidation that is dynamically coupled with cellular reducing systems allows oxidative protein modifications to regulate protein function, analogous to phosphoregulation. However, the relatively labile nature of many reversible protein oxidation states hampers the reliable detection and identification of modified proteins. Consequently, specialized methods to stabilize protein oxidation in combination with techniques to detect specific types of modification have been developed. Here, these techniques are discussed, and their sensitivity, selectivity and ability to reliably identify reversibly oxidized proteins are critically assessed.

Introduction
Cellular proteins can undergo a diverse array of post-translational modifications that include the addition of lipids, phosphate, sugars and oxidant moieties. Specific modifications can occur preferentially at select amino acids, but generally there is a propensity for such alterations to modify protein function, providing a potential mechanism of regulation.

In terms of oxidant-dependent modifications, cysteine thiols are a primary target. However, not all cysteine residues are redox-sensitive, as only those with a low pKₐ, which is dependent on their precise surrounding environment, tend to be reactive. Thus not all thiols are ‘created equally’, providing a basis for oxidant-selective signalling [1]. The presence of amino acids lysine, arginine or histidine in the circumambient space of a cysteine leads to deprotonation of its thiol. Such low-pKₐ thiols exist as a thiolate anion (S⁻) at neutral, cellular pH, making them more reactive with electrophilic oxidants compared with the protonated thiol state. Cysteine thiols can undergo a variety of oxidative modifications, many being reversible via the cellular reducing system. The key enzymes involved in reversal of protein thiol oxidation are Trx (thioredoxin) and Grx (glutaredoxin), which selectively reduce protein disulfides and glutathionylation respectively [2]. Oxidative modifications that are irreversible are often considered as damage, potentially contributing to disease progression. Such irreversible oxidation states generally include the sulfenic and sulfonic acid states, although in the specialized case of 2-cysteine peroxiredoxins, sulfinic acid formation can be reduced by the enzyme sulfiredoxin through an ATP-dependent reaction [3]. The formation of a disulfide is often through an intermediate oxidation state, which is commonly a sulfenic acid or S-nitrosothiol. When such reversible intermediate oxidation states are generated, they may be resolved by a vicinal thiol, a reactive thiol on another protein or by glutathione. This leads to the formation of an intra-disulfide, inter-disulfide or glutathione adduct respectively. These various oxidation states of the cysteine thiols are shown in Figure 1. As cysteine oxidation is not indiscriminate, but selective, and in many cases occurs reversibly to control the function of proteins, this provides a dynamic well-orchestrated mechanism of signalling to appropriately alter function in the setting of changes in cellular redox [4].

Cysteine thiol alkylation to preserve oxidative modifications and prevent artificial oxidation
The intracellular environment is reducing, owing to the abundance of reducing enzymes that maintain redox-sensitive proteins in a non-oxidized state. By lysing cells in the presence of air there is a risk that proteins will become artificially oxidized by molecular oxygen. Such artificial oxidations are more likely when procedures purify proteins from complex mixtures, owing to total withdrawal from reducing buffers. In addition, there is also potential for oxidative modifications to
be transferred from one protein to another within the lysate, leading to artificial protein oxidation. This can commonly occur via thiol–disulfide exchange or shuffling reactions. These potential pitfalls can be avoided by directly lysing tissue or cells into an alkylating agent that will bind to free thiols preventing air oxidation and transfer reactions. There are many different alkylating agents available with the most commonly used being iodoacetamide, maleimide, iodoacetate and methyl methanethiosulfonate. In addition to alkylating agents, the pH of lysis buffer can be lowered to prevent artefactual thiol oxidation. Utilizing acid conditions below the $pK_a$ of protein cysteine thiols ensure their protonation, lowering their reactivity and so likelihood of air oxidation after lysis.

**Biotin-switch assays for identifying S-nitrosothiols, sulfenic acid or glutathione-modified proteins**

The cysteine thiol modifications S-nitrosylation and sulfenation are generally intermediate oxidation states making them short-lived and therefore difficult to detect. However, some proteins may have these oxidized states in a stable form, owing to their inaccessibility to thiol-reducing agents. Indeed, in the case of stable S-nitrosylation, this modification has become accepted as a physiological modification that can regulate protein function. This is supported by a number of identified proteins that are resistant to denitrosylation and under basal conditions are stably S-nitrosylated [5].

