Biochimica et Biophysica Acta 1840 (2014) 830–837

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Gel-based methods in redox proteomics ☆

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ARTICLE INFO

Article history:
Received 28 February 2013
Received in revised form 12 April 2013
Accepted 16 April 2013
Available online 23 April 2013

Keywords:
Redox proteomics

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-sulfenylation, S-nitrosylation, S-thionylation and interprotein disulfide 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 and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

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-sulfenylation, S-nitrosylation and disulfide-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 sulfinic and sulfonic acids are often considered in terms of damage. However, it has been shown that in at least one case, a sulfinylated form of 2-cysteine peroxiredoxin can be reduced by the ATP-dependent protein sulfiredoxin [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)

☆ This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

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http://dx.doi.org/10.1016/j.bbagen.2013.04.021

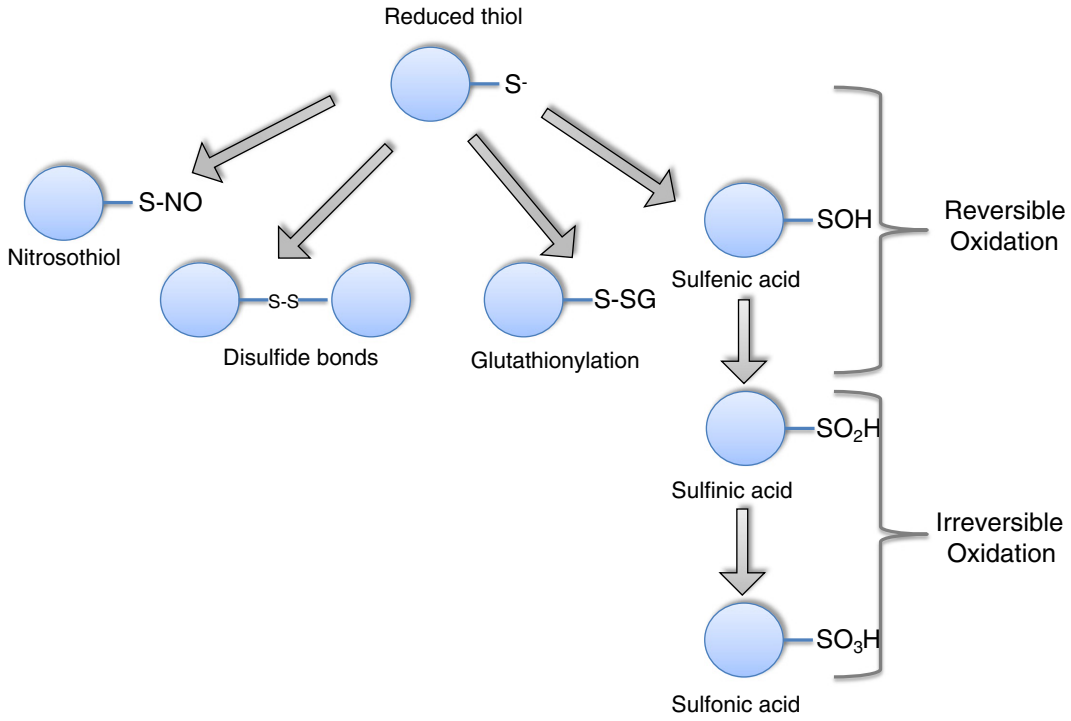

Fig. 1. Outline of potential thiol modifications. Protein thiols that are exposed to oxidation can form a variety of intermediates, nitrosothiols, sulfenic acid. These intermediates may be converted into more stable states such as inter- or intra-protein disulfides. These types of modifications are reversible,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

and thiosulfates. 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 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 disulfide 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 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.

There are a number of methods that utilise this "gain of signal" strategy, as outlined above, to identify specific forms of oxidation, for example methods that identify protein S-thionylation, S-sulfenylation, S-nitrosylation and intraprotein disulfide bonds.

