Chasing Cysteine Oxidative Modifications

Proteomic Tools for Characterizing Cysteine Redox Status

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Redox proteomics involves the large-scale analysis of oxidative protein posttranslational modifications. In particular, cysteine residues have become the subject of intensifying research because of their redox-reactive thiol side chain. Certain reactive cysteine residues can function as redox switches, which sense changes in the local redox environment by flipping between the reduced and oxidized state. Depending on the reactive oxygen or nitrogen species, cysteine residues can receive one of several oxidative modifications, each with the potential to confer a functional effect. Modification of these redox switches has been found to play an important role in oxidative signaling in the cardiovascular system and elsewhere. Because of the labile and dynamic nature of these modifications, several targeted approaches have been developed to enrich, identify, and characterize the status of these critical residues. Here, we review the various proteomic strategies and limitations for the large-scale analysis of the different oxidative cysteine modifications.

Reactive oxygen and nitrogen species (RO/NS) have been found to play a dual role in the cardiovascular system, acting both as second messengers in physiological redox signaling and as agents of oxidative damage, leading to pathological conditions.[1,2] This dual role is determined largely by the balance of oxidant production and the capacity of the cell's antioxidant defense. RO/NS in cardiomyocytes can be produced by several sources, including, in the mitochondria, nicotinamide adenine dinucleotide phosphate oxidases and NO synthases.[3–5] The levels of these species are held in check by antioxidant scavengers such as catalase, glutathione (GSH) peroxidase, superoxide dismutase, peroxiredoxin, and free GSH, which patrol the cell neutralizing them.[6] Changes in redox balance occur when the levels of RO/NS production exceed the local antioxidant capacity. Small changes in the concentration of some species (eg, superoxide, hydrogen peroxide, or NO) are more likely to participate in redox-signaling events with oxidative damage increasing as their levels rise, whereas other species (eg, hydroxyl radical) inflict damage even at low concentrations.[2,7]

A common mechanism for redox signaling is the oxidative posttranslational modification of cysteine (Cys) residues.[8,9] The reactive thiol side chain of Cys can function as a sensor or switch, flipping between the reduced and oxidized state in response to fluctuations in the various RO/NS.[10,11] Depending on their local concentration, RO/NS can react with Cys to form one of several reversible (S-nitrosylation [SNO], S-glutathionylation, sulfenic acid, inter- and intramolecular disulfide bonds, or S-sulfhydration) or irreversible (sulfininc or sulfonic acid) modifications (Figure 1).[1,12,13] The unique chemistry responsible for the formation of each Cys modification has been previously reviewed.[8,14] Such modifications have been found to affect cellular signaling, impacting both adaptive and maladaptive cardiac responses. For example, SNO of G-protein–coupled receptor kinase 2 has been found to decrease phosphorylation of β-adrenergic receptors, slowing receptor internalization and desensitization.[15] Alternatively, oxidation of histone deactylase 4 and heat shock protein, DnaJb5, can produce an intermolecular disulfide bond–linked dimer that is translocated out of the nucleus. This translocation prevents the inhibition of histone deactylase 4 of myocyte enhancer factor-2–dependent gene transcription, resulting in the initiation of hypertrophy.[16]

Identification and characterization of oxidative signaling pathways are crucial for understanding the diverse effects of RO/NS in the cardiovascular system. Because of the variety and, in some cases, unstable nature of the different Cys modifications, several techniques have been developed for their investigation. These techniques can be divided into 2 categories: general detection evaluates the overall modification status in a cell or tissue, whereas specific detection identifies all the individual amino acid residues modified. Identification of individual modified Cys residues and the particular redox modification have been achieved using proteomics techniques, often by coupling a specific enrichment strategy with high-throughput mass spectrometry (MS) analysis.[17] Here, we review the various proteomic approaches available to examine the redox modification state of Cys with particular emphasis on those techniques that facilitate the large-scale identification of individual modified residues. Several of these techniques show great promise for dissecting the signaling events and effects of redox regulation.