For detecting protein S-nitrosylation, specific antibodies have been generated; however, for many proteins this modification will not be stable enough for immunodetection. Indeed, there is some scepticism as to whether such antibodies truly detect S-NO adducts. Owing to such limitations, a new approach has become especially popular for the identification of S-nitrosylated proteins. This has been termed ‘the biotin-switch method’ and was developed by Jaffrey and Snyder in 2001 [6]. This method relies on stably labelling proteins containing a nitrosothiol [7]. This technique involves multiple steps, with the first being complete alkylation of all free thiols, which is aided by heating samples under denaturing conditions with SDS. This step is crucial as it stabilizes the oxidation state as described above. Subsequently, an unincorporated alkylating agent is removed by precipitating and rinsing proteins or by column desalting. The nitrosothiol modification is then selectively reduced using ascorbate with the resulting free thiol then being labelled with a biotin-conjugated alkylating agent. It has been recently reported that addition of copper during labelling enhances reduction of S-nitrosothiols improving the detection of modified proteins [8]. The exchange of the labile nitrosothiol modification for a stable biotin tag allows easy detection of modified proteins by Western blotting with avidin-based detection protocols. In addition, nitrosothiol-adducted proteins can be identified by using solid-phase avidin materials followed by MS analysis or immunoblotting for candidate targets. To improve purification of modified proteins, a reducible biotinylated alkylating agent can be used during the labelling step. This includes the use of biotin-HPDP $\{N$-$[6$-(biotinamido)hexyl]-3'-$(2'$-pyridyldithio)-propionamide\}$, which can be coupled to streptavidin–Sepharose after alkylation or more simply by using thiopropyl-Sepharose 6B, a reversible alkylating agent already coupled with Sepharose. This procedure allows modified proteins to be eluted from affinity matrices using 2-mercaptoethanol. The advantage of this approach is that it significantly decreases the elution of non-specific proteins that bind to the affinity matrix that would normally be detected if proteins were to be eluted using Laemmli buffer [9].

Although this method has been shown to be specific for nitrosothiol modifications, it is, however, relatively

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**Figure 1** Reactive protein thiolates can undergo oxidation to an intermediate state before being converted into a more stable disulfide

Disulfides may be reversed back to the basal reduced state by either Grx or Trx. In the presence of high levels of $H_2O_2$, sulfenic acids can undergo further oxidation (hyperoxidation) to the sulfinic and the sulfonic acid forms, which are generally irreversible in biological systems.
Identify the modification have been developed. Furthermore, the reactivity of the cyclic diketone dimedone for sulfenic acids using this technique. However, by exploiting the specific physiological or pathological conditions difficult to detect. This makes subtle changes in sulfenic acid formation under physiological conditions. Originally, the detection of modified proteins used radiolabelled dimedone and autoradiography or changes in optical absorbance. Subsequently, MS has been widely used to monitor dimedone reacting with sulfinates. More recently fluorescent or biotinylated dimedone derivatives have been generated, as shown in Figure 3. The biotin attached to dimedone makes the compound more cell-permeant and can also easily be used with avidin-based methods to capture target proteins. However, the need for a biotin tag or fluorophore for detecting modified proteins is no longer required, since the recent advent of antibodies that specifically recognize dimedone bound to sulfenic acid-modified proteins.

Identification of sulfenic acid-modified proteins using dimedone

The arsenite-dependent biotin-switch labelling of sulfinated proteins has limited sensitivity due to the time it takes to exchange the modification for a stable biotin tag, as intracellular sulfinates are rapidly redox-cycled. Furthermore, the labelling protocol is carried out under denaturing conditions that probably destabilizes many sulfinates that are normally stabilized by crucial local environments within the protein. This makes subtle changes in sulfenic acid formation under physiological or pathological conditions difficult to detect using this technique. However, by exploiting the specific reactivity of the cyclic diketone dimedone for sulfenic acids, several rapid, simple and sensitive methods for detecting this modification have been developed. Furthermore, the derivatization of sulfenic acids with dimedone forms a stable product that is not subject to further redox alteration during sample processing and can be carried out without denaturing conditions. Originally, the detection of modified proteins used radiolabelled dimedone and autoradiography or changes in optical absorbance. Subsequently, MS has been widely used to monitor dimedone reacting with sulfinates. More recently fluorescent or biotinylated dimedone derivatives have been generated, as shown in Figure 3. The biotin attached to dimedone makes the compound more cell-permeant and can also easily be used with avidin-based methods to capture target proteins. However, the need for a biotin tag or fluorophore for detecting modified proteins is no longer required, since the recent advent of antibodies that specifically recognize dimedone bound to sulfenic acid-modified proteins.