3.1. S-thionylation

S-thionylation is where a disulfide is formed between a protein and a low molecular weight molecule, such as cysteine, homocysteine or glutathione. One way in which S-thionylation can be detected involves a tagged disulfide compound being incorporated into the protein via thiol–disulfide exchange reactions. Some examples of such compounds are cysteine or glutathione disulfide (GSSG) combined with an amino-conjugated fluorescent or biotin tag [9], as shown in Fig. 2. Reduced cysteine or glutathione (GSH) can also be combined with an amino-conjugated tag to generate a redox probe that can be used within cells or biological systems to monitor S-cysteinylation or S-glutathionylation respectively during oxidative stress. Initially protein S-glutathionylation was studied by monitoring the incorporation of radiolabelled ^{35}S-cysteine into GSH which can then disulfide bond to proteins during oxidant stress. However disadvantages to this approach, include a requirement to inhibit protein synthesis to prevent incorporation of labelled cysteine into proteins, a lack of an affinity tag which makes identification of the target proteins technically challenging, and safety issues due to emission of ionising radiation. Subsequently, Sullivan et al. developed biotinylated glutathione ethyl ester (BioGEE), which is cell permeant and allows direct detection of S-glutathionylated proteins [10]. Once in the cell, BioGEE is de-esterified to biotin–glutathione and can form disulfides with proteins during oxidative stress. Proteins that contain the biotinylated glutathione can then be identified following avidin-capture, protein separation and mass spectrometry (MS).

3.2. S-sulfenylation

When exposed to oxidants, such as peroxide molecules, protein cysteines can also form a sulfenic acid (–SOH) which can be detected by "tagging" strategies involving dimedone (5,5-dimethyl-1,3-cyclohexanedione). Dimedone reacts with a sulphenic acid to form a stable derivatisation product. Dimedone bound to cysteine sulfenic acids was initially monitored using colourimetry and then MS [11,12]. However, several derivatives of dimedone have subsequently been produced, whereby fluorescent or biotin tags have been attached to dimedone [13,14]. These tags enable the modified proteins to be affinity purified and isolated [15]. Recently, chemical probes have been developed which label sulfenic acids in live cells [16,17]. Antibodies have also been produced that pan-specifically detect dimedone bound to sulfenic acid and these have helped identify proteins that form sulfenic acids [2,18].

3.3. S-nitrosylation

The 'biotin switch' assay was developed to detect S-nitrosylated cysteines [19]. S-nitrosylation involves the covalent addition of nitric oxide (NO) to a cysteine to form a nitrosothiol (–SNO). Free NO cannot directly react with thiols, but there are several possibilities as to how NO can become a nitrosating agent. NO may form a nitrosonium cation (NO^+), which if formed in the locality of a thiol may nitrosylate the thiol. Another option is that NO can be oxidised to form nitrogen dioxide (NO_2), which can combine with another molecule of NO to form dinitrogen trioxide (N_2O_3), which can nitrosylate thiols. Similarly, a thiol may be oxidised to a thiyl radical which can then react with NO. Trans-nitrosylation reactions whereby nitrosothiols, such as nitrosocysteine (CysNO) or nitrosoglutathione (GSNO), directly react with protein thiols to efficiently induce S-nitrosylation can also occur. As with other thiol modifications, S-nitrosylation can directly regulate protein and cell function. For example, protein S-nitrosylation can regulate cell cycle and epigenetics [20,21], transcription [22], the activity of proteins [23], and cellular transport as well as the folding and trafficking of the proteins and their degradation [24]. S-nitrosylation also plays a significant role in cell signalling in a wide range of diseases [25].

