ROSICS: CHEMISTRY AND PROTEOMICS OF CYSTEINE MODIFICATIONS IN REDOX BIOLOGY

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Post-translational modifications (PTMs) occurring in proteins determine their functions and regulations. Proteomic tools are available to identify PTMs and have proved invaluable to expanding the inventory of these tools of nature that hold the keys to biological processes. Cysteine (Cys), the least abundant (1–2%) of amino acid residues, are unique in that they play key roles in maintaining stability of protein structure, participating in active sites of enzymes, regulating protein function and binding to metals, among others. Cys residues are major targets of reactive oxygen species (ROS), which are important mediators and modulators of various biological processes. It is therefore necessary to identify the Cys-containing ROS target proteins, as well as the sites and species of their PTMs. Cutting edge proteomic tools which have helped identify the PTMs at reactive Cys residues, have also revealed that Cys residues are modified in numerous ways. These modifications include formation of disulfide, thiosulfinate and thiosulfonate, oxidation to sulfenic, sulfinic, sulfonic acids and thiosulfonic acid, transformation to dehydroalanine (DHA) and serine, palmitoylation and farnesylation, formation of chemical adducts with glutathione, 4-hydroxynonenal and 15-deoxy PGJ2, and various other chemicals. We present here, a review of relevant ROS biology, possible chemical reactions of Cys residues and details of the proteomic strategies employed for rapid, efficient and sensitive identification of diverse and novel PTMs involving reactive Cys residues of redox-sensitive proteins. We propose a new name, "ROSics," for the science which describes the principles of mode of action of ROS at molecular levels. © 2014 The Authors. *Mass Spectrometry Reviews* published by Wiley Periodicals Inc.

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I. INTRODUCTION

Reactive oxygen species (ROS) are the defense system that battles infections by engulfing and killing foreign microorganisms in phagocytic cells. In nonphagocytic cells, ROS generated by external stimuli, growth factors, cytokines, metabolites, and infections, act as signaling molecules for the oxidation of target proteins and for the regulation of the on-off switch proteins in the signaling pathways. The first group of proteins includes phosphatases, kinases and oxidoreductases, that contain ROS-sensitive cysteine (Cys) residues and the proteins of the second group promote various cellular processes such as proliferation, differentiation, migration, metastasis/angiogenesis, inflammation, and death (Fig. 1) (Chang et al., 2002; Giannoni et al., 2005; Kim et al., 2006b; Rhee, 2006). The relationship between physiological changes and molecular mechanisms involving ROS, a component of redox biology, is not well understood. Redox biology includes the processes for generation of cellular ROS, oxidative changes in ROS target proteins in terms of specific sites and modification species, their molecular activities and structures, changes in the interactome, and the effects of ROS on various signaling pathways. Identification of ROS target proteins and their modification sites and species, including the chemical properties of reactive Cys residues which are the major targets of ROS, are critical for understanding the molecular mechanisms in ROS-induced signaling pathways. Proteomic technology using mass spectrometry (MS) has provided invaluable cutting edge tools for identifying the molecular changes including modification sites and species in target proteins.

In this review, we briefly discussed selected aspects of redox biology and the proteomic tools employed to identify several oxidative modifications of proteins, and their biological functions, paying special attention to modifications of Cys sulfhydryl.

II. REDOX BIOLOGY

Cellular ROS affect many signaling pathways depending on the amounts and the location where ROS are generated. Small increases in ROS induce cell proliferation, differentiation,

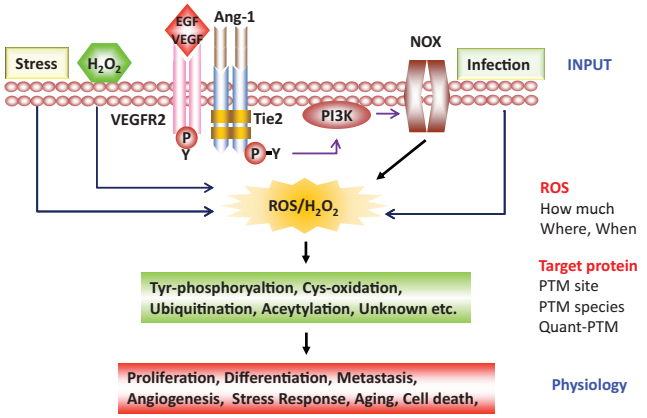


FIGURE 1. ROS-mediated signaling pathways. A variety of external stimuli induce the generation of ROS. Depending on the amount and localization of ROS generated, target proteins and modification species and amounts are varied, which cause diverse cellular physiology.

migration, and angiogenesis (Nishikawa, 2008; Owusu-Ansah & Banerjee, 2009; Arana et al., 2012). On the other hand, large increases in ROS, which can overwhelm cellular antioxidant defense system, elicit cellular oxidative stress and induce aging and cell death (Benhar, Engelberg, & Levitzki, 2002). The molecular mechanisms of these processes are still poorly understood although knowledge obtained from the human genome project and cutting edge technologies including proteomics and genomics, have begun to reveal novel ROS-induced signaling pathways operating in health and disease. Systemic analysis of cellular and molecular responses to ROS in terms of target proteins, modifications in Cys sites, nature of modification species, and quantitative analysis of the post-translational modifications (PTMs), can lead to discovery of novel targets for preventive, diagnostic and therapeutic strategies for cancer, type II diabetes, and cardiovascular, inflammatory, and neurodegenerative diseases, among others, that may result from unbalanced ROS homeostasis (Wyche et al., 2004; Gavazzi et al., 2006; Block, Zecca, & Hong, 2007; Liao et al., 2007; Sorce & Krause, 2009).

A. ROS Homeostasis: Generation and Elimination of ROS

Cellular homeostasis of ROS is well balanced by regulated production and elimination of ROS. Intracellular ROS can be generated via various ways. Extensive amounts of ROS are produced in phagocytic cells by innate immune system that defends the host from infections by killing foreign pathogens and microorganisms. In nonphagocytic cells, relatively low levels of ROS are produced continuously by NADPH oxidase (NOX) activated by various growth factors (e.g., EGF, PDGF, VEGF) and cytokines (e.g., TNFα, IL-1), oxidative phosphorylation in mitochondria, and oxidative protein folding in ER (Rhee, 2006; Bae et al., 2011; Gupta et al., 2012). Most cellular ROS originate from superoxide anion ($O_2^{\bullet -}$), which is readily converted to H_2O_2 by superoxide dismutase (SOD) family of enzymes, and then reduced to H_2O by catalase, glutathione peroxidase (GPx) or peroxiredoxin (PRDX) families of enzymes (Rhee, 2006).

B. ROS Target Proteins

ROS primarily oxidize Cys residues of proteins. The Cys residue, the least abundant of amino acids (1–2%), seems to have unique features that enable it to significantly influence protein structure and function, and its metal binding and its enzymatic activities (Marino & Gladyshev, 2012). The redox active Cys, believed to have a relatively low pK_a (Giles, Giles, & Jacob, 2003; Salsbury et al., 2008), is one of the major targets of ROS in biological systems (Kim et al., 2000) since the thiolate form (Cys-S^-) of Cys is required for the oxidation of Cys by ROS, as discussed later in part IV. Reactive sulfenic acid (Cys-SOH), the primary oxidation product of Cys (Cys-SH) by ROS, can readily form inter- or intra-disulfide bond (Cys-S-S-Cys) with a nearby Cys which can be reversible, or be oxidized further to sulfinic (Cys-SO_2H) and sulfonic acids (Cys-SO_3H). The kind of oxidative change that occurs in the protein structure, determines whether the protein will change in its biological activity, its interaction with other proteins, and its cellular localization. So far, only a limited number of proteins have been described they are sensitive to oxidation/reduction processes. A recent review notes that ROS directly interact with signaling molecules to initiate a wide range of cellular processes, such as proliferation and modulate: PTEN phosphatase; protein tyrosine phosphatase; PI3-kinase; MAP kinase; ROS homeostasis; antioxidant genes, thioredoxin (TRX), peroxidredoxin (PRDX), Ref-1, and Nrf-2; mitochondrial oxidative stress; apoptosis and regulate p66Shc; and ATM-regulated DNA damage (Rhee, 2006; Ray, Huang, & Tsuji, 2012). It was also reported that Hsp33 employs redox-sensitive cysteines, whose oxidation status controls its ability to unfold proteins as part of its chaperone function (Ilbert et al., 2007; Kumsta & Jakob, 2009; Reichmann et al., 2012).

III. CONTRIBUTION OF PROTEOMIC TECHNOLOGIES TO ROSics

Proteomics including tandem mass spectrometry (MS/MS) for analyzing protein sequences, identifying PTMs, sites modified, and quantifying modified peptides by combining protein digestion with trypsin and peptide sequencing with MS/MS (bottom up proteomcis) has been a valuable cutting edge analytical tool in ROSics (Seo et al., 2008). Employment of MS in conjunction with other techniques, has led to the identification of more than 900 PTMs, that have been listed in UniMod database (www.unimod.org). This list is rapidly increasing and now includes phosphorylation, acetylation, acylation, ubiquitination, sumoylation, glycosylation, methylation, and, Cys-modifications, as major PTMs occurring inside cells (Jeong, Lee, & Lee, 2012). PTMs play pivotal roles in various signaling cascades, and tryptic peptides containing the PTMs constitute an important, albeit minor fraction of the total protein milieu. Due to the low abundance of most PTMs, as well as the peptides that contain them, prior enrichment of modified peptides is necessary in MS analysis. Therefore large scale systemic analysis of each PTM can be carried out only after identification of the PTM and the enrichment of the modified peptides. Accomplishing this requires robust methods that can address the complexity and dynamic range of the cellular proteome.

Phosphorylation, the most extensively studied PTM, has been reported in more than 10,000 proteins, variously concerned

with, for example, specific signaling involved in cell cycle (Olsen et al., 2010), mitosis (Dephoure et al., 2008), cAMP- (Gunaratne et al., 2010) and VEGF/Angiopoietin-1 dependent processes (Kim et al., 2007a,b), lung cancer (Rush et al., 2005; Rikova et al., 2007), and heat shock (Kim, Song, & Lee, 2002). Recent studies demonstrated that kinases and phosphatases known to undergo Cys-based redox regulation and tyrosine phosphorylations of various growth factor receptors are enhanced by ROS (Truong & Carroll, 2012). Strategies employed for enrichment of phosphopeptides include immobilized metal ion affinity chromatography (IMAC), titanium dioxide (TiO_2) chromatography, and immunoaffinity chromatography with anti-pY antibodies (Thingholm, Jensen, & Larsen, 2009). Another approach was separating cellular proteins with or without prior treatments on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), detecting proteins phosphorylated on Tyr residues with anti-pY antibody, and analyzing each spot with tandem MS (Kim, Song, & Lee, 2002; Kim et al., 2007a,b). Separation of low-abundant modified proteins from abundant unmodified proteins on 2D-PAGE, can be useful in enriching only the modified proteins.

Acetylation is another abundant PTM. Positively charged peptides, which are readily acetylated at their Lys residues, interact with negatively charged DNA, thereby playing a key regulatory role in gene expression. For example, acetylation of p53 and histone, inhibits DNA binding and renders DNA more relaxed; deactylation reverses this process. A recent study demonstrates that Cys-oxidation of FoxO modulates the acetylation of FoxO by p300/CBP acetyltransferase (Dansen et al., 2009). Massive acetylation was detected by MS in human acute myeloid leukemia cell line (Choudhary et al., 2009), Drosophila (Weinert et al., 2011) and human liver tissue (Weinert et al., 2011), after enrichment of acetylated peptides employing immunoaffinity purification using anti-Ac-Lys antibody (Guan et al., 2010).

Ubiquitination and sumoylation are PTMs that contain small polypeptide ubiquitin and SUMO, covalently attached to Lys residue, which increases the bulk of proteins. Ubiquitination regulates protein degradation, signal transduction, intracellular localization, and DNA repair, depending on the nature and site of linkage. Recent studies showed that ROS inactivates deubiqutinase (Lee et al., 2013) and SUMO proteases (Yan et al., 2010), and regulates the ubiquitin pathway (Doris, Rumsby, & Morgan, 2012). Most common enrichment methods for ubiquitinated and SUMOylated proteins are immunoaffinity purification employing exogenously tagged ubiquitin and SUMO. Large scale purifications with enrichment and MS identifications of ubiquitinated proteins in human osteosarcoma cells (Danielsen et al., 2011) and sumoylated ones in HEK293 cells (Blomster et al., 2010; Bruderer et al., 2011; Galisson et al., 2011) have been performed.

Glycosylation results in heterogenous populations of proteins with varying molecular weights. They play key roles as receptors that facilitate protein localization on membrane surface because of their hydrophilicity and altered surface charge. Ser and Thr residues modified by O-linked β-N-acetylglucosamine (O-GlcNAcylation) were identified by MS in cytokinesis which is crosstalked with phosphorylation (Wang et al., 2010) and in postsynaptic density preparations after enriching O-GlcNAc peptides employing lectin immobilized affinity chromatography (Vosseller et al., 2006).