Identification of thiolated proteins using immunoblotting or tag-based chemistry

Pan-specific antibodies have been generated for S-glutathionylated and S-homocysteinylated proteins. However, the selectivity and sensitivity of these antibodies is unclear and the method often does not work well for immunoblotting complex protein mixtures. Using such antibodies in conjunction with immunoprecipitation of target proteins can yield clearer results with complex proteins mixtures. However, this requires a known target and the ability to cleanly immunocapture it. Another approach for monitoring protein S-thiolation, particularly in the pursuit of identifying new substrates, involves the use of tagged derivatives of low-molecular-mass thiols which may be incorporated into proteins via disulfide bond formation. Reduced forms of these labelled molecules include cysteine (disulfide bond cysteine dimer) with an N-terminal fluorescent tag or GSSG disulfide with an N-terminal biotin tag that can be used for the detection of thiols susceptible to S-homocysteinylation or glutathionylation specifically. These probes, because of their oxidized disulfide, can undergo exchange reactions with reactive cysteine thiols, resulting in S-thiolation by the tagged derivative. Reduced forms of these probes have also been used and can exchange with protein thiols that are basally oxidized or modify proteins when oxidants are present that can drive S-thiolation reactions via S-nitrosylation or sulfination intermediates. These approaches allow detection, quantification and, with some tags, the purification and identification of S-thiolated proteins. For GSH, a biotinylated ethyl ester analogue has been generated for improved cell permeability.

Intraprotein disulfide and vicinal thiol analysis

The formation of an intramolecular disulfide in some proteins can cause a small shift on non-reducing SDS/PAGE gels detected after immunoblotting. This is due to a change in structure that decreases SDS-dependent protein linearization. However, other modifications including phosphorylation

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**Figure 2** The biotin-switch assay relies on blocking free thiols with alkylating agent and then labelling modified cysteine residues with a biotin tag following selective reduction.

This technique can be used to identify S-nitrosylated, sulfenic acid or glutathionylated proteins using different forms of selective reduction.

**Figure 3** Different approaches for identifying sulfenic acid-modified proteins using dimedone.

Proteins derivatized by dimedone can be detected using a specific antibody that recognizes only proteins modified by this compound. Alternatively, a biotinyalted derivative can be used for detection and purification, or a fluorescent conjugated form of dimedone can also be used for the detection of modified proteins.
Figure 4 | Detecting proteins susceptible to S-thiolation using tagged thiolating agents

Oxidized tagged dimeric thiolating agents can directly react with susceptible protein thiols. In addition, reduced tagged thiolating agents can exchange with oxidative modifications on protein thiols or modify reduced thiols in the presence of oxidants that induce this type of modification.

and S-thiolation can also cause a change in protein migration, making this an unreliable readout for intraprotein disulfide formation. Generally, the formation of intraprotein disulfides occurs between vicinal thiols that are located in close proximity in the linear protein sequence. The formation of such disulfides usually occurs through intermediate oxidation of one of the thiols (usually the most reactive with the lowest pKₐ), which is then reduced by a neighbouring resolving thiol, leading to disulfide formation. Several compounds are known to selectively react with and cross-link vicinal thiols, including PAO (phenylarsine oxide) and DBB (dibromobimane) as shown in Figure 5. A PAO–Sepharose matrix has been developed for trapping proteins with vicinal thiols that can then be identified by immunoblotting for specific targets or using MS analysis [25]. Many of the proteins that bind to this compound are likely to be sensitive to intramolecular disulfide formation. The fact that formation of a disulfide will block protein binding to PAO can also be exploited to monitor this form of oxidation of select targets under different physiological or pathological conditions [26]. In addition, DBB can be used for monitoring protein intramolecular disulfide formation by measuring the fluorescence that is generated when this compound binds to vicinal cysteine pairs located within 3–6 Å (1Å = 0.1 nm) of each other [27]. Fluorescence can be detected for complex mixtures or purified proteins on gels by using the fluorogenic properties of this compound upon replacement of both bromine atoms.