Jaffrey and Snyder developed the biotin switch assay to detect protein S-nitrosylation. The assay comprises a series of steps, which help stabilise the relatively labile –SNO modification, before selectively reducing and labelling it. Initially free (non-S-nitrosylated) thiol groups are blocked using methyl methane thiosulfonate (MMTS) which alkylates all the free thiols. This conversion is done in the presence of SDS and heat which helps to denature the proteins and facilitates exposure and efficient alkylation of the thiols present in the interior of the protein. The excess, unincorporated alkylating agent is subsequently removed by precipitation using acetone and then washing the proteins, or by column desalting. The nitrosylated thiols are then converted to free thiols by adding ascorbate, a –SNO selective reducing agent. The final step involves attaching a biotin tag to the free thiols. Biotin-HPDP (N-[6-(biotinamido)-hexyl]-3V-(2V-pyridyldithio) propionamide) is used to tag the free thiols in the protein which can then be separated by SDS-PAGE and detected using a streptavidin-HRP or biotin-HRP conjugated antibody. The tagged proteins can also be purified by avidin-capture, eluted, separated by SDS-PAGE and then detected by immunoblotting. Proteins eluted in a non-reducing buffer can be detected by a biotin-HRP antibody and if eluted in a reducing buffer can be detected by an antibody towards the protein of interest. MS analysis can also be used to identify the tagged proteins (Fig. 3). The original biotin switch method has evolved over time. Firstly, NEM has largely replaced MMTS as the alkylating agent. Alkylating via MMTS blocks thiols by forming a disulfide, which can be easily reduced with DTT, whilst NEM blocks thiols by forming a thioether bond which is generally irreversible. Secondly, biotin-HPDP was originally used to tag the free thiol via a disulfide bond. Similar to MMTS, a disulfide bond is

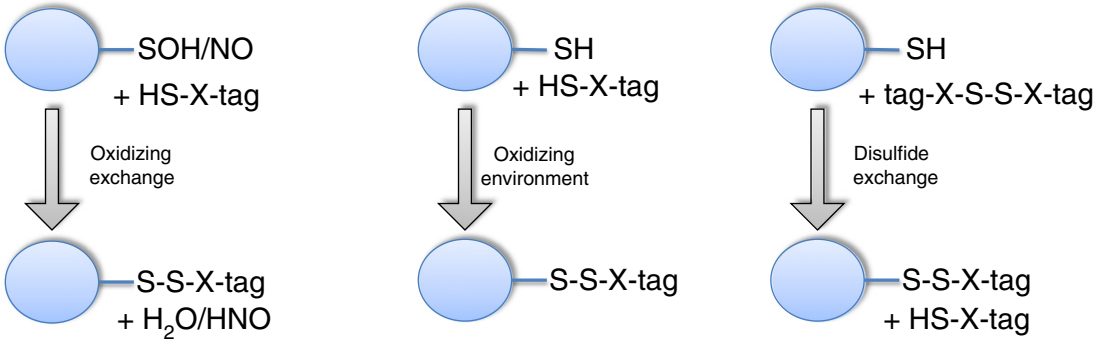

Fig. 2. Tagged thiolating agents can be used as probes for detecting protein S-thionylation. Disulfide based probes can undergo exchange reactions with oxidative modifications on protein thiols. When oxidants are present, these probes can also modify reduced thiols.