Reversible Thiol Modifications

S-Nitrosylation

SNO, also known as S-nitrosation, results from the covalent addition of a nitrosonium ion equivalent to a Cys thiol (Figure 1). This modification has been found in a variety of dynamic redox-signaling/regulation events in the heart and

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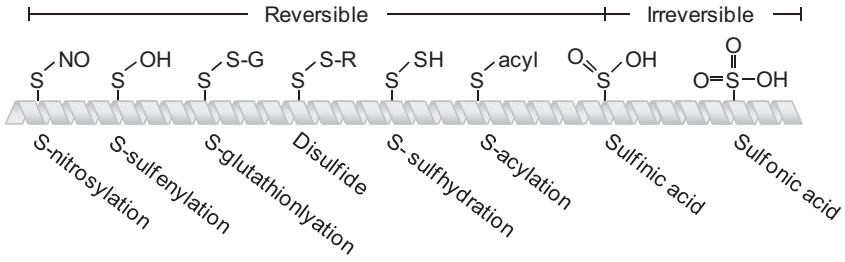

Figure 1. Oxidative cysteine modifications. Listed are several common physiologically reversible and irreversible oxidative posttranslational modifications that can occur to the thiol side chain of a cysteine (Cys). Sulfinic acid has been placed at the border between reversible and irreversible because of some examples where it has been found to be enzymatically reversed.[12,13]

has been reviewed extensively.[18–20] Among its various effects, SNO modification has been implicated in cardioprotection by several studies.[21–24] Treatments of hearts with NO donors to increase SNO, particularly in the mitochondria, before an ischemic insult have been found to reduce infarct size.[24,25] A leading hypothesis for this protective effect is that reversible SNO modifications induced before an ischemic event protect critical Cys from subsequent irreversible oxidation.[18]

Biotin Switch Assay. Because SNO modifications are very labile, they are challenging to study using traditional biochemical techniques.[26] In 2001, Jaffrey et al [27,28] introduced the biotin switch assay, making detection and enrichment of these modified sites feasible in complex mixtures. The assay uses a replacement strategy where free thiols are blocked (usually with n-ethylmaleimide, S-methyl methanethiosulfonate, or iodoacetamide), SNO modifications are specifically reduced with ascorbate, and the newly exposed thiols are simultaneously labeled with a thiol-reactive biotin. Once the modified sites are labeled with the stable biotin group, they can be easily detected or enriched by streptavidin affinity chromatography. This approach has been expanded to include individual Cys identification by introducing a tryptic (or other protease) digestion of biotin switch–labeled proteins before capture.[29,30] This results in the specific enrichment of only biotinylated peptides that can be analyzed by MS to determine the proteins modified and the individual modified Cys.

Since the original biotin switch description,[28] numerous variations have emerged which improved the assay as well as expanded it to other reversible Cys modifications (discussed below) (Figure 2). For SNO detection, the majority of biotin switch options primarily revolve around the choice of thiol-labeling reagent (Table). Selecting the best label for an experiment depends on 2 factors: the need for a permanent mass tag in the MS analysis and the ability for relative quantification. The original biotin switch protocol used N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP) which forms a mixed disulfide with the previously SNO-modified Cys residue through reaction with its pyridyldithiol group. Other groups have simplified labeling by using a thiol-reactive resin (known as SNO-resin-assisted capture [SNO-RAC]) which directly binds free Cys to the matrix, eliminating the need for a streptavidin capture step required for the enrichment of biotinylated proteins or peptides.[31] Although highly effective, these approaches have a limitation; elution requires reduction of the mixed disulfide bond that forms between biotin-HPDP or the thiol-reactive resin and the peptide. This results in an unmodified peptide proceeding to MS analysis. Without a signature mass label at the modified Cys, it can be difficult to assess nonspecific binding in the capture or make site determinations for peptides with >1 Cys. As an alternative, biotin-HPDP or other permanent biotin-based reagents, such as biotin-maleimide or biotin-PEO-maleimide, have been used in combination with reversibly binding monomeric avidin where biotin-labeled peptides can be captured, eluted under acidic conditions, and analyzed by MS while maintaining the biotin label.[15,32] These labels produce a detectable mass shift in the fragmentation spectrum, indicating the modified Cys.[33] The irreversible nature of the maleimide-based labels also allows samples to be reduced and alkylated before digestion, which will open any endogenous disulfide bonds to improve digestion efficiency and eliminate any potential disulfide-linked peptides that would be lost in the analysis.[33]