Identification of intermolecular disulfide protein complexes using diagonal gel electrophoresis

To monitor potential intermolecular disulfide formation between candidate proteins a non-reducing SDS/PAGE gel of samples can be run and then immunoprobed using antibodies to candidate proteins that are oxidized in this way. For example, non-reducing SDS/PAGE has been used to monitor disulfide formation in protein kinases A and G [28–30], as well as peroxiredoxins in response to oxidant stress [31]. It is important to consider that some antibodies may decrease or increase their affinity for proteins if they form disulfide complexes as a result of an impact on the epitope. To identify new intermolecular disulfide complexes, diagonal gel electrophoresis can be used [32,33], as outlined in Figure 6. This technique relies on running a sample on a non-reducing SDS/PAGE gel to preserve any disulfide-bound proteins, which will resolve as a higher-molecular-mass complex. The resolved sample lane is then excised and exposed to 2-mercaptoethanol to reduce any disulfides, and then this lane is resolved a second time on a new gel by placing it horizontally as the staking layer. Proteins that were originally in a reduced state will run at the same mass as before, forming a diagonal smear across the gel that can been visualized with total protein stains such as Coomassie Blue. However, proteins that were originally oxidized will now migrate to a lower-molecular-mass than before and so will run off the diagonal of the gel. These proteins can be identified by cutting out gel spots and analysing them by MS. Newly identified targets of interprotein disulfide formation can be subsequently studied more routinely using standard non-reducing SDS/PAGE gels and immunoblotting with antibodies to specific proteins.

Detection of thiol lipid modifications

In addition to oxidation, cysteine thiols can also be modified by numerous different lipids that include 4-HNE (hydroxynonenal), palmitate, PGs (prostaglandins) and nitro...
Figure 6 | SDS/PAGE gel-based assay for identifying proteins susceptible to intermolecular disulfide bond formation

The principle of this method is that oxidized proteins will run higher on non-reducing SDS/PAGE gels than their individual components. By cutting out the resolved lane and running it on a fresh gel under reducing conditions, disulfides will be lost and proteins will run at their normal mass. Proteins that were originally disulfide-bound will now run at a lower molecular mass than before and will therefore run off the diagonal of the gel.

Figure 7 | Modification of cysteine thiols by palmitoyl-CoA, HNE or 15d-PGJ₂
fatty acids. The addition of palmitate to cysteine thiols is catalysed by palmitoyl acyltransferase that uses palmitoyl-CoA as a substrate. The palmitate moiety is attached through a thioester bond that can be readily hydrolysed by protein acyl thioesterases [34]. This reversible modification has been shown to be important for shuttling proteins to different cellular compartments and for stable anchoring to lipid bilayers. Protein palmitoylation can be detected by immunoprobing for a specific target or performing MS. Purified using streptavidin–Sepharose, and specific targets proteins then being detected in homogenates or lysates [35]. Modified proteins can then be identified by affinity-capturing biotinylated proteins and then either immunoconjugating for a specific target or performing MS.

Many of the PGs that can specify cysteine residues including 15d-PGJ2 (15-deoxy-Δ12,14-PGJ2) and Δ12-PGJ2 as well as HNE and nitro fatty acids modify cysteine thiols through a Michael addition reaction. This is the reaction of a nucleophilic cysteine thiolate anion with an α,β-unsaturated carbonyl enone or with a conjugated nitroalkene. For detecting proteins modified by HNE, there are several commercial antibodies available. However, the sensitivity and selectivity of these antibodies are questionable, with the added complexity that antibodies may preferential recognize different modified residues, as HNE can also adduct to lysine and histidine as well cysteine residues. For investigating proteins that can be modified by PGs, a biotinylated form of 15d-PGJ2 has been developed [36]. This cell-permeant lipid can be added to cells or tissue, with the modified proteins then being detected in homogenates or lysates by Western blotting with avidin-based detection protocols [37] (Figure 7). Alternatively, modified proteins can be purified using streptavidin–Sepharose, and specific targets can be detected using immunoprecipitation or new targets can be identified using MS.

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**References**

26. Benitez, L.V. and Allison, W.S. (1974) The inactivation of the acyl thioester bond by HNE and nitro fatty acids. The addition of palmitate to cysteine thiols is catalysed by palmitoyl acyltransferase that uses palmitoyl-CoA as a substrate. The palmitate moiety is attached through a thioester bond that can be readily hydrolysed by protein acyl thioesterases [34]. This reversible modification has been shown to be important for shuttling proteins to different cellular compartments and for stable anchoring to lipid bilayers. Protein palmitoylation can be detected by using the biotin-switch assay described above (Figure 2) using hydroxylamine during the labelling step to selectively hydrolyse the bond between palmitate and modified cysteine residues [35]. Modified proteins can then be identified by affinity-capturing biotinylated proteins and then either immunoconjugating for a specific target or performing MS. Purified using streptavidin–Sepharose, and specific targets proteins then being detected in homogenates or lysates [35]. Modified proteins can then be identified by affinity-capturing biotinylated proteins and then either immunoconjugating for a specific target or performing MS. Purified using streptavidin–Sepharose, and specific targets proteins then being detected in homogenates or lysates [35].


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