Oxidative modification of Cys residues is a major PTM involved in ROS-mediated cellular signaling pathways. Modifications in reactive Cys residue are diverse and include sulfenic acid, sulfinic acid, sulfonic acid, disulfide, chemical adduct formations, and acylation, among others (Table 1). Enrichment methods for these modifications have not yet been developed and large scale identification was possible only for Cys modifications which can be enriched. 4-Hydroxy-2-nonenal (HNE), generated during lipid peroxidation, modifies Cys residues forming 4-HNE adducts. These adducts are commonly enriched by immunoaffinity chromatography or solid phase hydrazide enrichment (Roe et al., 2007; Mendez et al., 2010). Biotin-15-Deoxy-D12,14-prostaglandin J2 (15d-PGJ_2) was employed to label the reactive Cys, and the labeled peptides were enriched using streptavidin affinity chromatography in neuroblastoma samples (Aldini et al., 2007). 15d-PGJ_2, one of the terminal products of the cyclooxygenase-2 (COX-2) pathway, is a cyclopentenone-type prostaglandin with potent anti-inflammatory effect because it antagonizes the activities of pro-inflammatory transcription factors, such as NF-κB, STAT3, and AP-1 by stimulating anti-inflammatory transcription factor Nrf2 (Surh et al., 2011). Recently, methodology for enriching the Cys oxidation product, sulfenic acid, was developed using its specific label, dimedone. Purifications of sulfenic acid containing proteins in Hela cells and renal medulla of hypertensive rats, were carried out on a large scale after enriching peptides containing sulfenic acid, labeled with biotin-dimedone, using streptavidin affinity chromatography (Leonard, Reddie, & Carroll, 2009; Seo & Carroll, 2009; Tyther et al., 2010; Giron, Dayon, & Sanchez, 2011).

A. An Approach for Comprehensive Identification of ROS-Induced PTMs: SEMSA Technology

Existing proteomic approaches involving a combination of affinity-based enrichment and mass spectrometric analysis, have helped in identifying only single PTMs or PTMs of a particular kind. However, increasing number of proteins exhibit diverse cellular functions, exist in several forms, and contain different PTMs at various sites, a comprehensive identification of multiple PTMs in a single protein is required to understand if, and how, the PTM's influence the protein's functions. For example, ROS can modify cellular proteins in numerous ways, by inducing a variety of PTMs which modulate the activities of various enzymes, such as tyrosine phosphatase PTP1B and PTEN (Lee et al., 1998; Lee et al., 2002), MAP kinase phosphatase (Kamata et al., 2005), histone deacetylase HDACs (Ago et al., 2008), SUMO ligase (Bossis & Melchior, 2006) and protease (Xu et al., 2008), and ubiquitin E1, E3 ligases (Obin et al., 1998; Zhang et al., 2004; Yao et al., 2004). The activity changes of these enzymes result from ROS-caused generation of PTMs discussed above. However, because of the dynamic complexities of PTMs *in vivo*, and their low abundance, comprehensive identification of PTMs in a single protein remains a challenging problem in proteomics. A 100% peptide coverage with MS/MS is required for identifying all the PTMs in the protein.

As already noted, the low abundant multiple PTMs can only be identified if their populations can be enriched. 2D-PAGE which separates proteins based on their charge (isoelectric points) and molecular mass, can be useful to separately purify different populations of a single protein. Comprehensive identification of

TABLE 1. List of identified modifications at Cys residues

PTM type at Cys	Monoisotopic mass change	Remarks
Oxidation		
Dehydroalanine	-33.987721	
Cys to Ser	-15.977156	
Disulfide	-2.0145	Cys-S-S-Cys
Sulfenic acid	+15.994915	Cys-SOH
Sulfinic acid	+31.98983	Cys-SO_2H
Sulfonic acid	+47.984745	Cys-SO_3H
Thiosulfonic acid	+63.961901	Cys-SO_2-SH
Selenylation	+79.916520	Cys-S-SeH
S-nitrosylation	+28.990164	Cys-S-NO
Acylation		
Cysteinylation	+119.004099	
Glutathionylation	+305.068156	
Farnesylation	+204.187801	
Palmitoylation	+238.229666	
Geranyl-geranylation	+272.250401	
Diacylglycrol	+576.511761	
S-guanylation	+344.039610	
Chemical Adduct		
4-hydroxynonenal	+156.115030	HNE
Cyano	+24.995249	Cys-SCN
PGJ2	+320.5	15-deoxy-Δ12,14-prostaglandin-J2 (15d-PGJ_2)
Artifactual adduct		
Propionamide	+71.037114	Cys-SH Acrylamide adduct in PAGE
	+87.0365	Cys-SOH Acrylamide adduct in PAGE
	+103.0359	Cys-SO_2H Acrylamide adduct in PAGE
Carbamidomethyl	+57.021464	Cys-SH Alkylation adduct of iodoacetamide
	+73.0209	Cys-SOH Alkylation adduct of iodoacetamide
	+89.0203	Cys-SO_2H Alkylation adduct of Iodoacetamide
DeStreak	+75.998285	Cysteine mercaptoethanol
N-ethylmalemide	+125.0477	Cys-SH Adduct of N-ethylmaleimide
	+141.0471	Cys-SOH Adduct of N-ethylmaleimide
	+157.0465	Cys-SO_2H Adduct of N-ethylmaleimide
Unknown	+134, +150, +284	

PTMs in multiply modified proteins can be accomplished by combining 2D-PAGE with MS/MS for peptide sequencing, although this approach may have limitation in terms of throughput analysis (Hwang et al., 2009). For example, phosphorylated, oxidized, and acetylated proteins move in acidic direction on 2D-PAGE, ubiquitinated, sumoylated, glutathionylated proteins move upward because of increases in molecular mass, while disulfide crosslinked proteins and chemical adducts move either

upward or downward depending on their size, chemical charge and polarity.

We developed a strategy for rapid, efficient and comprehensive identification of PTMs occurring in biological processes *in vivo*. This strategy employs a *s*electively *e*xcluded *m*ass *s*creening *a*nalysis (SEMSA) of unmodified peptides during LC-ESI-q-TOF MS/MS through replicated runs of a purified protein on 2D-PAGE (Seo et al., 2008). Precursor ion list of unmodified peptides with high mass intensities was obtained during the initial run followed by exclusion of these unmodified peptides in subsequent runs. Exclusion list can grow as long as replicate runs are iteratively performed (Fig. 2). The SEMSA strategy enables the identifications of modified peptides with precursor ions having low intensities by selecting for MS/MS sequencing, even though the sample amounts and analysis times required are two- to threefold. SEMSA approach in combination with PTM search algorithm MOD[i] (Kim et al., 2006a; Na et al., 2008) was applied to GAPDH protein modified by oxidative stress *in vivo* and separated on 2D-PAGE. Diverse PTM populations were identified in various peptides. For example, using this approach, it was found that peptide ^{146}IISNASCTTNCLAPLAK^{162} containing active site CxxxC showed 12 kinds of modified peptides including 6 MS/MS spectra (Table 2 and Fig. 3). Multiple modifications including minor modifications of cellular GAPDH were identified, and by raising the peptide coverage (>92%) to include 19 types of modifications on 42 sites, additional potential for finding novel modifications such as transformation of Cys to Ser was validated. Based on precursor ion *m/z*, label-free quantitative analysis of PTMs was performed for identifying molecular changes in heterogeneous protein populations. These studies showed that PTMs *in vivo* are more complicated and heterogeneous than previously reported. This strategy has been applied for systematic characterization of multiple PTMs in functional proteomics.

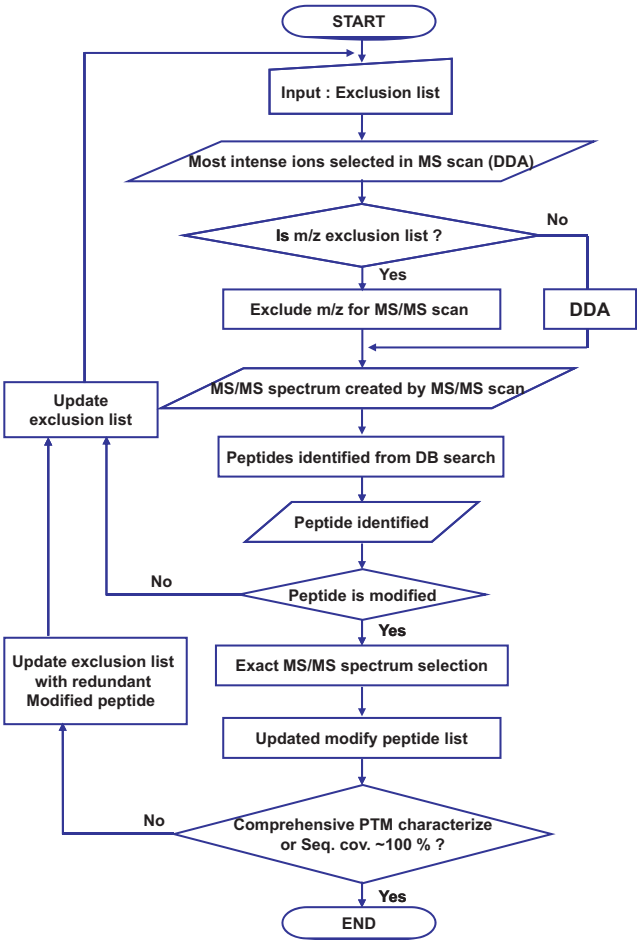


FIGURE 2. Scheme of SEMSA for comprehensive PTM characterization using MS/MS. After survey scanning of the first LC-MS run the MS/MS data were submitted to the Mascot and MODi searching for peptide identification. The list of identified peptides in the first run, the candidate precursor ions of MS/MS acquisition were compared to the exclusion list, and the same procedure was followed. The new peak list was used in the second MS/MS run. In replicate runs, accumulated peak lists established a PTM-specific peptide library (Seo et al., 2008).

B. Proteo-Informatic Tools for ROSics

Mass spectrometric algorithm tools help extract information on PTMs from MS/MS sequencing. However, most existing searching algorithms have limitations to detect multiple and unknown modifictions and searches had therefore to be restricted to a few types of PTMs. MS-Alignment (Tsur et al., 2005) predicts PTMs expected in a sample by comparing spectral alignment between database peptide and a spectrum followed by InsPecT search (Tanner et al., 2005). ModifiComb introduced a ΔM histogram between unassigned spectra and base peptides in a database (Savitski, Nielsen, & Zubarev, 2006). However, these tools have the drawback that they miss low abundant PTMs and those observed infrequently. MOD[i], pronounced "mod eye," is a searching algorithm that can find all known types of PTMs, as well as a multitude of modified sites in a peptide, very rapidly and simultaneously and is not limited or restricted in this regard, like most other algorithms (Kim et al., 2006a; Na et al., 2008). MOD[i], can also uniquely deal with computational complexities and finds multiple PTMs in a peptide, is therefore a useful tool to discover novel PTMs. MODa, a novel recently improved "multi-blind" spectral alignment algorithm, allows unrestricted PTM searches with high speed and with no limitation on the number of modifications per peptide (Na, Bandeira, & Paek, 2012). In our hands, combination of SEMSA technology for MS analysis with algorithms MOD[i] or MODa, offered a powerful tool to find unexpected and novel PTMs having low abundance in cells.

C. Identification of Disulfide Bond

Identifying the sites of disulfide bonds in a protein is essential for thorough understanding of the protein's tertiary and quaternary structures and its biological regulation. Disulfide bond formation reversibly regulates diverse signaling pathways induced by ROS. Disulfide linked peptides are usually identified indirectly by labeling free sulfhydryl groups with alkylating agents, followed by chemical reduction and mass spectral comparison or by detecting the expected masses of disulfide linked peptides on mass scan level. Conventional identifications of disulfide linked peptides are ambiguous when the protein is highly bridged and modified. Standard MS search engines such as Mascot (Perkins et al., 1999) or SEQUEST (Eng, McCormack, & Yates, 1994) cannot match the masses observed from disulfide linked peptides with the masses of peptide pairs from a database, and thus, are not useful in identifying interpeptide disulfide bonds. An algorithmic solution for the

TABLE 2. Modification list of GAPDH peptide (^{146}IISNASCTTNCLAPLAK^{162}) containing active site cysteine (Seo et al., 2008)

Modifications	m/z	Mr (calc) mass
I I S N A S C T T N C L A P L A K	861.0301	1720.0445
I I S N A S C T T N C L A P L A K propionamide	895.9611	1789.9066
I I S N A S C T T N C L A P L A K propionamide propionamide	931.4797	1860.9437
I I S N A S C T T N C L A P L A K phosphorylation propionamide	935.9443	1869.8729
I I S N A S C T T N C L A P L A K phosphorylation propionamide propionamide	971.4629	1940.9101
I I S N A S C T T N C L A P L A K propionamide deamide propionamide	931.9717	1861.9278
I I S N A S C T T N C L A P L A K cysteic acid propionamide	919.9535	1837.8914
I I S N A S C T T N C L A P L A K cysteic acid deamide propionamide	920.4459	1838.8762
I I S N A S C T T N C L A P L A K substituted S propionamide	887.9725	1773.9294
I I S N A S C T T N C L A P L A K phosphorylation substituted S propionamide	927.9557	1853.8958
I I S N A S C T T N C L A P L A K 2 phosphorylations substituted S	932.4203	1862.8250
I I S N A S C T T N C L A P L A K disulfide bond	859.4347	1716.8538

analysis of tandem mass spectra of disulfide bonded peptides under non-reducing conditions was recently published (Choi et al., 2010), and this should facilitate accurate identification of disulfide linked peptides. This new algorithm called "DBond" analyzes disulfide linked peptides based on specific MS/MS features of disulfide linked sites (Choi et al., 2010). DBond takes into account fragmentation patterns of disulfide linked peptides in nucleoside diphosphate kinase (NDPK) as a model protein, with fragment ions including Cys, Cys thioaldehyde (−2 Da, C^T), Cys persulfide (+32 Da, C^S) and dehydroalanine (DHA, −34 Da, C^Δ) (Fig. 4A). Using this algorithm, a homodimer and a heterodimer in a dozen novel disulfide bonds were successfully identified in secretagogin, methionine sulfoxide reductase, and PDI (Fig. 4B; Denoncin et al., 2010). DBond, which recognizes the disulfide bond fragmentation characteristics and PTMs, offers a novel approach for automatic identification of unknown disulfide bonds and their sites from tandem mass spectra. Glutathionylation is a typical disulfide linkage in proteins and massively glutathionylated proteins were identified in oxidatively stressed T-lymphocytes (Fratelli et al., 2002), and a less glutathionylated minor peptide was identified in NDPK, employing SEMSA and DBond searching algorithm (Lee et al., 2009).