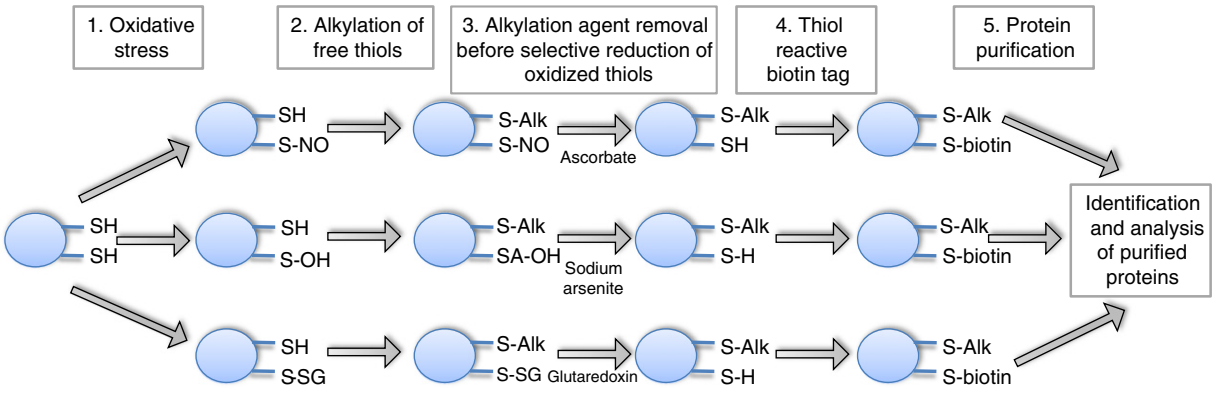

Fig. 3. The biotin-switch method to identify and purify S-nitrosylated, S-sulfenylated or S-glutathionylated proteins. This method initially blocks free thiols, prior to selective reduction and labelling of modified cysteine residues. By using different forms of selective reduction, this method can be used to identify S-nitrosylated, S-sulfenylated or S-glutathionylated proteins.

weaker than a thioether bond, therefore biotin-maleimide, and biotin-iodoacetamide are other possible tagging agents. Similarly, another variation, termed the His-tag switch method, replaces the biotin-HPDP with a His-tag peptide. This peptide is able to irreversibly bind to free thiols and allows purification and identification of the modified cysteines [26].

The biotin switch is not commonly used to detect S-nitrosylation of small biomolecules and has relatively poor sensitivity, detecting nanomole levels of S-nitrosylation per milligramme of protein [27]. Therefore, other variations of the method have been suggested to try and improve the sensitivity. The addition of reduced copper (Cu^{2+}) increases the sensitivity of the assay [28]. This is because the Cu^{2+} reduces −SNO to −SH with the formation of cupric ions which can then be regenerated by ascorbate. Thus the conventional biotin switch method may be relatively insensitive, with low concentrations of ascorbate perhaps providing inadequate reduction [29], whereas at higher concentrations it may be non-selective and reduce other thiol oxidation states and so generate false positives [30]. Sinapinic acid, which also selectively reduces S-nitrosylated proteins without reduction of disulfide bonds, is an alternative to ascorbate [31]. The biotin switch method has also been modified by using different reducing agents to detect other types of thiol modifications. For example, sodium arsenite or glutaredoxin can be used instead of ascorbate to reduce and selectively label S-sulfenylation and S-glutathionylation respectively. Dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) can be used to reduce disulfide bonds, but these will also reduce S-sulfenylated and S-nitrosylated proteins.

Several reagents have recently been developed to monitor S-nitrosylation. Wang et al. have shown how triarylphosphine reagents are capable of forming reductive ligation reactions with S-nitrosothiols [32]. Subsequently a coumarin-based phosphine molecule has been produced [33], which reacts with S-nitrosylated thiols to generate a fluorescent product that can be detected fluorometrically. This compound has been shown to specifically detect S-nitrosothiols with no reactivity towards disulfides [33,34]. Overall this compound is of great use to detect and quantify S-nitrosylation in vitro. However, this compound does not identify which protein thiols have been S-nitrosylated, it simply quantifies the amount of the S-nitrosylation.

A water soluble phosphine reagent has also been developed by Bechtold et al. [34]. This phosphine reagent reacts with S-nitrosothiols to generate stable S-alkylphosphonium adducts which can be detected by NMR and MS analysis. However, further work is required to develop a reagent that contains an affinity tag. Subsequently, another phosphine compound has recently been shown to react with S-nitrosylated thiols. A phosphine-thioester group is able to react with S-nitrosothiols to generate a stable disulfide which is linked to a biotin tag to aid detection [35]. These phosphine reagents react with S-nitrosothiols in a different manner to the ones highlighted above. They work via Staudinger-type ligation reactions, whereby the phosphine reacts with the S-nitrosothiol to generate a stable sulphenamide-based product. However, the selectivity of these thioester compounds for S-nitrosothiols is questionable, as they also react with disulfides and sulfenic acids [4]. Whilst these compounds offer a novel way of monitoring and detecting S-nitrosylation, further work is required to make them "gel-friendly", for example by the addition of affinity tags.