In addition to detection and site mapping, the biotin switch technique has been combined with traditional proteomic quantitative methods such as iTRAQ,[34] stable isotope labeling by amino acids in cell culture,[35,36] or label-free quantification to measure the extent of SNO labeling at each modified Cys. iTRAQ and stable isotope labeling by amino acids in cell culture reagents have isotopic versions that allow different samples to be combined before MS analysis so that the ratio at each modified site can be compared. The iTRAQ reagent reacts with a peptide's N-termini and can be applied after elution from a standard biotin switch procedure[31] so that samples from up to 8 different conditions can be combined, although it has only been demonstrated successfully using the SNO-RAC technique. SNO can also be quantified by combining the biotin switch assay with stable isotope labeling by amino acids in cell culture.[37] In this case, cells are metabolically labeled with heavy or light versions of lysine and arginine, and SNO can be measured by comparing the parent mass intensity of the heavy and light peptides. Although iTRAQ and stable isotope labeling by amino acids in cell culture labeling allow for relative quantification of SNO between samples, neither option directly labels the modified Cys, and therefore they can only be used in conjunction with another biotin switch method. Label-free quantification does not require isotope-labeled reagents and is achieved by measuring the signal intensity for each precursor ion determined to be an SNO-modified peptide. This approach has been used effectively to identify >950 SNO-modified sites in cardiac tissue.[38,39]

An alternative for measurement of SNO modification is a labeling reagent that combines direct Cys labeling, enrichment, and quantification. Isotope-coded affinity tag (ICAT)[40] and SNO-capture (SNOCAP)[32] are isotope-coded, thiol-reactive reagents that are coupled to a biotin group for enrichment.[32,41,42] They come in heavy and light isotopic versions to compare