D. Novel and Diverse Cys Oxidations

Redox sensitive Cys sulfhydryl (Cys-SH) having low pK_a is the major target of ROS in biological systems. It regulates protein function by undergoing oxidation to sulfenic (Cys-SOH), sulfinic (Cys-SO_2H), sulfonic acids (Cys-SO_3H), nitrosoCys (Cys-S-NO), and inter- and intra-disulfide bond formation (Cys-S-S-Cys) (Table 1). The primary oxidation product of Cys by

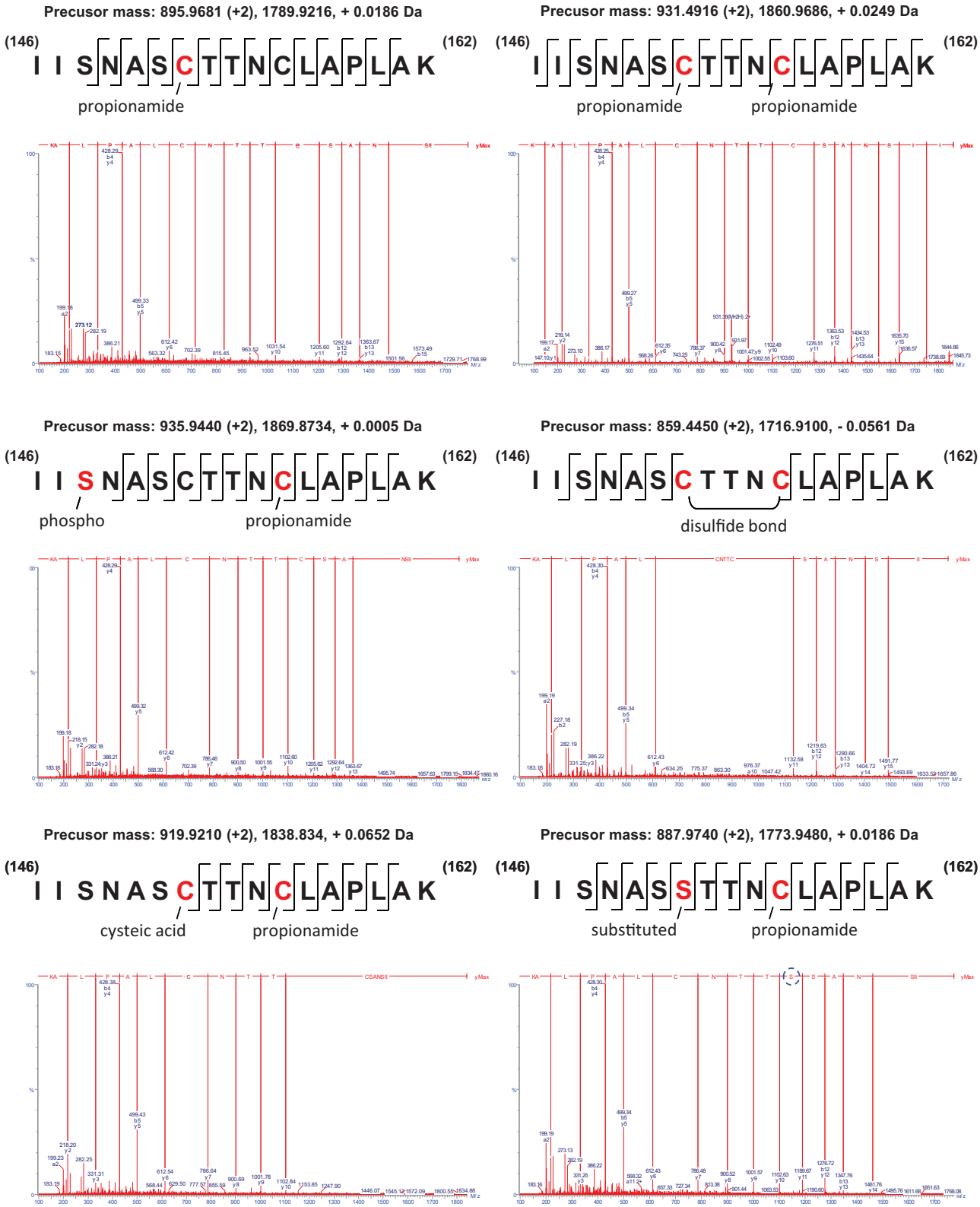


FIGURE 3. Examples of ESI-MS/MS spectra of modified peptides of GAPDH containing active site cysteine (^{146}IISNASCTTNCLAPLAK^{162}) (Seo et al., 2008). The gel spots were destained and digested with trypsin, or proteins in solution were digested with trypsin and the resulting peptides extracted, evaporated to dryness in SpeedVac and redissolved in 10% ACN containing 0.1% formic acid. The dissolved peptides were desalted on line prior to separation using trap column (i.d. 180 μm × 20 mm, Symmetry® C18) cartridge, and separated on a C18 reversed-phase 75 μm i.d. × 200 mm analytical column (1.7 μm particle size, BEH130 C18, Waters Co., Milford, MA, USA) with integrated electrospray ionization PicoTip™ (±10 μm i.d., New Objective, Wobum, MA, USA) using nanoAcquity™/ESI/MS (SYNAPT™ HDMS™, Waters Co.) (Lee et al., 2009).

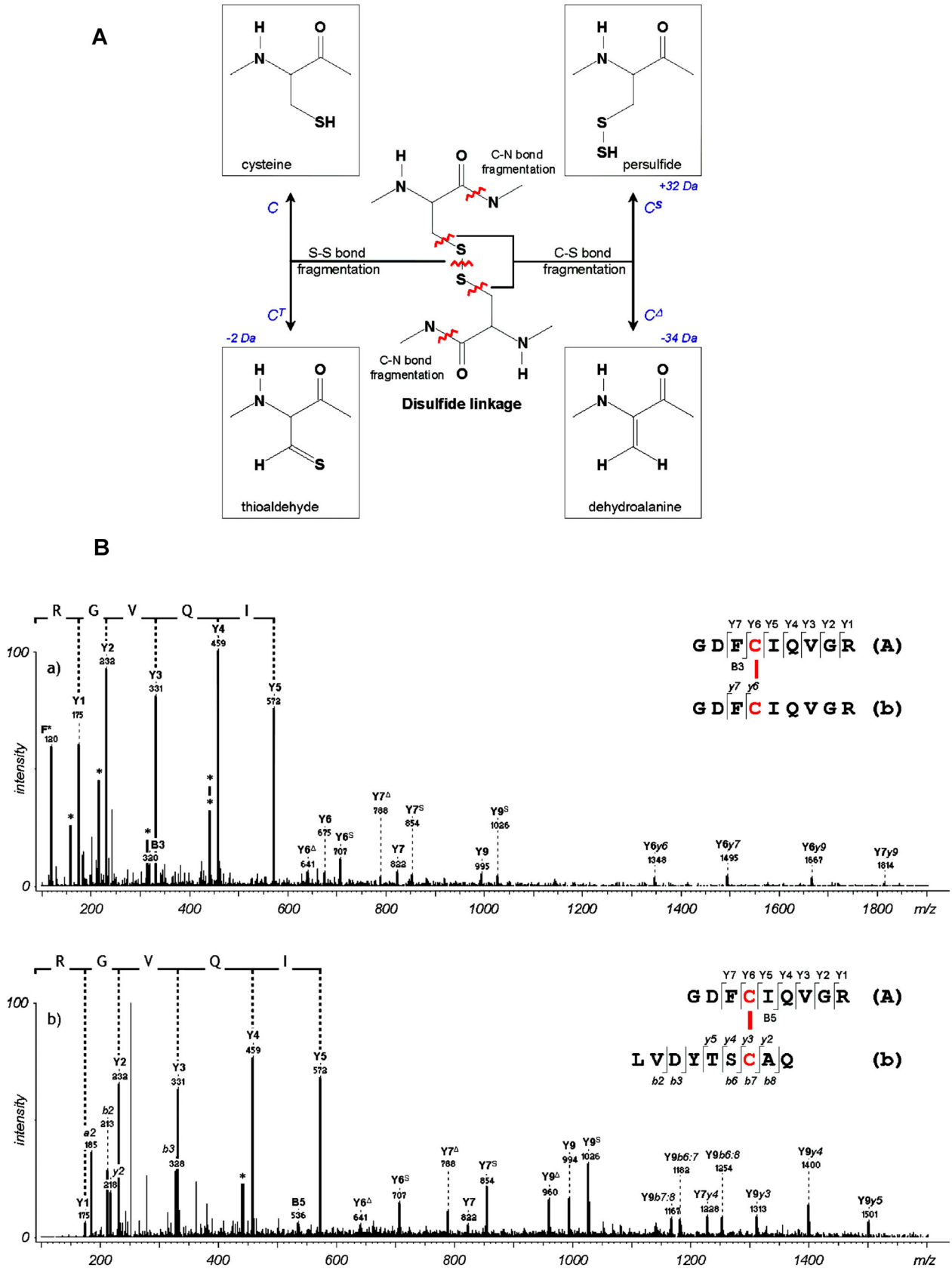


FIGURE 4. **A**: Possible products of fragmentation at the disulfide linkage. **B**: MS/MS spectra of peptide GDFCIQVGR-GDFCIQVGR and GDFCIQVGR-LVDYTSCAQ disulfide linkage were interpreted. The spectra show fragmentation characteristics of disulfide linkage. Ion annotations used: "Δ," dehydroalanine ion (Choi et al., 2010).

ROS is sulfenic acid (Cys-SOH), an intermediate form, easily oxidized further to disulfide bond (Cys-SS-Cys), sulfinic (Cys-SO_2H) and sulfonic acids (Cys-SO_3H). Sulfinic acid is a comparatively stable intermediate, which can be reversibly reduced to Cys-SH by sulfiredoxin (Biteau, Labarre, & Toledano, 2003) or oxidized to sulfonic acid (Cys-SO_3H), the most extremely oxidized irreversible derivative of Cys.

Iodoacetamide (IAM) and N-ethylmaleimide (NEM) have long been known to react only free Cys-SH, by a different mechanism discussed in the next section. Biotin-tagged IAM (BIAM) (Kim et al., 2000) including isotope coded affinity tag (ICAT) (Han et al., 2001) were used for detecting or enriching the free Cys-SH. Also most free sulfhydryls can be easily labeled by generating acrylamide adducts (propionamide, C^{aa}, $\Delta m = +71.0359$ Da) in acrylamide gel electrophoresis.

A previous study reported that alkylating agents react with oxidized Cys (Cys-SOH and Cys-SO_2H) as well as free thiol (Cys-SH) (Jeong et al., 2011). When hydrogen peroxide-treated recombinant proteins such as GAPDH, NDPK, or peroxiredoxin 6 (PRDX6) were subjected to various alkylating agents known to capture Cys-SH including IAM or NEM, sequencing with UPLC-ESI-q-TOF tandem MS, MS/MS spectra after trypsin digestion showed signals corresponding to chemical adducts of sulfenic and sulfinic acids as well as free sulfhydryls (Fig. 5 and