Another chemical strategy to detect S-nitrosylated thiols utilises organomercury compounds [36,37]. These compounds react directly with S-nitrosylated thiols, forming a stable thiol–mercury bond. By using a solid phase resin or via affinity capture, S-nitrosylated proteins can be captured and subsequently identified. The method is analogous to the biotin switch method in that it contains a series of steps: firstly free reduced thiols are blocked with MMTS; secondly S-nitrosylated proteins and peptides are captured by the phenylmercury compounds either by solid phase capture or by affinity capture, captured proteins can then be released either with β-mercaptoethanol or performic acid; and lastly the S-nitrosylated thiols are identified. Samples can be analysed by SDS-PAGE and other associated methods to detect, quantify or identify (by MS or Western immunoblot) modified proteins.

3.4. Intra-molecular protein disulfides

Intraprotein disulfides have mainly been identified using proteomic strategies. However, there are several compounds that are known to cross link or 'tag' vicinal thiols. Phenylarsine oxide (PAO) and dibromobimane (DBB) are examples of compounds that are capable of selectively reacting with vicinal thiols. In a method similar to the biotin switch assay, PAO can be used to selectively adduct to reduced vicinal thiols, after which the remaining non-vicinal reduced thiols are chemically alkylated. The PAO-tagged thiols are then reduced and subsequently labelled with a thiol-reactive reagent to aid identification either via immunoblotting or using proteomic methods. DBB generates a fluorescent signal when it is bound to a pair of reduced vicinal thiols and is lost if they are oxidised to the disulfide state [38], a feature that can be combined with gel proteomics utilising quantitative fluorescent imaging to identify protein spots that lose signal under conditions of oxidant stress.

4. Antibody-based detection of thiol modifications

The use of antibodies to detect cysteine oxidation offers a relatively straightforward way of monitoring changes in protein thiol oxidation states. Antibodies have been used to monitor the thiol redox status of proteins including those that detect protein S-glutathionylation, S-nitrosylation and S-homocysteinylation [39–41]. Such antibodies

have been developed so they pan-specifically detect such modifications, meaning they recognise a number of different proteins that have been modified by a specific oxidative modification. For example a commercially available antibody that detects S-glutathionylated proteins is able to detect multiple modified proteins in a biological sample [42]. An obvious advantage of such antibodies is that the modification can be routinely monitored using widely available, robust methods that are routine in many laboratories. However, it is important to note that such antibodies may not detect all modified proteins as detection depends on how the antibody was raised and what epitope the antibody recognises. Antibodies that recognise dimedone derivatised S-sulfenylated proteins have also been generated [2,18]. These pan-specific antibodies can be combined with immunoprecipitation techniques to determine the thiol redox status of specific candidate proteins.

Protein thiols may also be modified by reactive, oxidising lipids such as 4-hydroxynonenal (4-HNE), malondialdehyde, prostaglandins and nitrated fatty acids. Pan-specific antibodies have been generated against 4-HNE or malondialdehyde-modified proteins which are commercially available.

Antibodies to oxidised forms of specific proteins have also been produced. For example, sulfinated and sulfonated forms of peroxiredoxins can be detected by antibodies that recognise these specific oxidation states [43]. Similarly antibodies against oxidised states of glyceraldehyde phosphate dehydrogenase or protein tyrosine phosphatase 1B have also been developed [44–46].