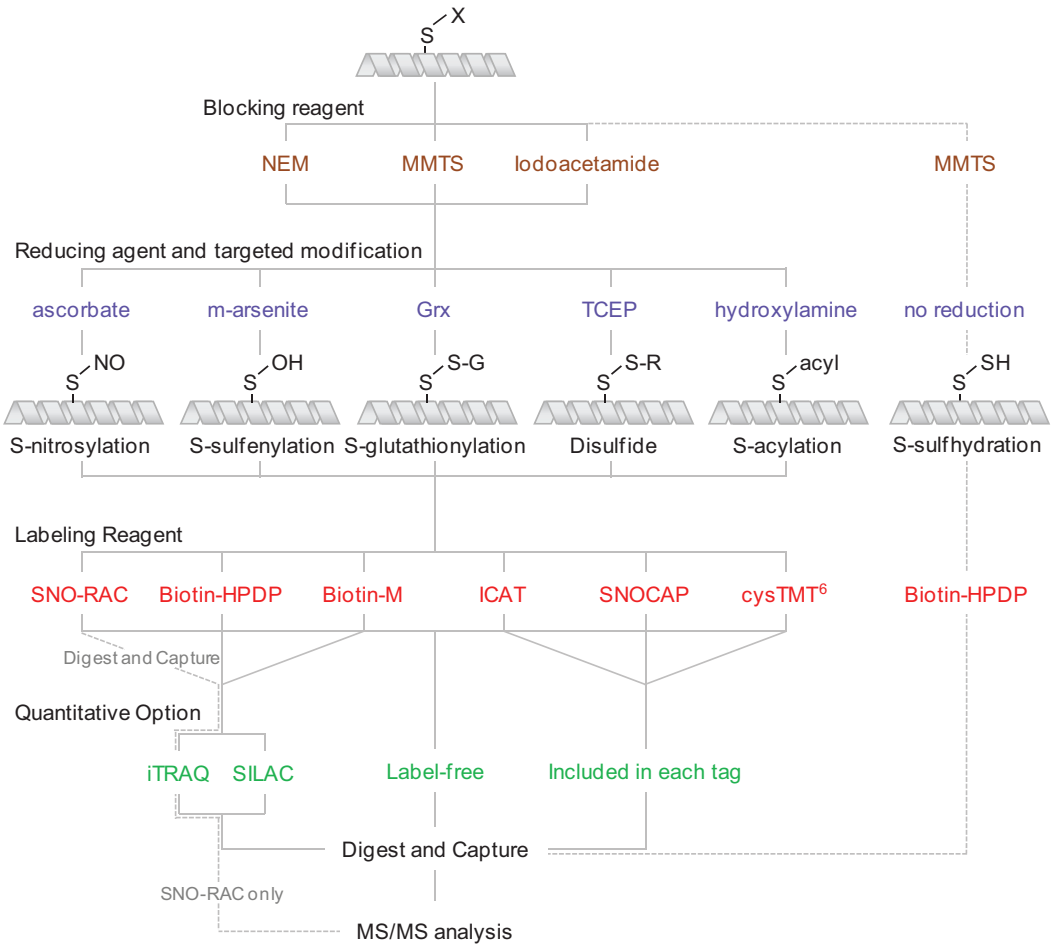

Figure 2. Options for identifying reversible thiol modifications in the biotin switch assay. The various options and developments available for the biotin switch assay have made it a very flexible tool for detection, identification, and site mapping of proteins with reversible thiol modifications. Steps in the protocol can be customized depending on the target modification and goals of the investigation. NEM, n-ethylmaleimide; MMTS, S-methyl methanethiosulfonate; SNO-RAC, S-nitrosylation resin assisted capture; Biotin-HPDP, N-[6-(Biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide; ICAT, isotope coded affinity tag; SNOCAP, S-nitrosylation capture; cysTMT, cysteine-reactive tandem mass tags; iTRAQ, isobaric tags for relative and absolute quantitation ; and SILAC,stable isotope labeling by amino acids in cell culture.

the MS parent ion intensities between 2 samples. Although the reagents have the same experimental use, they have different thiol-reactive groups; ICAT uses an iodoacetamide-based reaction whereas SNO-capture has a pyridyldithiol group, which can change the chemistry and efficiency of the reaction. Additionally, ICAT has an acid cleavable linker between

Table. Characteristics of the Biotin Switch Labeling Reagents

Labeling Reagent	Thiol-Reactive Group	Mass Difference to Modified Cys, Da	Commercially Available	Quantitative Potential
Biotin-HPDP	Pyridyldithiol	428.19	Yes	Label-free
Biotin-M	Maleimide	452.54	Yes	Label-free
Modified sepharose-NHS	Pyridyldithiol	NA	Yes	Label-free
ICAT	Iodoacetamide	227.13/236.16	Yes	2-plex
SNOCAP	Pyridyldithiol	345.09/349.09	No	2-plex
cysTMT6	Pyridyldithiol	304.17	Yes	6-plex
Nonthiol labeling reagents				
iTRAQ	NA	NA	Yes	8-plex
SILAC	NA	NA	Yes	2-plex

ICAT indicates isotope coded affinity tag; SNOCAP, S-nitrosylation capture; cysTMT, cysteine-reactive tandem mass tags; Biotin-HPDP, N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; NHS,N-hydroxysuccinimide; iTRAQ,isobaric tags for relative and absolute quantitation; SILAC, stable isotope labeling by amino acids in cell culture; and NA, not applicable.

the biotin group and the isotopic region, which reduces the overall size of the mass tag, improving detection of larger peptides.[43–45] The newest quantitative option is the Cys-reactive tandem mass tags (cysTMT),[6] which is a thiol-reactive version of the popular amine-reactive TMT reagent.[46,47] This label combines a permanent mass tag at each individual Cys with greater quantitative potential than the other options. When used in a biotin switch style assay, instead of a biotin-avidin–based enrichment, labeled peptides are reversibly captured by an immunoaffinity resin leaving the mass tag in place. The cysTMT[6] has 6 isotopically balanced versions that release reporter ions (126–131 Da) during fragmentation to compare the extent of modification at each site across samples.[47]

It should be noted that a common critique of the biotin switch assays has been the use of ascorbate as the specific SNO-reducing agent. In a few instances, ascorbate has been implicated in the unintended reduction of disulfide bonds or other oxidative modifications.[48–50] However, a strong case has been made that the reduction of protein or mixed disulfides by ascorbate is not thermodynamically favorable, and that most likely the reported artifacts are the result of accidental light exposure or other contaminants.[51,52] We recently demonstrated, using the cysTMT[6] reagent, that it was possible to discriminate the labeling of disulfides from SNO sites and found the number of inadvertently labeled disulfides to be a small fraction (≈3%) of the total sites identified.[47]

Alternative to the Biotin Switch. In addition to the biotin switch assay, some groups have pursued alternate chemistries to specifically target SNO modifications. A recent report outlines a technique using organo-mercury compounds coupled to biotin which targets SNO modifications. Doulias et al[53] report detecting 328 endogenous SNO-modified sites from mouse liver, which is the largest number of endogenous modified sites detected to date. One of the limiting factors of the ascorbate-based biotin switch has been sensitivity, particularly for endogenous detection.[54] This latest innovation may be valuable in increasing the sensitivity of SNO detection and thus expand our understanding of the regulatory potential of these modifications; however, the organo-mercury compounds have not been subject to the same scrutiny as ascorbate regarding specificity of SNO-modification detection and should be approached with caution.[51,52,54]