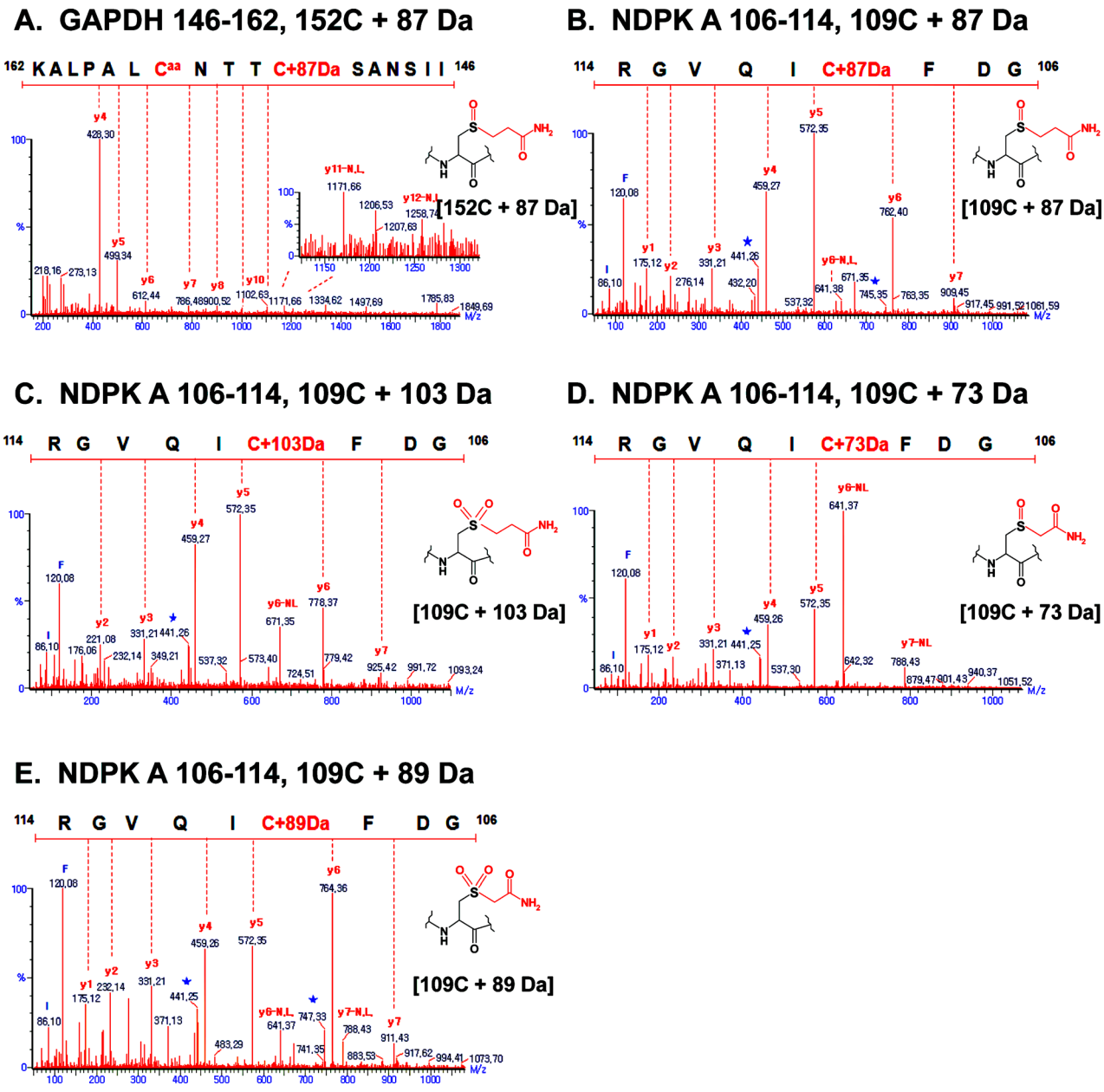


FIGURE 5. MS/MS spectra showing mass shifts of sum of alkylating agent and oxygen at Cys residues in GAPDH and NDPK-A. **A**: Cys152 of GAPDH has +87 Da shift corresponding to sum of acrylamide adduct propionamide (+71 Da) and one oxygen (+16 Da). **B**,**C**: Cys109 of NDPK-A has +87 and +103 Da shifts matching to sum of propionamide (+71 Da) and one (+16 Da) or two oxygens (+32 Da). **D**,**E**: Cys109 of NDPK-A has +73 and +89 Da shifts of sum of iodoacetamide adduct acetamide (+57 Da) and one (+16 Da) or two oxygens (+32 Da). In MS/MS spectra, star indicates fragment ion which was lost H_2O or NH_3 (Jeong et al., 2011).

TABLE 3. Predicted element composition of mass shift of Cys in GAPDH and NDPK A (Jeong et al., 2011)

	Mass shift	Predicted element composition
71	71.0371	Propionamide
87	87.0365	O + Propionamide
103	103.0359	O_2 + Propionamide
57	57.0215	Alkylation adduct with iodoacetamide
73	73.0209	O + Alkylation adduct with iodoacetamide
89	89.0203	O_2 + Alkylation adduct with iodoacetamide
125	125.0477	N-ethylmaleimide (NEM)
141	141.0471	O + N-ethylmaleimide
157	157.0465	O_2 + N-ethylmaleimide
143	143.0582	NEM hydration (NEMhyd)
159	159.0576	O + NEMhyd
175	175.0570	O_2 + NEMhyd

Table 3). The +71 Da shift (C_3H_5NO) at Cys residue indicates acrylamide adduct of Cys-SH and the +87 Da and +103 Da shifts indicate acrylamide adducts of Cys-SOH and Cys-SO_2H respectively. These reactions occur with IAM and NEM having different mass shifts. These results show that Cys-SOH and Cys-SO_2H can easily react with acrylamide, IAM and NEM as well as Cys-SH. Therefore, for identifying reduced Cys sulfhydryl it is necessary to develop reagents that react only with Cys-SH, and not Cys-SOH and Cys-SO_2H.

Cys-SH is oxidatively modified by ROS to sulfenic acid (Cys-SOH), which is easily transformed to disulfide bond, sulfinic acid (Cys-SO_2H) or sulfonamide (Cys-SNO). Methodology for specifically labeling sulfenic acid (Cys-SOH) with dimedone, was recently established. Large scale identifications of sulfenic acid in Hela cells and renal medulla of hypertensive rat were carried out by enriching peptides containing sulfenic acid, and labeling them with biotin-dimedone, using streptavidin affinity chromatography (Leonard, Reddie, & Carroll, 2009; Seo & Carroll, 2009; Tyther et al., 2010; Giron, Dayon, & Sanchez, 2011). This is one possible way to identify ROS sensitive Cys residues. However, because Cys-SOH is an intermediate, transient form of protein oxidation, it cannot fully serve as a biomarker of protein oxidation.

In addition to known Cys oxidations, a novel low abundant Cys modification was detected in cellular GAPDH purified on 2D-PAGE employing SEMSA for nanoUPLC-ESI-q-TOF tandem MS analysis, in conjunction with MOD[i] and MODmap algorithm (Kim et al., 2006b; Na et al., 2008). Unexpected mass shifts ($\Delta m = -16, -34, +64, +87$, and +103 Da) at redox-active Cys residue were detected in cellular GAPDH, oxidized NDPK, PRDX6, and mitochondrial proteins. Mass differences of −16, −34, and +64 Da are presumed to reflect the conversion of Cys to Ser, DHA, and Cys-SO_2-SH (thiosulfinic acid), respectively shown in Figure 6 and Table 4 (Jeong et al., 2011). In efforts to determine the possible pathways leading to the formation of these products, thiosulfinate (Cys-S-SO-Cys) and thiosulfonate (Cys-S-SO_2-Cys) intermediates were identified with DBond in NDPK. These model compounds were synthesized and hydrolysis and hydration of thiosulfonate (Cys-S-SO_2-Cys) either to DHA ($\Delta m = -34$ Da) or Ser along with Cys-SO_2-SH ($\Delta m = +64$ Da) in weak base was examined. These findings suggest that oxidations take place at redox-active Cys residues in cellular proteins, with the formation of novel modifications including thiosulfonic acid, Cys-SO_2-SH, and DHA, and conversion of Cys to Ser, in addition to sulfenic, sulfinic and sulfonic acids of reactive Cys. Figure 7 presents a proposed oxidative modification pathway for reactive Cys.

Various external stimuli including heat shock and various growth factors generate ROS through NOX and simultaneously phosphorylate various proteins at their tyrosine residues. Cellular phosphorylations increase because of the inhibition of phosphatases by the ready oxidation of their active site Cys residues. By combining 2D-PAGE and MS, tyrosine phosphoproteome was identified in cells treated with various growth factors (VEGF and Ang-1) and stresses (heat shock and H_2O_2), mimicking ROS-mediated signaling pathways (Kim, Song, & Lee, 2002; Kim et al., 2006a, 2007a,b). Recently, protein-tyrosine phosphatome and redoxome were studied in order to understand the biological crosstalk between phosphorylation and oxidation (Karisch et al., 2011). Phosphatase expressions and their oxidations were quantitatively analyzed in cancer cells and tissues, and it was found that they display distinctive phosphatases and oxidized forms. Clues on the connection of oxidation with phosphorylation and ubiquitination were disclosed (Kim, Song, & Lee, 2002; Kim et al., 2011). Arguably, altered redox regulation seems to add yet another factor to complicate physiological processes.

E. Chemical Modifications of Cys Residues

Cys residues in proteins react with various chemicals. Palmitoylation is an acylation at Cys residue, which facilitates membrane

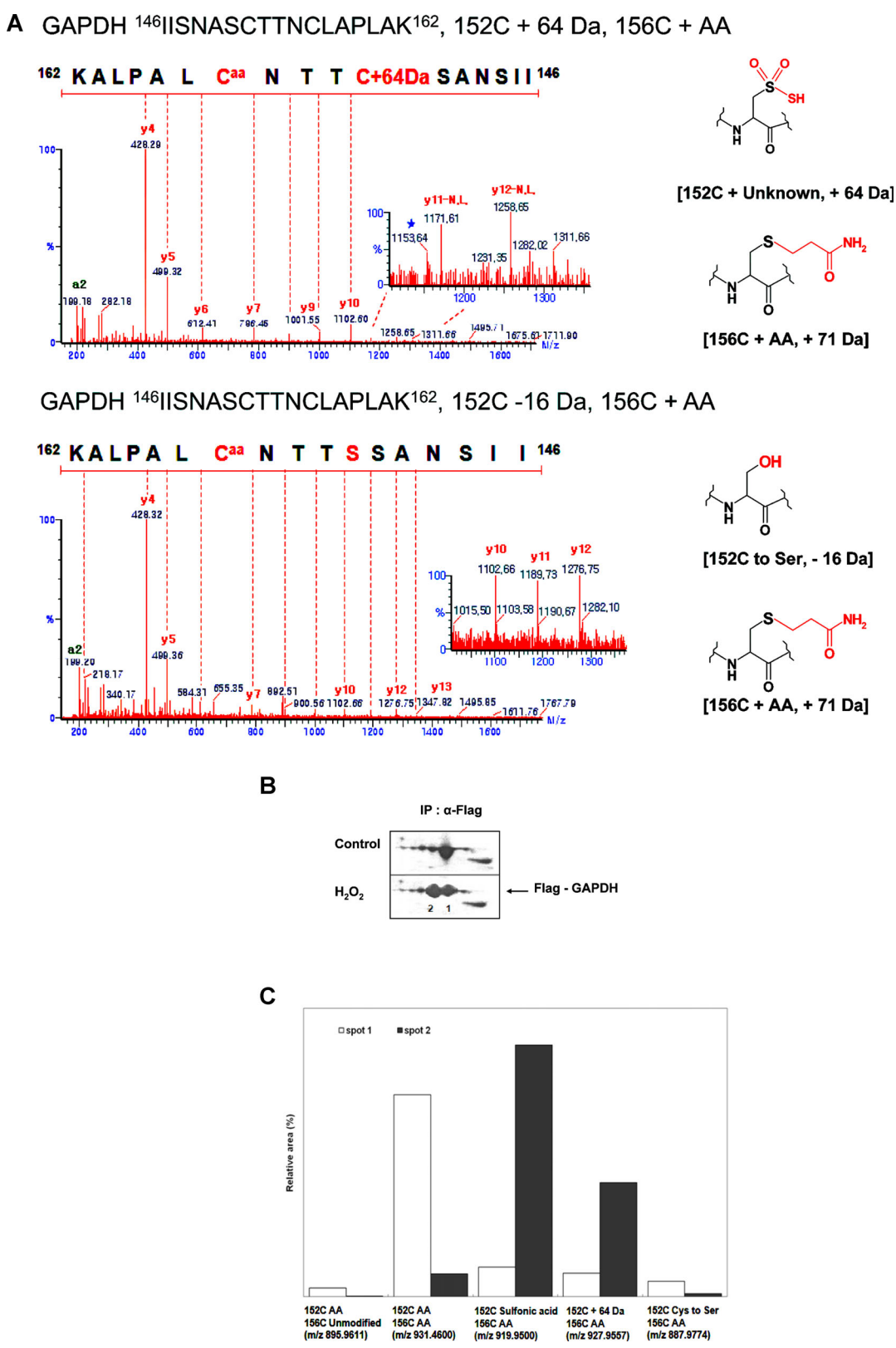


FIGURE 6. Novel modifications of active site cysteine detected in GAPDH. **A**: MS/MS spectra of active site "CXXXC" containing unknown mass shift +64 and −16 Da. C^{aa} indicates acrylamide adduct of Cys residue and star indicates fragment ion which was lost H_2O or NH_3. Presumed chemical structure of each mass shift at Cys residue was presented in right side. **B**: The immuno-precipitated GAPDH was separated on 2D-PAGE and detected with coomassie staining. **C**: Quantitative analyses of modifications in peptide ^{146}IISNASCTTNCLAPLAK^{162} based on precursor ion intensities using Glu-fibrinopeptide as an internal standard (Jeong et al., 2011).

TABLE 4. Summary of cysteine modifications observed in cellular GAPDH purified from HEK293 cells by immunoprecipitation and by tandem MS spectrometry

Site/Sequence	m/z	Mr (calc) mass	Nominal mass difference (Da)	Position	Modification
146-162 IISNASCTTNCLAPLAK	895.9611	1789.9066	+71	152C	Acrylamide
	931.4797	1860.9437	+71/+71	152/156C	Acrylamide/Acrylamide
	919.9535	1837.8914	+48/+71	152/156C	Sulfonic acid/Acrylamide
	927.9557	1853.8958	+64/+71	152/156C	Unknown/Acrylamide
	887.9774	1773.9398	-16/+71	152/156C	Substitute C to S/Acrylamide
	859.4370	1716.8536	-2	152/156C	Intra disulfide
235-248 VPTANVSVVDLTCR	737.3900	1472.7656	0	·	Unmodified
	772.9092	1543.8028	+71	247C	Acrylamide
	753.3901	1504.7645	+32	247C	Sulfinic acid
	761.3830	1520.7504	+48	247C	Sulfonic acid
	720.3968	1438.7780	-34	247C	Dehydroala C

Peptide *m*/*z* and calculated mass, and detected nominal mass changes at 152C/156C and 247C residues were presented (Jeong et al., 2011).

association and proper subcellular localization of the protein. Systematic profiling of palmitoylated proteins has been done after labeling the protein with biotin-labeled palmitic acid analog 17-octadecynoic acid in combination with stable-isotope labeling of amino acids in cell culture (SILAC) (Martin et al., 2011) and endothelial cell palmitoylproteomics was examined (Marin et al., 2012). As mentioned previously, HNE, generated during lipid peroxidation, modifies Cys of proteins (Roe et al., 2007; Mendez et al., 2010), and 15d-PGJ_2, one of the terminal products of the COX-2 pathway, was used to label Cys of various proteins employing biotin-15d-PGJ_2 (Aldini et al., 2007). Biological effects of these and other chemical modifications are the focus of many on-going studies.