Antibodies to specific proteins that are known or suspected to be sensitive to oxidation can be used with isoelectric focusing (IEF) gel electrophoresis to determine their oxidation state. Oxidation of a thiol modifies the protein charge, hence altering its migration on an IEF gel. Following IEF separation and immunoblotting with a specific antibody to the candidate protein, a band shift may be observed. Similarly band-shifts may be observed and subsequently detected with specific protein antibodies following the incorporation of thiol-reactive tags. As discussed earlier, maleimide, PEG-maleimide and IAM can be used to chemically derivatise protein thiols, for example by adding a defined molecular weight to the protein. By analysing such labelled samples by SDS-PAGE and immunoblotting, a specific band shift may be observed depending on the redox state of the protein.

Antibodies have also been used to monitor the thiol disulfide redox state of specific proteins. For example, following non-reducing SDS-PAGE with immunoblotting, protein kinase A, protein kinase G and peroxiredoxins have been shown to form an interprotein disulfide after oxidant stress [47,48]. In this case a band corresponding to the dimer weight is observed with a concomitant loss of the corresponding monomer band. However, this approach is only applicable to candidate proteins where a suitable antibody is available [49].

5. Diagonal gel electrophoresis for detection of intermolecular protein disulfides

Interprotein disulfide bond formation is one of many potential oxidative thiol modifications that occur during oxidative stress. This modification may result in a change in conformation and therefore the functional activity, or its association with other protein. Formation of disulfides could be intra-molecular or inter-molecular. As discussed above, proteins that form intra-molecular and inter-molecular disulfides can be detected using non-reducing gels [50]. Diagonal gel electrophoresis coupled with MS can be used to identify new proteins that form inter-protein disulfide complexes [51]. It has been useful in the identification of disulfide complexes formed by protein kinases A and G, and recently in identifying the disulfide bond formation by tropomyosin with oxidative stress during myocardial infarction [52].

As shown in Fig. 4, initially samples (prepared under non-reducing conditions in SDS sample buffer) are run on a non-reducing gel SDS-PAGE gel. The lane in which the sample is run is then excised and incubated in DTT or 2-mercaptoethanol to reduce any disulfides. After incubation the excised section of the gel is placed as the stacking gel on another gel which is then run under reducing conditions. Proteins that were originally interprotein disulfides are now reduced and so run at a lighter molecular weight than before. Consequently, these proteins run off the diagonal line (observed when the gel is stained for total protein) formed by the proteins which were not in disulfide complexes. Proteins below the diagonal line can be excised and identified using MS (Fig. 4).

6. DIGE analysis

Generally redox proteomics involves a separation step, usually 2-D electrophoresis (2-DE) followed by an identification step, typically involving MS. 2-DE is a gel-based method routinely used to resolve proteins and map difference in protein expression. 2-DE separates proteins based on two physical characteristics, isoelectric point and size, which allows resolution of numerous proteins. After separation proteins are visualised by staining, the image is then analysed and subsequently protein spots are excised and subjected to MS analysis. However, 2-DE is known to have a number of limitations and difference in gel electrophoresis (DIGE) was developed due to problems with inter-gel variation in 2-DE [53]. DIGE also allows more than one sample to be resolved on a single gel (multiplexing) [54,55]. In general, unmodified thiols from protein samples are blocked with an alkylating agent, such as NEM, modified thiols are then selectively reduced and subsequently reduced free thiols are fluorescently labelled with thiol alkylating reagents that add different coloured tags. These labelled samples are then combined into a single sample and run on the same gel. Using a fluorescence plate scanner, differences between protein samples are compared and quantified. If a protein is thiol-oxidised it will then have less available thiols and will therefore incorporate less of the fluorescent probe used to label the samples under oxidant stress. This decrease will be detected and quantified, and excised for MS identification. Several mitochondrial proteins which are sensitive to oxidation have been identified, using redox DIGE methods including acyl-CoA, VDAC-1 and creatine kinase [56,57]. Fu et al. have also identified a number of cardiac proteins that are sensitive to oxidation using redox DIGE, including NF-κB repressing factor and malate dehydrogenase [58]. Recently, several groups have combined the selective reduction of S-nitrosothiols with DIGE, in a method called SNO-DIGE (S-nitrosothiol DIGE) [59,60], identifying proteins that are susceptible to S-nitrosylation. Using SNO-DIGE a number of proteins have been identified as being targets of S-nitrosylation. These include several mitochondrial proteins such as aconitase, creatine kinase, malate dehydrogenase and heat shock protein 60 [59,61]. DIGE offers reproducible and confident protein identifications and is suitable for large scale screening. However, this method can also struggle to identify hydrophobic proteins which often do not resolve well during the first IEF step [56].