S-Sulfenylation

Similar to SNO, sulfenylation, also known as sulfenic acid modifications, are reversible, labile moieties that have been challenging to study directly with traditional techniques. Sulfenic acid is the first oxoform generated by hydrogen peroxide oxidation of a thiolate forming an R-S-OH group (Figure 1), the further oxoforms being sulfinic and sulfonic acid.[55] Proteomic analysis of sulfenylation has been approached using a few different methodologies. Initially, the oxidative (Ox)ICAT technique was used by the Cohen group to compare the state of oxidation between peroxide-stimulated and peroxise-unstimulated samples.[56,57] OxICAT used the ICAT reagent to observe the extent of oxidative modification on individual Cys by measuring the decrease in labeling between the heavy and light versions of the tag at the modified sites. This early attempt lacked a mechanism to specifically target sulfenic acid–modified sites, and instead gave an indication of the extent of modification in a sample that had significant levels of sulfenylation.[58,59]

Since this initial endeavor, several sulfenylation-targeted approaches have emerged. The Eaton group [60] have developed a biotin switch style assay which used m-arsenite in place of ascorbate to specifically reduce sulfenic acids (Figure 2). In their study, they were able to demonstrate a progressive loss of sulfenated signal with increased peroxide treatment, indicating m-arsenite is specific to sulfenic acid and not the further oxoforms.[61] Using this technique, they successfully identified sulfenated proteins in isolated hearts treated with hydrogen peroxide.[61] Although this approach has not been widely adopted, it could be easily combined with any of the previously discussed biotin switch tools (ICAT, cysTMT,[6] etc) to allow multiplex quantification at each modified site.

More commonly, sulfenic acid modifications have been targeted using dimedone chemistry. Dimedone-based probes, conjugated with biotin or azide, have been reported to stably and specifically label sulfenylated thiols.[62,63] This reaction has been shown to directly target SOH groups and does not require the blocking step or specific reducing agent necessary for biotin switch style approaches. Once labeled, the biotin group or, in the case of azide conjugated probes, a click chemistry addition of a biotin moiety can be used for enrichment and detection. The Carroll group [64–66] has also developed heavy and light isotopic versions of the probes to allow comparison of modification states in 2 conditions by MS. Using these tags, they performed a proof-of-principle experiment with yeast GSH peroxidase and demonstrated relative quantification of protein sulfenic acid modifications.[65] They have since shown that the dimedone-based probes can also be effective for labeling sulfenic acid modifications in living cells, demonstrating both their quantitative potential and spatial and temporal resolution for detecting and characterizing the modifications in vivo.[66]

S-Glutathionylation

In addition to RO/NS, there are other potential thiol-reactive agents like GSH, which can result in oxidative posttranslational modifications. GSH is a Cys-containing tripeptide (glutamate-Cys-glycine), which can be an electron donor for oxidative species and form stable but reversible mixed disulfide bonds with available Cys (Figure 1).[67] The selective addition of a GSH group to a protein has been found to regulate protein function. For example, S-glutathionylation modifications can regulate intercellular calcium concentration by increasing sarcoendoplasmic reticulum calcium transport ATPase activity or reversibly uncouple NO synthase, shifting production of NO to superoxide activity effecting vasodilation.[68,69] Cellular levels of S-glutathionylation increase during oxidative stress, and it has been proposed that S-glutathionylation functions as a temporary thiol cap, protecting critical Cys from potentially damaging irreversible oxidative modifications.[70]

S-glutathionlyation modifications have been enriched and identified using a protocol similar to the biotin switch assay outlined earlier. For this application, GSH modifications are specifically reduced by glutaredoxin (Figure 2).[71–75] After enzymatic reduction, newly exposed thiols can be labeled and detected using any of the various thiol-reactive tags discussed

earlier in MS analyses. The assay has been performed in vitro and in situ, where glutaredoxin was overexpressed in cells, so reduction and labeling could occur before lysis.[72,73] After reduction, newly exposed thiols can be labeled and detected in a similar manner to SNO modification.