Cyano-Cys (+25 Da change, -S-CN) was detected in many reactive Cys residues in GAPDH, NDPK, and PRDX6 among others (Jeong et al., 2011). However, it is not known where cyano-Cys modification occurs inside the cells. Several unknown PTMs of active site Cys47 of PRDX6 were identified in liver tissues from two inbred mouse strains. Two of the PRDX6 modifications are different in the two mouse strains, as shown in Figure 8A. Quantitative analysis of PTMs in active Cys revealed, in addition to known modifications, significant unknown mass changes (+134, +150, +284 Da) at Cys47 of PRDX6 (Fig. 8B and Table 5), whose origins also could not be explained. Distinct differences in Cys47 modification were observed in each spot on 2D-PAGE. Acidic spot PRDX6 from C3H/HeJ mouse is enriched by peptide containing sulfonic acid, cyano, unknown +134 and +150 Da mass changes, while basic PRDX6 from C57BL/6J mouse is enriched by peptide containing DHA, acrylamide adduct of sulfinic acid and unknown +284 Da mass change (Jeong, Lee, & Lee, 2012). These observations indicate that many unknown PTMs may have not been previously detected in MS/MS sequencing, because of lack of algorithms that help their characterization. Our studies discussed here show that MOD^i and MODa can be used to cast a wider net for identifying low abundant new PTMs.

Proteomic technology using MS/MS analysis can also be applied to find the target proteins of various chemical inhibitors and help understand their modes of action. For example, N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), a serine protease inhibitor, has recently been reported to inhibit the expression of inflammatory mediators by blocking NF-κB activation. It was also found that TPCK inhibits NF-κB activation by forming the chemical adduct with Cys179 of IKKβ and Cys38 of p65/RelA detected by MS/MS (Ha et al., 2009).

IV. CYSTEINE CHEMISTRY

Unexpected massive modifications of reactive Cys residues may play roles in redox biology. These unexpected modifications can be identified only by peptide sequencing with MS/MS analysis, but knowledge of chemical reactivity of Cys residues necessary to accept the unexpected Cys modifications detected by MS analysis. It is important therefore, to understand the fundamentals of the chemistry of Cys residue. Among the amino acid residues in proteins, Cys is by far the most reactive since the sulfur atom in Cys is not only nucleophilic but is also prone to oxidation, leading to various reversible and irreversible PTMs of Cys in proteins (Nagy & Winterbourn, 2010; Fig. 7). Most of these modifications have parallels in organic chemistry while a few of them may not have been described in organic chemistry textbooks. Nevertheless, all of the identified Cys modifications follow accepted reaction mechanisms. Oxidation of Cys thiol to sulfenic acid expands Cys modification by adding electrophilicity to the sulfur atom. Detailed chemical mechanistic pathways for various nucleophilic, oxidative and electrophilic modifications of Cys and derivatives are summarized in this chapter.

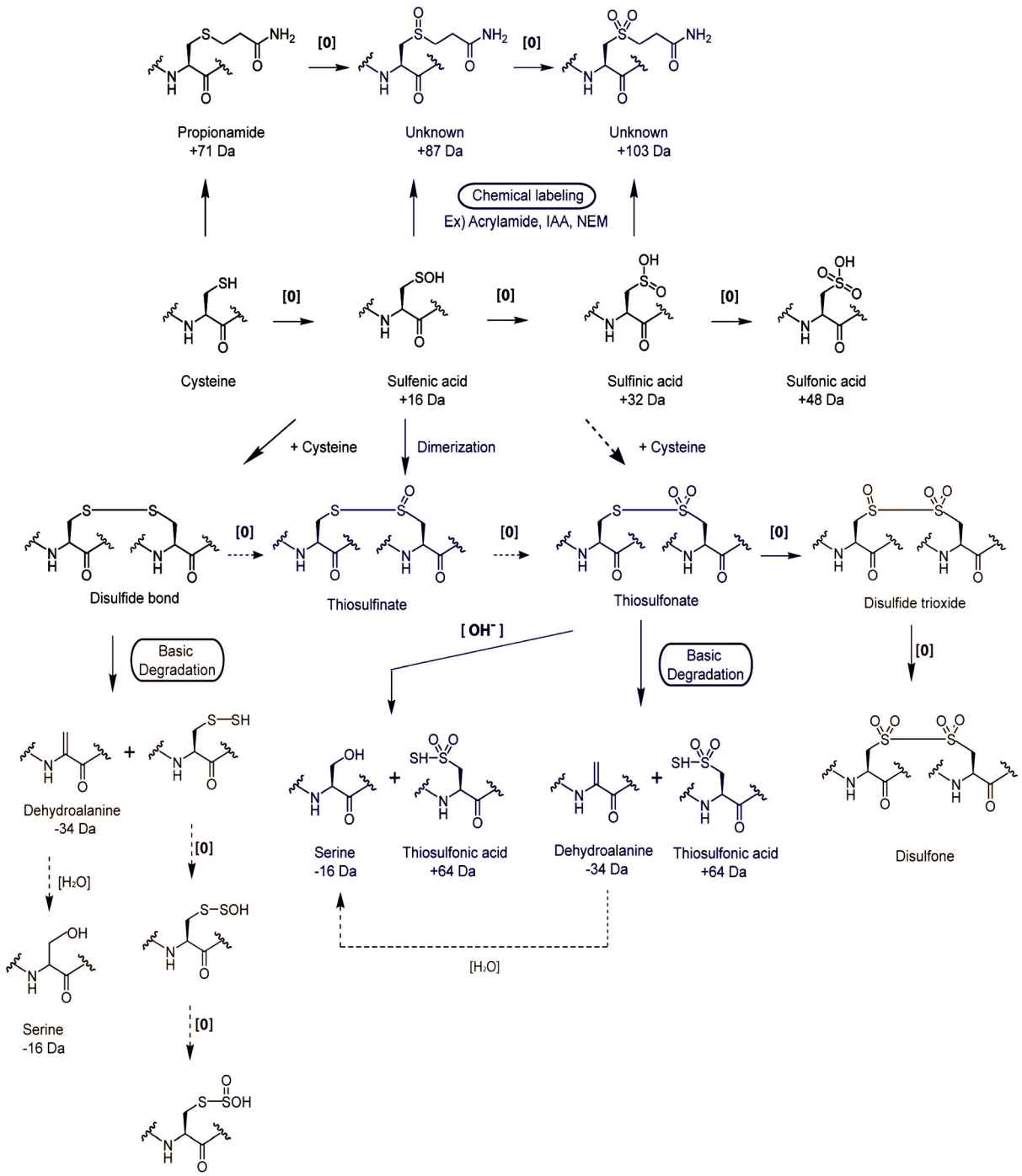


FIGURE 7. A mechanism to explain novel cysteine modifications. Known pathways (black line) and newly identified pathway (blue line).

A. Substitution Reaction

As nucleophiles, thiolates (RS^-) are more reactive than thiols (RSH). Thus pK_a of Cys plays an important role in the nucleophilicity of Cys. The pK_a of Cys is 8.4, but pK_a of Cys residue in proteins are greatly varied 4–9 affected by protein environment. Basic residues close to –SH of Cys in protein structure lower the pK_a of Cys and readily ionize to thiolates (RS^-). Cys thiolates irreversibly undergo nucleophilic substitution, during lipidation. Cys thiol produces two major classes of lipidated proteins: S-palmitoylated and S-prenylated proteins. Palmitoylated proteins are obtained from a 16-carbon saturated fatty acyl CoA (Scheme 1a) and palmitoylated Cys sites ($\Delta m = +238.2$ Da) were detected with MS in isomerohydrolase (Takahashi et al., 2009). Carbon atom on carbonyl group is attacked by Cys residues (thiolate, RS^-) to generate labile thioether bond (acylation) with elimination of CoA-SH which can be catalyzed by palmitoyl-S-protein transferases. Cys thiolate nucleophiles

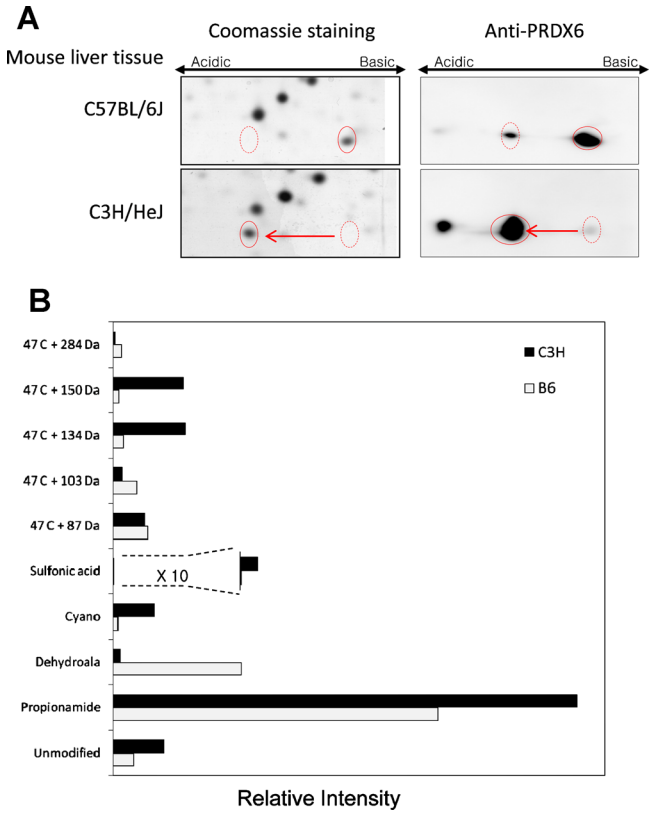


FIGURE 8. Profiles of Peroxiredoxin 6 from livers of C57BL/6J and C3H/HeJ inbred strains of mice. **A**: Cell lysates of PRDX6 from liver tissue of C57BL/6J and C3H/HeJ mouse strains were separated on 2D-PAGE and detected with coomassie staining (left) and with Western analysis using anti-PRDX6 antibody (right). **B**: Quantitative analysis of each modified peptides in each PRDX6 spot from liver tissue of C57BL/6J and C3H/HeJ mouse is summarized (Jeong, Lee, & Lee, 2012).

react with prenyl electrophiles through S_N1 type substitution reaction (Scheme 1b; Casey, 1995; Walsh, Garneau-Tsodikova, & Gatto, 2005). Prenyl pyrophosphates readily lose diphosphates to form primary carbocations that are stabilized by allylic resonance.

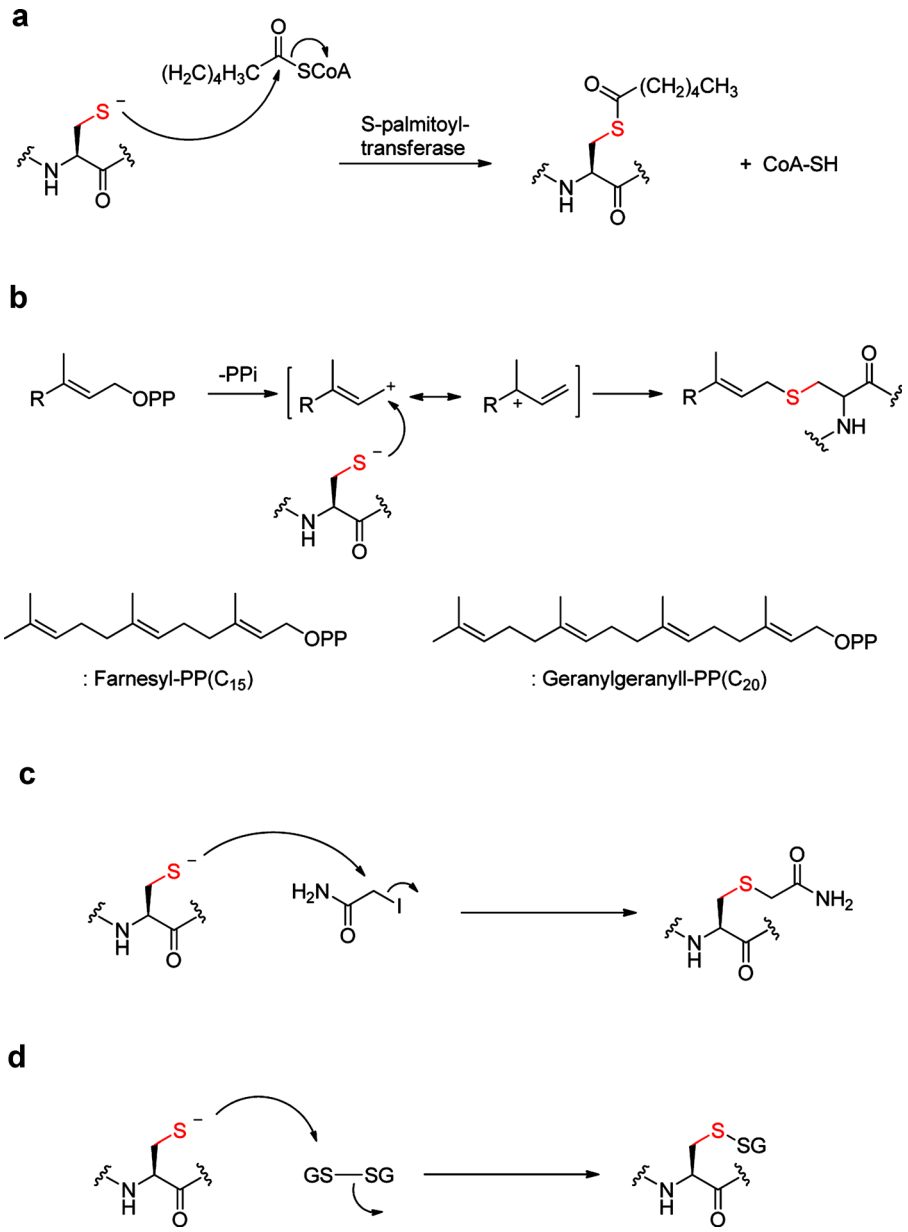
Cys thiolates can undergo S_N2 type substitution reaction with external electrophiles such as iodoacetamide (IAM). Since thiolates react much faster than thiols in the S_N2 type substitution reaction, IAM substitution reaction can be used to distinguish the reactivity of Cys residues in the proteins (Scheme 1c; Weerapana et al., 2010). Cys thiolate also reacts with disulfides through S_N2 type substitution reaction to form disulfide bonds and the outcome of this substitution reaction is one mode of Cys oxidation reaction. Glutathionylated Cys was detected with MS combining Dbond algorithm in NDPK (Scheme 1d; Lee et al., 2009).