7. Non-gel based methods

There are non-gel based methods in redox proteomics which are capable of identifying proteins susceptible to oxidation. These techniques have been reviewed previously [62,63] and will be discussed in detail in another review in this special edition. Briefly, Mud-LC (multidimensional liquid chromatography) coupled to electrospray ionisation tandem MS whereby protein mixtures are digested in solution provides an alternative to gel-based methods for identifying proteins that are susceptible to oxidation. More recently several other techniques, which chemically or metabolically label proteins, have been used to monitor thiol changes in the redox proteome following oxidative stress. Stable isotope labelling by amino acids in cell culture (SILAC) was first described by Mann, whereby cells are grown in culture containing light or heavy stable isotopes of lysine/arginine [64]. The heavy isotope amino acids are then incorporated into the protein

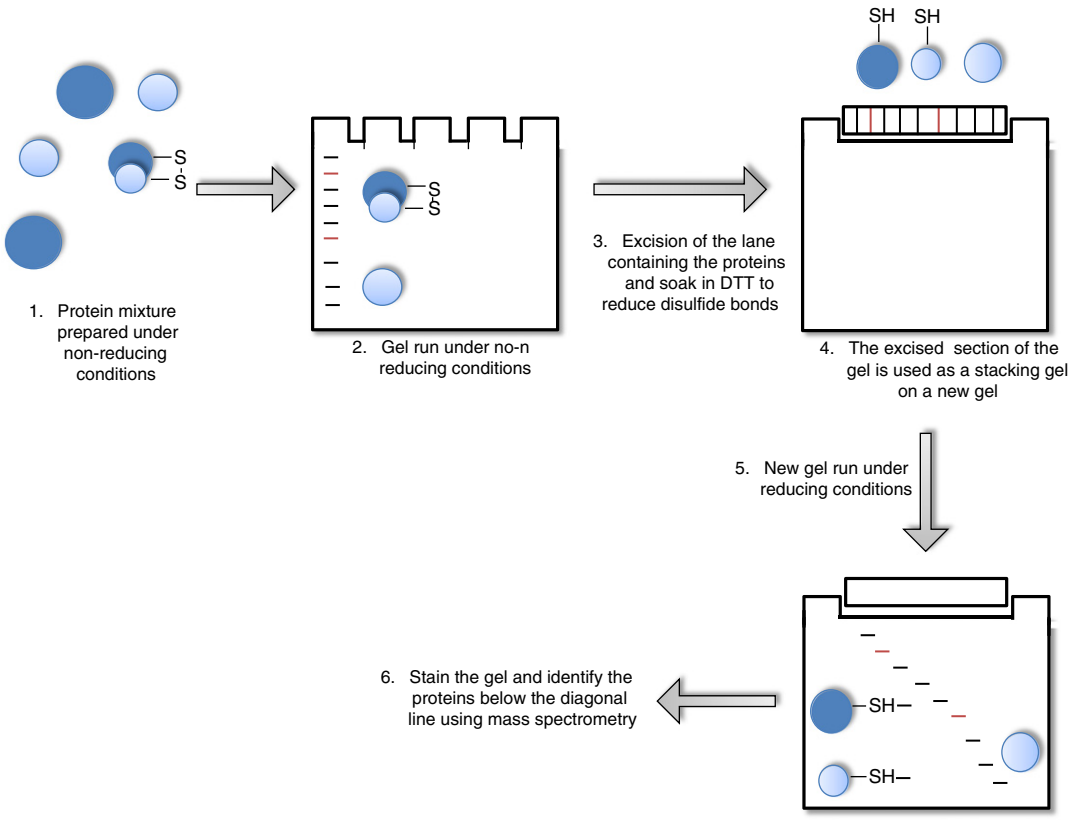

Fig. 4. Diagonal gel electrophoresis to identify proteins that form disulfide bonds. Proteins are initially run under non-reducing conditions. Those proteins that form a disulfide bond will consequently run at a higher molecular weight than their individual weights. However, after the lane is excised and re-run under reducing conditions, the proteins will run at their normal monomeric weight. As a result, any proteins that were initially in a disulfide dimer complex now run at a lower molecular weight compared to the initial gel and so run off the diagonal. These proteins can then be identified using mass spectrometry.

pool. Subsequently, the heavy labelled proteins are mixed with the light labelled proteins in a single sample and subjected to SDS-PAGE, tryptic digestion and then MS analysis. Proteins can also be chemically labelled in techniques such as ICATs (isotope-coded affinity tags [65,66]). Differential isotope thiol-specific tags are used to label proteins, which allow samples to be combined and digested. These tags contain a biotin residue which enables labelled peptides to be enriched by avidin affinity capture, with subsequent identification by MS.

8. Strengths and limitations of gel-based methods

The main strength of gel-based methodologies is that they involve technically simple, robust techniques. Many laboratories will have the necessary equipment for running standard SDS-PAGE gels, meaning specialised, expensive equipment does not generally have to be procured. Gel-based methods also generally provide a visual representation of the results, following either Western blotting or protein staining of a gel. In contrast with many MS-based methodologies, results are often heavily reliant on data processing which can distance the user from the raw data and make judgements on data quality more complex. Some gel methods, such as IEF or diagonal gels have greater complexity compared to standard SDS-PAGE methods. They are more technically challenging, requiring specialist knowledge on how to set up, run these gels and interpret the data. However, pre-cast IEF gels are widely commercially available now, making this technique more accessible and potentially reproducible.