Compared with other oxidative posttranslational modifications, S-glutathionlyation is a relatively stable modification with a unique mass, making direct analysis is also possible. S-glutathionylation can be specifically targeted through the use of a biotin-labeled GSH ester.[76–78] This cell-permeable reagent can be loaded into cells, and, when harvested, the proteins and individual Cys that received the biotinylated GSH is easily captured with avidin and identified by MS. This technique has been used to identify 15 S-glutathionylated proteins in rat cardiomyocytes.[76] One downside to this technique is that loading a cell with the biotinylated GSH ester can artificially shift the ratio of GSH and GSSG in the cell, which can promote S-glutathionylation. If this concern can be controlled for, direct labeling of S-glutathionylated proteins provides a faster and more direct approach to identifying modified residues than the biotin switch style approaches.

Another potential option is the use of antibodies to detect and enrich S-glutathionylated proteins. As mentioned earlier, this modification is sufficiently stable, and antibodies raised against a GSH epitope can be used for detection in immunobased assays (eg, ELISA, immunohistochemistry, Western blots). Although there has been success in using antibodies to target and assay the modification status of individual protein,[79–81] there has yet to be any examples of large-scale proteomic analysis using an immuno-based enrichment for S-glutathionylation resulting in site identifications. Additionally, comparison of this approach with GSH ester treatment found immuno-enrichment to be far less specific and less sensitive.[76]

Disulfide Bonds

Cys can also be modified through reaction with another available Cys forming a protein disulfide bond. These modifications have historically been seen as static structural features that occur during protein folding in the oxidizing environment of the endoplasmic reticulum, which persist throughout the life span of the protein. More recently, a small but growing number of disulfides have been found to be dynamic in response to changes in the cell's redox environment.[82–84] The introduction of new disulfide interactions has the potential to significantly alter protein conformation or association, affecting function. Several cardiac proteins have been found to form disulfide-linked complexes under conditions similar to those of the oxidative stress experienced in ischemia reperfusion injury.[85] Analysis of these modifications presents a particular problem; the relatively large number of static disulfides can make isolation/detection of the smaller pool of dynamic bonds difficult. Several strategies have been used to detect and identify regulatory disulfides.

One of the longest-standing and most successful techniques for examining intermolecular disulfide bonds has been 2-dimensional diagonal gel electrophoresis.[86,87] In this approach, samples are separated in the first dimension under nonreducing conditions, either in a glass tube or gel lane. For the second dimension, proteins in the gel matrix are treated with a reducing agent to cleave any disulfides. Samples are then electrophoresed in a perpendicular direction and stained.[85,88] Proteins that had been involved in a disulfide-linked dimer or trimer and the like complex will run at their monomeric molecular weight in the second dimension, and thus fall below the diagonal line formed by the migration of most proteins. These protein spots can be excised and identified by MS.[89] This technique has been used successfully, resulting in the identification and characterization of several redox-sensitive disulfide-bonded protein complexes after oxidative insult.[85,90,91] One notable recent example was the characterization of a peroxide-induced disulfide that resulted in dimerization of protein kinase G, producing a cyclic guanosine monophosphate-independent mechanism of activation to increase vasorelaxation.[92]

Although diagonal gel electrophoresis can be an effective technique for identifying disulfide interactions, determining the particular Cys residues involved can be difficult. Current approaches favor site-directed mutagenesis at predicted target sites to determine whether the interaction is lost under equivalent stimuli. For an unbiased assessment of the general disulfide proteome, bulk capture techniques have been used although they have not been widely pursued by the proteomics community.[82] In this case, disulfide-engaged Cys can be captured and identified using an activated thiol sepharose.[93,94] This approach involves blocking any available Cys in a sample and then treating with a reducing agent, such as tris(2-carboxyethyl)phosphine or dithiothreitol, to expose the disulfide-bonded Cys. The sepharose resin containing pyridydithiol groups reacts with the newly free thiols in the lysates, capturing them. The bound proteins can be digested, washed, and eluted for MS analysis to identify the peptides which contain the disulfide-bonded residues. A more popular approach has been the use of the ICAT reagent to quantitatively assess redox-sensitive disulfide bonds in response to oxidative stress.[58,95,96] This approach could be combined with any of the biotin switch tags discussed earlier (Figure 2).