B. Addition Reaction for Formation of Chemical Adducts of Cys

Nucleophilic nature of Cys thiolate allows it to react with Michael acceptors. Typically, maleimides, vinyl sulfones, acrylamides, and α,β-unsaturated ketones are the most widely used Cys probes as Michael acceptors of Cys (Scheme 2a; Chalker et al., 2009). Chemical adducts of Cys with Michael acceptors were identified by MS (Jeong et al., 2011). Certain prostaglandins (PGs), especially, A- and J-type PGs, that contain reactive cyclopentenone rings are known to react both enzymatically and nonenzymatically with thiols (Uchida & Shibata, 2007). Cys modification sites of 15d-PGJ_2 were recently identified by MS in UCH-L1 related to Parkinson's disease (Koharudin et al., 2010) and in Ras, in cancer (Renedo et al., 2007). In the case of 15-deoxy-Δ12,14-prostaglandin-J2 (15d-PGJ_2) with two Michael acceptor positions, addition of Cys thiolate is chemoselective and occurs at the endocyclic β-position selectively (Scheme 2). This selectivity can be explained by the fact that the LUMO of 15d-PGJ_2 has a higher coefficient at the endo-β-position than

TABLE 5. Modification list of peptide containing active site cysteine in PRDX6, identified by UPLC-ESI-q-TOF tandem MS in liver tissue of C57BL/6J (B6) and C3H/HeJ (C3H) mouse strains (Jeong, Lee, & Lee, 2012).

Sequence /Peptide	Observed	Mr (expt)	Delta mass (Da)	Modifications	C57BL/6J (B6)	C3H/HeJ (C3H)
42-53 DFTPVCTTELGR	652.8200	1303.6400	-34	Dehydroala	O	O
	669.8029	1337.5912	0	·	X	O
	682.2950	1362.5910	25	Cyano	X	O
	693.7725	1385.5304	48	Sulfonic acid	X	O
	705.3291	1408.6436	71	Acrylamide adducts of Cys-SH	O	O
	713.3170	1424.6330	87	Acrylamide adducts of Cys-SOH	O	O
	721.3160	1440.6310	103	Acrylamide adducts of Cys-SO_2H	X	O
	736.7880	1471.5760	134	+ 134 Da (unknown)	X	O
	744.7990	1487.5980	150	+ 150 Da (unknown)	X	O
	811.8730	1621.7460	284	+ 284 Da (unknown)	O	X



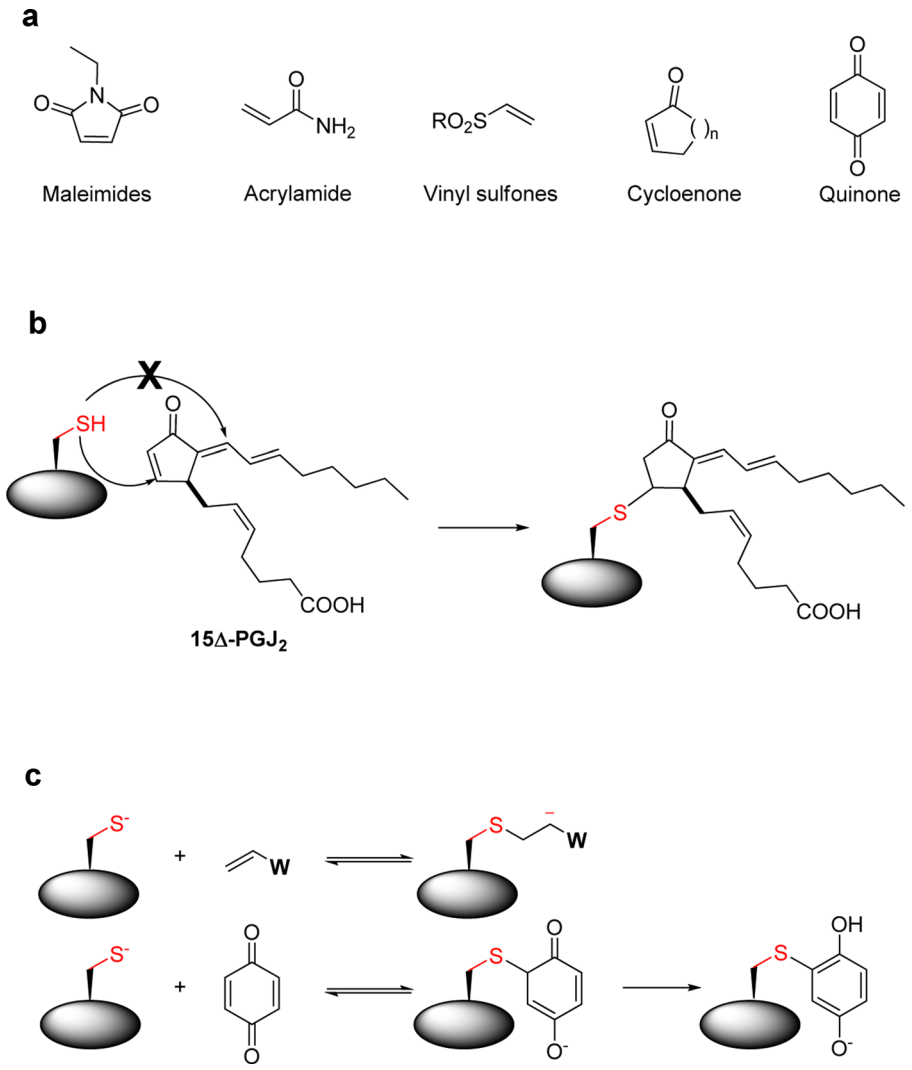
SCHEME 1. Mechanisms of lipidation reactions in (**a**) S-palmitoylation, (**b**) S-prenylation (S_N1 type reaction), (**c**) Capturing cysteine probe (S_N2 type reaction), and (**d**) Disulfide bond formation (S_N2 type reaction).

exo-β-position and more positive charge develops at the endo cyclic β-position (Suzuki et al., 1997). This selectivity also reflects the reversible nature of Michael type addition reaction. The Michael acceptors undergo addition reactions with Cys reversibly and, depending on the nature of Michael acceptor, the addition reaction could be less efficient or less selective as the Cys probe. Quinone type Michael acceptors undergo Michael addition reaction followed by the keto-enol tautomerization that forms hydroquinones irreversibly.

C. Oxidation of Cys

Cys is susceptible to oxidative stress as its thiol can be readily oxidized initially to sulfenic acid or mostly to disulfide through several oxidation routes reversibly (Scheme 3).

Most Cys residues in protein are deeply buried by other amino acids under hydrophobic environment. Exposed Cys residues interact with hydrogens of water molecules and various polar groups on protein surfaces. These interactions can have effects on the pK_a value of Cys. The reactivity of Cys, especially its oxidative transformation, is therefore largely determined by the protein's structural environment and the pK_a value of Cys residue (Brandes, Schmitt, & Jakob, 2009). Cys reactivity to electrophiles is associated to the deprotonation step of Cys residue, generating deprotonated form, thiolate (RS^-). Under basic conditions, Cys generates thiolate (RS^-) which reacts faster with ROS (i.e., hydrogen peroxide, H_2O_2) through nucleophilic reaction and thus Cys residues having lower pK_a values are oxidized faster (Winterbourn & Metodiewa, 1999; Marino & Gladyshev, 2012). Nevertheless, chemical oxidation



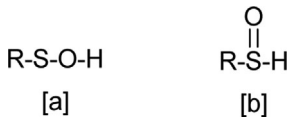
SCHEME 2. Mechanism of addition reaction (**a**) Michael acceptors of Cys, (**b**) selective addition to 15Δ-PGJ_2, (**c**) reversibility of the Michael addition reaction.

rates remain low when the pK_a is low because of a high activation barrier. Also how H_2O_2 oxidizes target Cys is not clear (Roos & Messens, 2011). Moreover, definitive protocols are needed to predict pK_as of Cys residues.

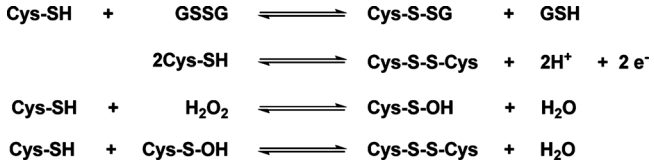
Sulfenic acid formed by oxidative stress creates a plethora of further transformations into higher oxidation forms and other Cys derivatives, some are reversible by reductive enzymes (Scheme 4).

1. *Sulfenic Acid and Related Oxidation Products*

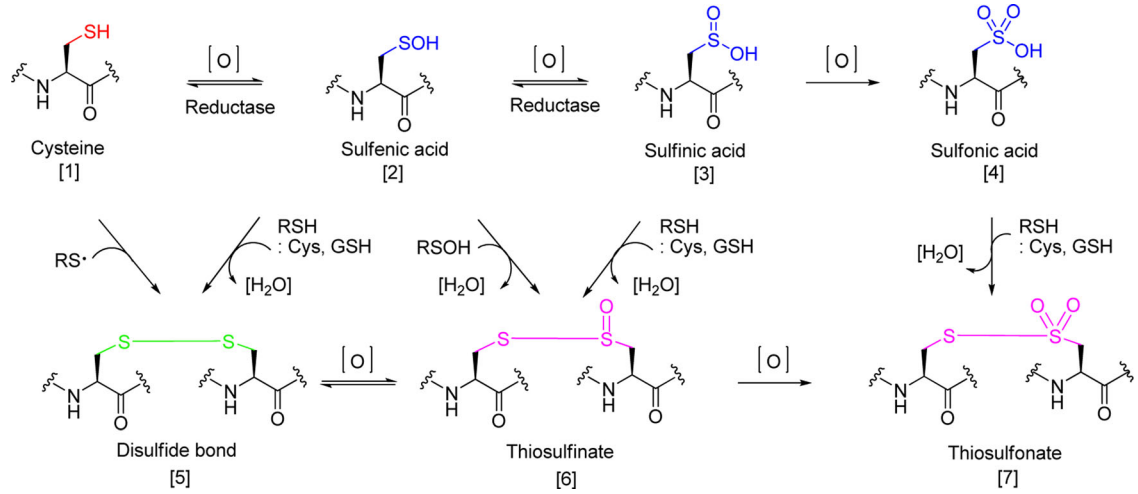
Cys thiol group (oxidation state of $S=-2$) can be oxidized by H_2O_2 or other available ROS to the sulfenic acid (**[1]**, -SOH, oxidation state of $S=0$). The sulfenic acid can be further oxidized to the higher oxidation state, sulfinic acid (**[2]**, -SO_2H, oxidation state of $S=+2$), and sulfonic acid (**[3]**, -SO_3H, oxidation state of $S=+4$). Sulfenyl Cys which is deprotonated by base plays the crucial role for the oxidation of thiol to sulfenic acid (Nagahara et al., 2009). Wood, Poole, and Karplus (2003a) proposed that, in peroxiredoxin, Cys thiol was deprotonated to form the highly reactive thiolate that is stabilized by the arginine residue and solvent (e.g., water). The thiolate undergoes nucleophilic attack on the peroxide to generate sulfenic acid. The sulfenic acid has two tautomeric structures. One has the proton attached on the oxygen [a] and the other has the proton attached on the sulfur [b].



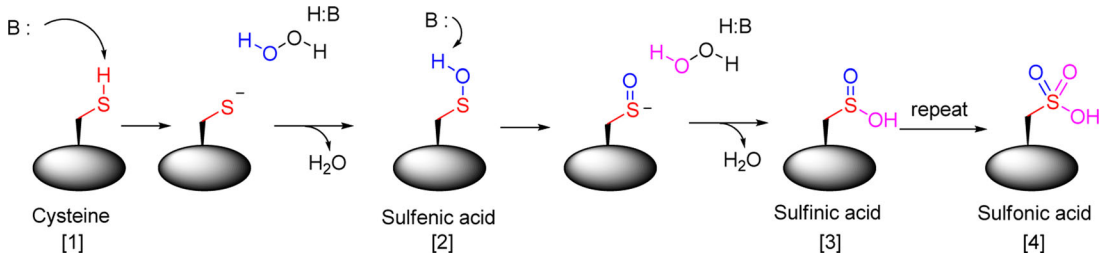
Mostly sulfenic acids exist as the R-SOH form where the proton is attached to the oxygen atom (Penn, Bolock, & Revelle, 1978) that allows the sulfur atom of the sulfenic acid to



SCHEME 3. Redox transformation of Cys.



SCHEME 4. Oxidative Cys thiol modifications in nature.