Western blotting involving the use of antibodies to detect changes in protein thiol oxidation state offers a quick and easy way of detecting such changes. However, the quality of antibodies can vary enormously from batch to batch and so results may not be consistent between experiments. Similarly not all antibodies may recognise the different redox states of proteins. For example we find that some antibodies to PKG or PKA (which as discussed above, form a disulfide dimer running at a higher molecular weight upon oxidation) selectively detect one redox state over the other [47,48]. Another thing to consider for antibody-based methods is their availability. Whilst antibodies suitable for Western blotting are generally commercially available, more specialised products that detect specific redox-modifications are not.

2-DE is a relatively simple method with a number of strengths and advantages, 2-DE is relatively quick, provides a lot of information from a single experiment, and numerous proteins can be separated due to its high chromatographic resolution. It does however have some limitations which include; gel-to-gel variation, relatively poor detection sensitivity, difficulty in resolving hydrophobic proteins or those with a high pI, labour-intensity, and training of personnel personal to achieve reproducible results. As discussed above DIGE was developed to overcome the shortcomings of conventional 2-DE. DIGE allows multiple samples to be analysed simultaneously, overcomes the gel-to-gel variation observed with 2-DE, and enables problematic proteins, such as low abundance proteins, to be detected.

There are a number of non-gel based methods which have been used to try and identify thiol changes in the redox proteome following oxidative stress. Whilst such methods overcome some of the limitations of gel-based methods, they too have limitations. For example a "shot-gun" approach involving a long LC separation to try and improve the resolution may still miss a lot of information. However the resolution may be improved with the use of a 2D-gel prior to the LC–MS analysis. MS based methods also require the use of highly specialised, expensive equipment which may not be accessible for all researchers. However, both gel-based and non-gel-based methods have their merits

and can provide similar information on thiol changes in the redox proteome. Perhaps the best approach for these methods is using them in conjunction with one another, so complementary information is collected.

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