An alternative to the various indirect labeling strategies for mapping disulfide bonds described earlier is direct identification of disulfide-linked peptides by MS. This is challenging, but it offers the most conclusive evidence of a disulfide interaction. In the majority of MS studies, Cys are reduced and alkylated before analysis, removing any possibility of disulfide observation. Disulfide-linked or looped peptides can be difficult to ionize and have been found to be more resistant to fragmentation in collision-induced dissociation.[97,98] When fragmentation does occur, it can induce splitting of the disulfide bond, making specific determination of the interaction difficult.[99] Recently, 2 approaches have been presented, which attempt to improve this situation. Electrospray ionization coupled with collision-induced dissociation can produce an asymmetric cleavage of the disulfide bond resulting in Cys with a disulfohydral and dehydroalanine residue across the cleavage site, representing signature masses that can be used for unambiguous identification of the residues involved.[100] In an alternate approach, disulfide bonds are cleaved electrolytically immediately before analysis.[101] The disulfide bonded peptides can be recognized by the loss of ionization energy compared with a control sample.

Unfortunately, neither of these approaches is currently suitable for complex samples or high-throughput analysis; however, software is being developed that will aid in this endeavor.[99]

It is important to note that in dealing with disulfide bonds or other thiol modifications, many MS protocols perform trypsin digestion in ammonium bicarbonate at pH 8.5.[102] This may be above the pKa of many Cys, causing their conversion to more reactive thiolates which can lead to disulfide rearrangement during sample processing.[103,104] When dealing with Cys-sensitive samples, it is recommended to use a lower pH buffer, such as ammonium acetate (pH 6.5), for better disulfide bond fidelity.[103]

S-Sulfhydration

An emerging redox modification is S-sulfhydration, which involves the reaction of the diffusible gas hydrogen sulfide (H_2S) with a free thiol to form a hydropersulfide moiety (R-SSH). This modification is also reversible and is rapidly being appreciated as an important regulatory mechanism in a variety of physiological processes, such as vasodilation, inflammation, and ischemia/reperfusion injury among others.[105–108] As the hydropersulfide group is similar in character to a disulfide bond, it can be difficult to discriminate between these 2 modifications for detection and enrichment. The Synder group has developed a modification of the biotin switch assay that is able to target these modifications.[109,110] Their procedure involves blocking the free thiols with MMTS, which they report specifically modifies free thiols (-SH) but not the hydropersulfides.[109] After blocking, biotin-HPDP is applied without any additional reducing agent to modify the remaining free hydropersulfides. The authors did not establish the nature of this interaction but suggest that it may result in the formation of a disulfide (R-SS-Biotin) or trisulfide (R-SSS-Biotin) moiety, linking the protein with the biotin group.[109] Once labeled, the proteins can be digested and the S-sulfhydrated peptide captured and analyzed following the standard biotin switch procedure. As biotin-HPDP, cysTMT, and the thiol-reactive resin used in SNO-resin-assisted capture technique have the same pyridyldithiol reactive group, it is reasonable to assume that these other reagents could also be used to label S-sulfhydration, although this configuration has not been reported.

In addition to a biotin switch strategy, S-sulfhydrated modifications can also be detected directly by MS/MS analysis. A 32-Da mass shift can be observed in the fragmentation spectrum of a hydropersulfide-modified peptide from a purified protein.[111] S-sulfhydration modifications have also been assessed using a maleimide labeling technique, also developed by the Snyder group.[112] Although not specifically a proteomic enrichment strategy to identify modified sites, this approach offers an elegant general assessment of protein sulfhydration using fluorescent probes and differential reduction. In this case, proteins were treated with maleimide-red, which irreversibly labels free thiol (R-S-maleimide-red) and hydropersulfides (R-SS-maleimide-red). In this labeling, hydropersulfides are converted to mixed disulfides which can be reduced by DTT, removing the red label. The extent of sulfhydration can be determined globally by comparing the loss in maleimide-red signal by electrophoresis with a nonreduced sample. In their initial report, the authors went one step further, also reducing any SNO modifications with ascorbate and labeling with maleimide-green before treating with DTT to examine the relationship between S-sulfhydration and SNO in hydrogen sulfide–stimulated nuclear factor-κB activation.[112]