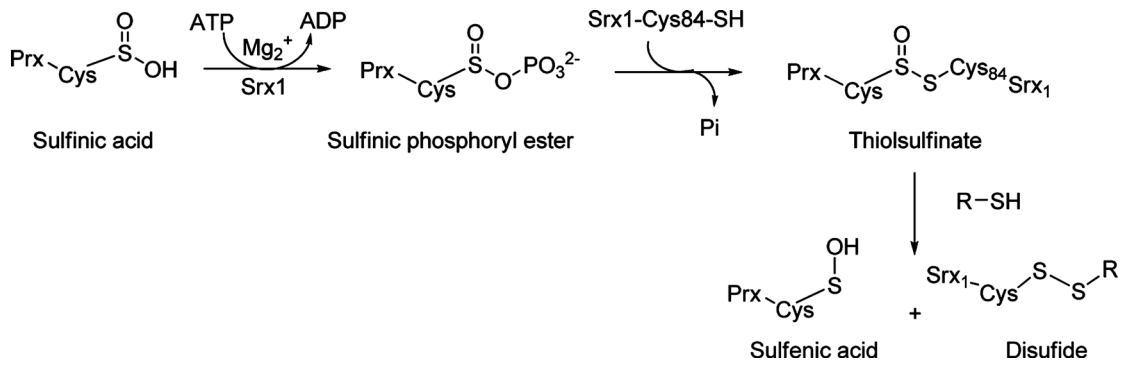
SCHEME 5. The mechanism of sulfenic acid and hyperoxidation by hydrogen peroxide.

serve an electrophile as well as a nucleohile. Sulfenic acid is generally unstable and is further oxidized to a stable sulfinic acid **[3]**, or forms a disulfide **[5]** with a nearby thiol group (Nagahara et al., 2009). The detailed mechanism of the oxidation was believed to require the nucleophilic attack of Cys-thiolate anion to the sulfur atom of sulfenic acid. The anion of sulfenic acid **[2]** that can be generated by base treatment reacts with peroxide to produce sulfinic acid **[3]**. In the same manner, sulfinic acid can be oxidized to sulfonic acid **[4]** (Scheme 5).

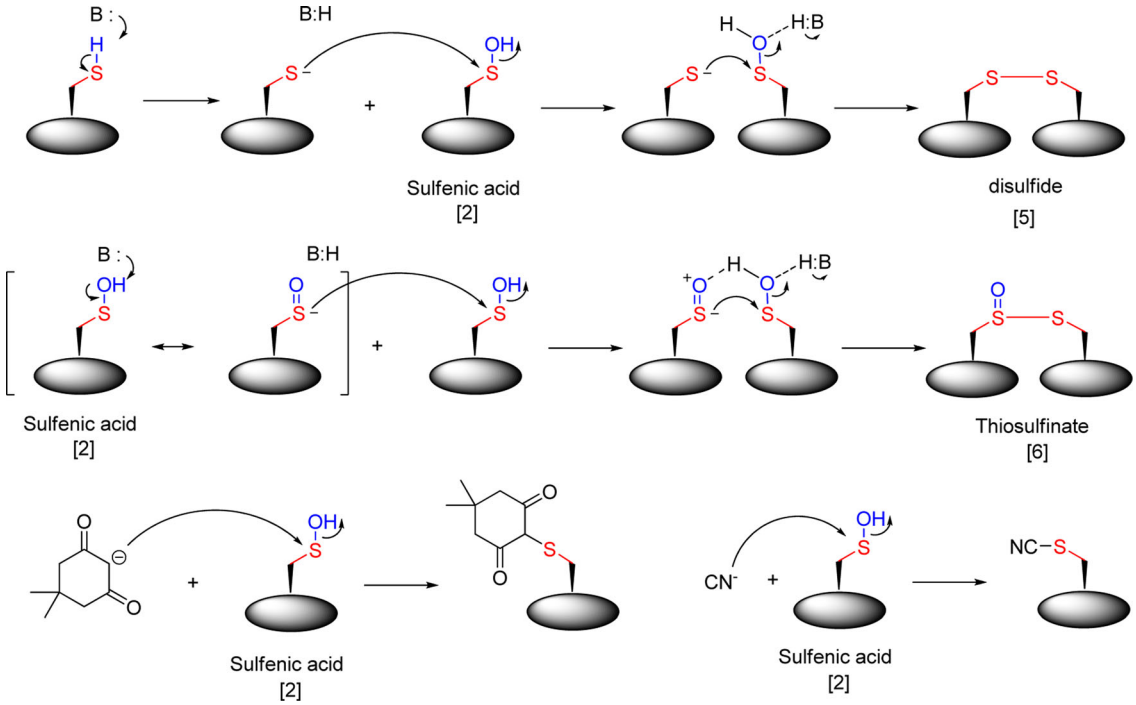
Initially, formation of Cys-sulfinic acid **[3]** and sulfonic acid **[4]** has been thought to be biologically irreversible. However, Cys-sulfinic acid can be reduced by sulphiredoxin (Srx1) that involves activation by phosphorylation followed by a thiol-mediated reduction step (Scheme 6, Biteau, Labarre, & Toledano, 2003). On the other hand, formation of sulfonic acid in biological systems is clearly irreversible (Poole, Karplus, & Claiborne, 2004).

Sulfenic acid can be an electrophile. The sulfur atom of sulfenic acid can act as an electrophile as well as a nucleophile. This characteristic is key to the formation of disulfide bond and thiosulfinate, detected in NDPK by MS **[6]** (Scheme 7, Jeong et al., 2011). The Cys-SOH can also react with other nucleophiles such as dimedone or cyanide.

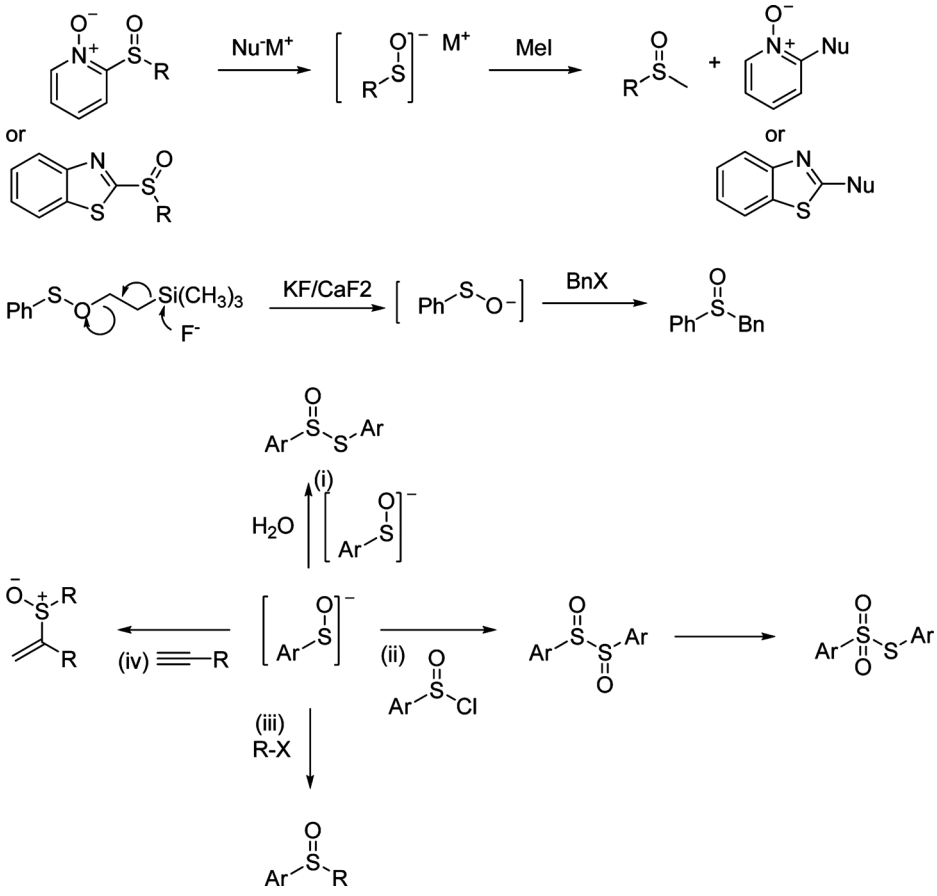
Sulfenic acid reacts with endogenous thiols, such as glutathione or other Cys residues, intra- or intermolecularly



SCHEME 6. A Hypothetical model for the reduction of the Cys-sulfinic acid by Srx1.



SCHEME 7. Mechanism of sulfenic acid reactions.



SCHEME 8. Generation and reactions of sulfenic acids.

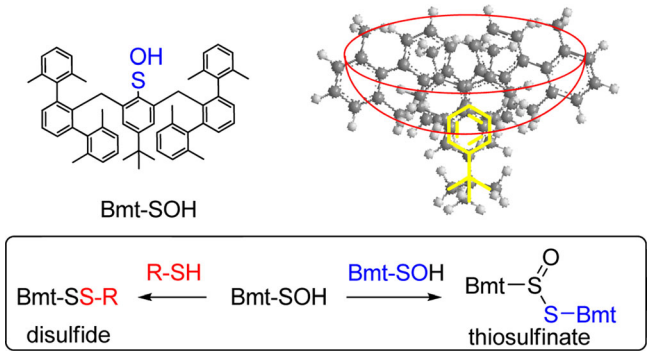
(Wood et al., 2003b). Thus disulfide bond can be formed by ROS and other cellular oxidants.

Sulfenate anions can act as weak nucleophiles as well as soft electrophiles. The Cys sulfenic acid under basic environment can attack another electrophilic sulfenic acid to form Cys thiosulfinate **[6]**. Similarly, Cys sulfinic acid can react with sulfenic acid to produce Cys thiosulfonate **[7]** (Jacob et al., 2003). The same modifications can be obtained through oxidation of disulfides by oxidants such as hydrogen peroxide or singlet oxygen (Finley, Wheeler, & Witt, 1981; Clennan et al., 1997). The nucleophilicity of sulfenic acid is influenced by its pK_a value. The pK_a of a sulfenic acid, estimated to be in the range of 4.5–12.5, is regulated by neighboring basic amino acids of the active site Cys in the protein (Paulsen & Carroll, 2013). Treating substituted sulfenates with reactive alkyl halides (e.g., MeI, BnBr) ensures alkylating forms outcome (Furukawa et al., 1992).

Scheme 8 depicts the generation and reactions of sulfenic acids as discussed previously (Vinkler & Klivényi, 1973). It was shown that sulfenate anions react: (i) in water to generate thiosulfinates; (ii) with arylsulfinyl chlorides to give thiosulfonates after rearrangement; (iii) with alkyl halides to give sulfoxides; and (iv) with enynes to give sulfinyldienes (Aversa et al., 1992).

Previous studies have not been able to elucidate the exact mechanism of these modifications *in vivo* because the Cys sulfenic acid, thiosulfinate and thiosulfonate are reactive and thus are not easy to isolate or detect. With MS/MS analysis, chemical adducts of sufenic acid with IAA, NEM, and acrylamide were identified as were those of Cys-SH as shown in Table 1 (Jeong et al., 2011).

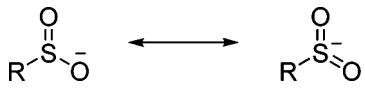
The high reactivity of Cys sulfenic acid prevents its isolation or *in vitro* studies of Cys sulfenic acid. Goto designed and synthesized a model system that contains a sulfenic acid sterically protected by bowl shaped environment (Bmt-SOH)



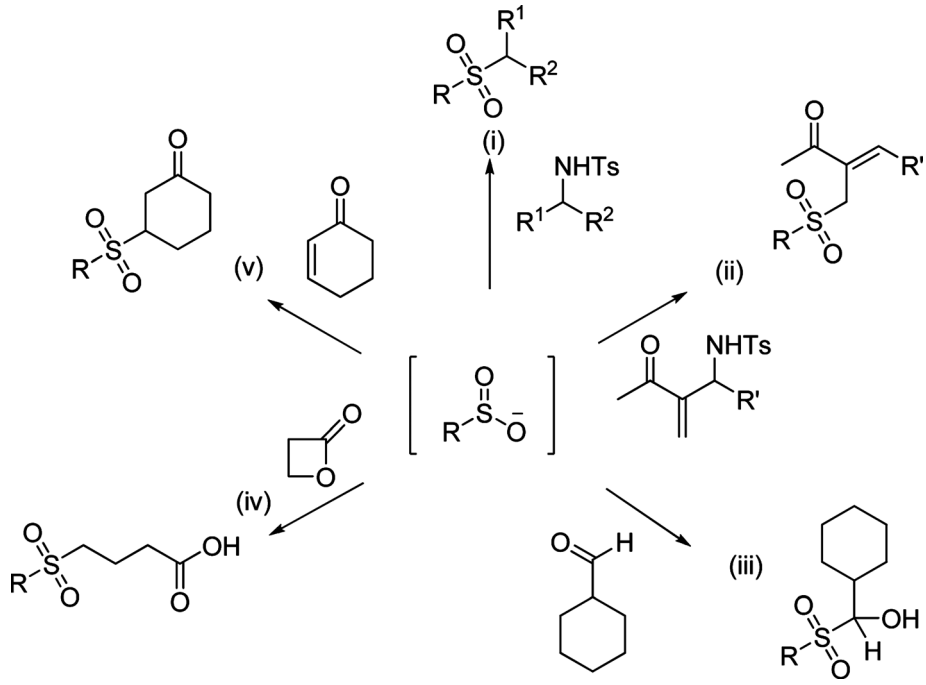
SCHEME 9. Reactions of sulfenic acid.

that is stable enough to permit study the reactions of sulfenic acid (Goto, Holler, & Okazaki, 1997). Both the formation of the symmetrical disulfide (Bmt-S)$_2$ from Bmt-SOH and Bmt-SH, and self-condensation reaction of two Bmt-SOHs that generate thiosulfinate were demonstrated (Scheme 9). *In vivo* thiosulfinate was detected in NDPK by MS/MS combined with DBond algorithm (Jeong et al., 2011).

Sulfinic acids, in contrast to sulfenic acids, are fully deprotonated at physiologic pH (p$K_a = \sim 2$), and are relatively stable. Sulfinic acids can exist in two resonance forms.



The sulfur atom of sulfinate anions can undergo nucleophilic addition to electrophiles to generate sulfone thermodynamically more stable than sulfinate ester (Baiday, Kobayahsi, & Mayr, 2010). Similarly, sulfur of a sulfinic acid attacks (i) carbocation to undergo S_N1 reaction (alkylation), as well as



SCHEME 10. Reactions of sulfinic acids.