S-Acylation

Although not a redox modification, S-acylation is another reversible and regulatory thiol modification that has been the subject of targeted proteomic investigation. This modification involves the covalent addition of a fatty acid acyl group to a Cys through a thioester bond.[113] Most commonly, a palmitic acid is added which has led to this modification being generally referred to as palmitoylation; however, this is not always the case.[114] S-acylation has been implicated in regulating protein localization and signaling, often temporarily anchoring protein to cell membranes. There are many examples of S-acylated proteins including various small G proteins, viral membrane glycoproteins, lipid raft–associated proteins, and histone variants.[115–118] Recently, a biotin-based labeling technique has been developed, which uses hydroxylamine to specifically cleave the thioester bond revealing a reactive thiol group at the modification site (Figure 2).[119,120] Using a biotin switch style approach, including blocking of unmodified Cys, Drisdel et al have found this to be a sensitive and specific approach for detecting S-acylation.[121,122] Forrester and colleagues adapted their resin-assisted capture approach and detected 93 sites of S-acylation from 10 to 20 mg of HEK293 cells, including several previously established sites.[122] No large-scale proteomic study has been done on the heart; however, both the expression of Kv1.5 and the cellular localization of Src kinases have been found to be regulated by changes in their S-acylation status, indicating this modification may have a role in heart function and failure.[123–125]

Irreversible Thiol Modifications

Sulfinic and Sulfonic Acid

Unlike the previously discussed thiol modifications, sulfinic and sulfonic acid are largely biologically irreversible and have generally been regarded as oxidative damage instead of signaling modifications.[9] In some cases, regulatory sulfinic acids have been found, which can be enzymatically reverted to the thiol form.[126–128] These modifications arise from the additional oxidation of a sulfenic (RS-OH) to form a sulfinic ($RS-O_2H$) and subsequently a sulfonic group ($RS-O_3H$) (Figure 1).[129] Because of their relative stability compared with the reversible thiol modifications, they do not require a replacement strategy and can be detected directly by MS; however, this presents its own complications.

Sulfonic acid–modified residues can be captured using a polyarginine resin. This technique was demonstrated by Chang et al [130] in 2010 by preparing a nano diamond resin conjugated with polyarginine, polyethylene glycol, and polylysine. They report that coordination between the positive amine groups of the arginine and negatively charged sulfo-groups resulted in a sensitive and specific capture of sulfonated peptides, but not significant amounts of the similarly charged phosphopeptides. As proof of principle, they successfully purified and detected sulfonic acid–containing peptides from a 5000-fold excess

ovalbumin digest[130]; however, the selectivity has not been tested in complex cell lysate. There is currently no enrichment strategy in use for sulfinic acid modifications. Recently, the Carroll group has been pursuing sulfinic acid ligation reactions using aryl-nitrosos compounds. Although only an initial assessment of the chemistry has been presented so far, they report that the approach reacts rapidly in aqueous conditions and is highly selective for sulfinic acid modifications, including being orthogonal to free Cys thiols.[131] The 2-nitrososbenzoic ester derivative they examined is more stable and specific at neutral pH than aryl diazonium salts.[131] With further development, this chemistry has great promise for the detection of sulfinic acid modifications in complex biological systems

Once these irreversible modifications have been enriched, detecting them by either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization MS can be difficult. The high negative charge associated with the sulfo-group can inhibit ionization by MALDI.[132] To improve detection, groups have included diammonium hydrogencitrate in the matrix which they found can improve ionization.[132] Additionally, sulfinic and sulfonic acid fragmentation can be difficult to distinguish from the fragmentation patterns of other posttranslational modifications, necessitating high-resolution MS instruments to differentiate them. Both sulfinic acid and hydropersulfide modifications result in a 32-Da mass shift, whereas sulfonic acid and phophorylation can experience an 80-Da neutral loss by collision-induced dissociation.[133] The loss of the sulfo-group can make determination of the specific Cys difficult.[133] Electron transfer dissociation is preferred to electron capture dissociation for sulfonic acid–containing peptide fragmentation to prevent the neutral loss of the moiety.[134] Also, it has been reported that sodium adducts can stabilize the SO_3 groups, further helping to prevent loss and improving site assignment.[134]

Conclusions

During the past decade, tremendous progress has been made in the field of redox proteomics. The catalog of modified and modifiable sites has increased exponentially along with our understanding of the role these oxidative modifications have in protein regulation and cellular signaling in the heart. The current methodologies have focused heavily on various switch chemistries, replacing a labile redox modification with more stable and versatile labels. These approaches have been effective, but they have yet to present a complete picture of the redox proteome. A greater emphasis on sensitivity for the detection of endogenous modification will be required to provide the most meaningful insights into the complex role of redox signaling in the cardiovascular system under physiological and pathological conditions.

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None.

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KEY WORDS: biotin switch assay ■ cysteine modification ■ mass spectrometry ■ redox-proteomics

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