(ii) activated terminal alkene (Liu et al., 2009), (iii) aldehydes, (iv) lactones (Gresham et al., 1952), (v) α,β-unsaturated compounds (Ogata, Sawaki, & Isono, 1969) (Scheme 10). Therefore, chemical adducts of sufinic acid with IAA, NEM, and acrylamide were also detected by MS/MS as shown in Table 1 (Jeong et al., 2011).

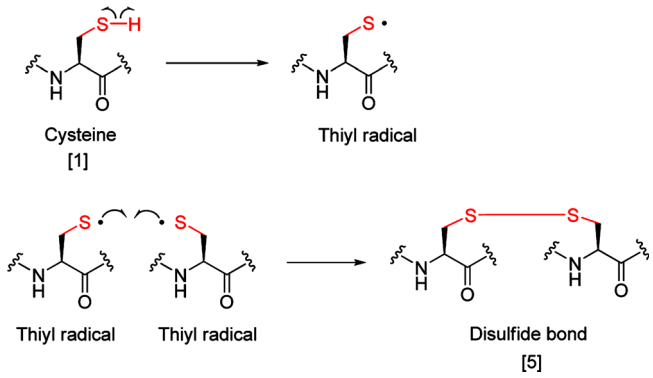
2. *Disulfide*

Disulfide is the main oxidation product of Cys and its formation is necessary to change protein structures by oxidation. However, direct identification of disulfide bond was not possible. A recent study showed that MS/MS analysis using DBond algorithm makes it possible to directly identify the crosslinked peptides (Choi et al., 2010). Structural changes by disulfide formation in NDPK were characterized by hydrogen/deuterium exchange with MS (Kim et al., 2013). Disulfide bond is known to be generated by nucleophilic attack of Cys thiolate leading to the formation of sulfenic acid, but there are several possible pathways to produce disulfide bonds from Cys thiol.

Cys thiolate can undergo nucleophilic attack on the disulfide bond, to form a new disulfide bond, as in GSSG (G = Glutathione) for example. The initial disulfide works as the electrophile. Thiosulfinate and thiosulfonate are more reactive electrophiles forming disulfide with the Cys thiolate (Scheme 11).

Cys thiol can also be oxidized through typical radical pathways. The thiyl radical is generated via single-electron transfer and hydrogen-atom transfer reactions. The thiyl radical is highly unstable as it instantly dimerizes to form a disulfide bond (Scheme 12). This thiol/thiyl radical redox coupling reaction occurs in enzymes, such as the ribonucleotide reductase (RNRase) (Jacob et al., 2003).

Also, in sulfonucleotide reduction, Cys-thiosulfonate (E-S-SO_3^-) is attacked by Cys-thiolate generating disulfide bond (Carroll et al., 2005). This reaction occurs in TRX. This

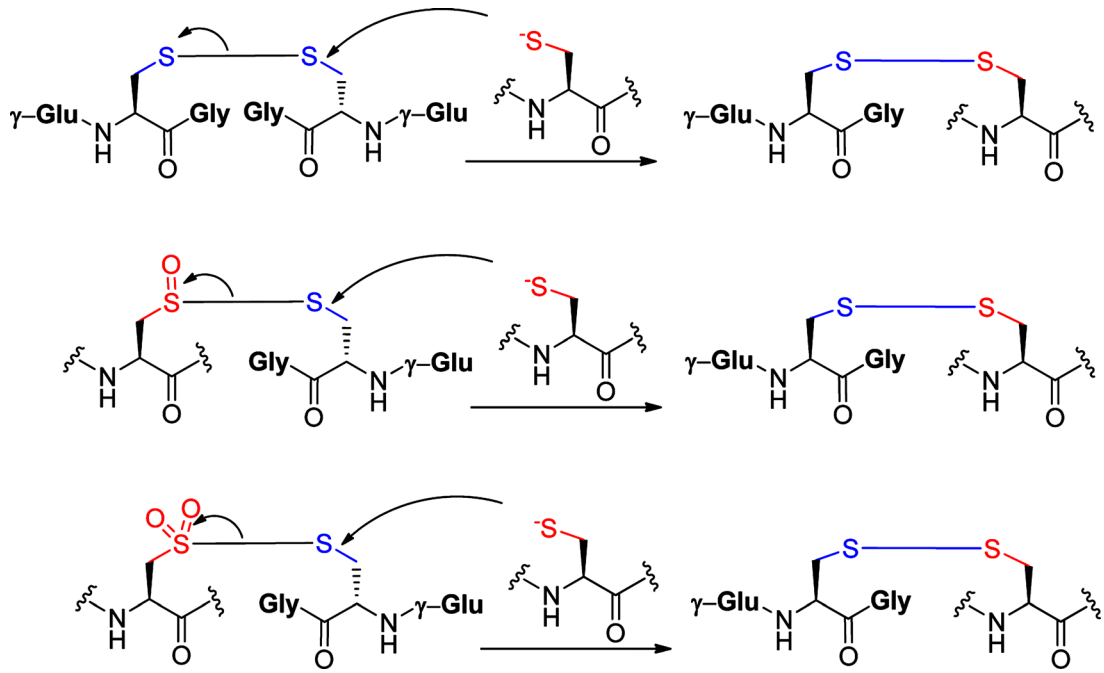


SCHEME 12. Radical pathway to disulfide. Radical is formed by single-electron transfer pathway.

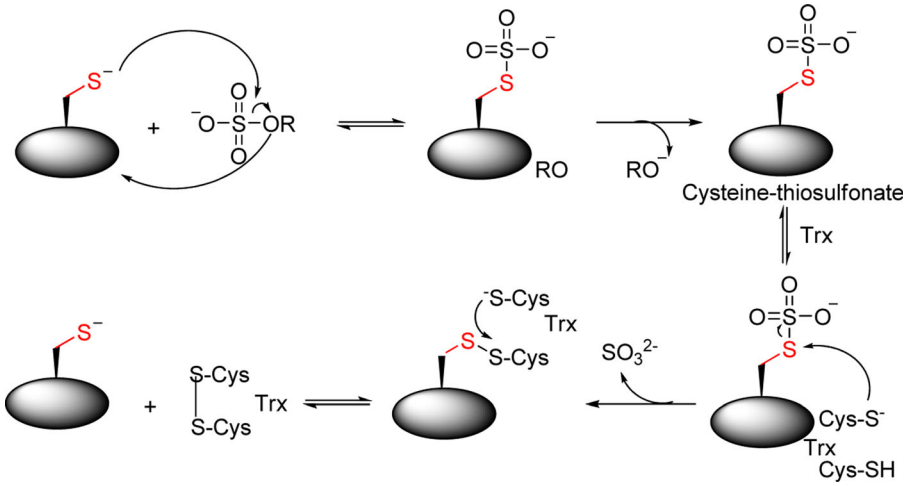
mechanism can be divided into two steps. In the first step, an enzyme-thiosulfonate intermediate is produced by nucleophilic attack of Cys residue to a sulfate compound. In the second reaction, more reactive N-terminal TRX Cys would carry out nucleophilic attack upon the sulfonucleotide reductase catalytic Cys. This reaction would result in the formation of a mixed disulfide between TRX and the sulfonucleotide reductase and concomitantly, release sulfite. Subsequent thiol/disulfide exchange with the second TRX thiolate would yield oxidized TRX and regenerate reduced sulfonucleotide reductase. Evidence for this mechanism has not been obtained yet (Holmgreen, 1989) because it involves it's a highly fast reaction (Scheme 13).

D. Elimination Reaction

Dehydroalanine (DHA) at Cys residue ($\Delta m = -34$ Da) has been found by MS/MS in many proteins after oxidation (Jeong

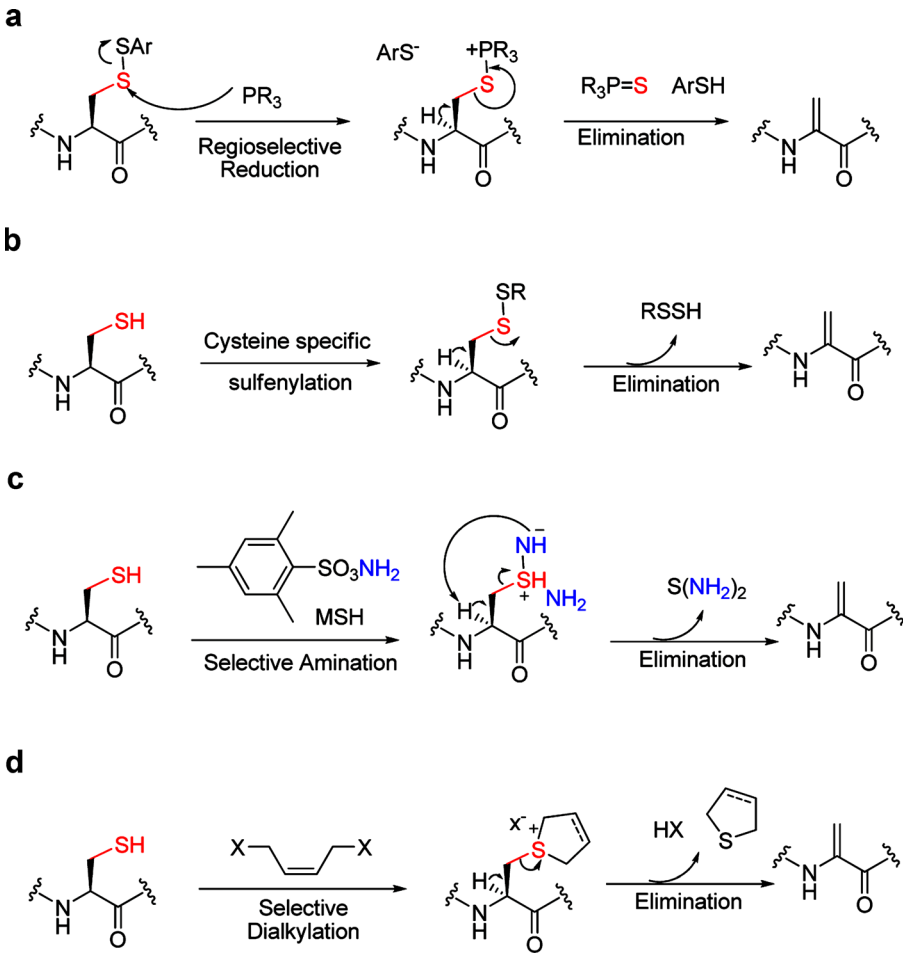


SCHEME 11. Nucleophilic reactions of thiolate to form disulfide.

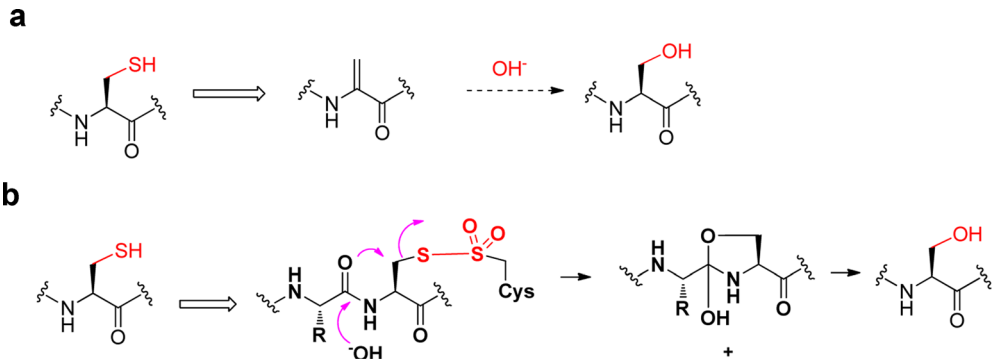


SCHEME 13. A Plausible mechanism for sulfonucleotide reduction in enzymes (Carroll et al., 2005).

et al., 2011; Jeong, Lee, & Lee, 2012), and also in lanthionine-containing antibiotic peptides-lantibiotics where it is formed by the enzymatic dehydration of serine (Willey & van der Donk, 2007). Synthetic DHA is a useful chemical precursor to a range of PTMs and their analogues by the conjugate addition of a thiol. Four alternate chemical routes are possible for Cys to be converted to DHA: the reduction-elimination of Cys disulfides, base-mediated elimination of Cys disulfides and related



SCHEME 14. Four alternate modes of elimination of Cys to dehydroalanine (DHA) (**a**) reduction-elimination of Cys-disulfides to DHA, (**b**) base-mediated elimination of Cys-disulfides to DHA, (**c**) oxidative elimination of Cys to DHA, (**d**) bis-alkylation elimination of Cys to DHA.



SCHEME 15. Plausible pathways to Ser from Cys, (**a**) Addition of OH- to DHA, (**b**) Intramolecular substitution of thiosulfinate to Ser.

derivatives, oxidative elimination of Cys, and the Bis-alkylation-elimination of Cys (Chalker et al., 2011; Scheme 14). None of these modes of elimination was found in natural Cys dehydration process. The reduction-elimination of Cys disulfide mechanism is mediated by electron rich phosphine. Phosphine attacks the disulfide bond. The phosphonium salt can undergo elimination to give DHA. Wang, Zhang, and Xian (2009) observed such eliminations when on treating with tris-(dimehtylamino)phosphine. Under basic conditions, using Ellman's reagent disulfide bond is eliminated. However, disulfide can change sulfinic acid in the presence of hydroxide and this method is limited to organic solvents. Therefore this method is not suitable for protein modification. Instead Mukaiyama's reagent is well-suited for generating DHA and the yield is moderate. This method can proceed under highly basic condition (>pH 10). Oxidative elimination of Cys to DHA using *O*-mesitylenesulfonylhydroxylamine (MSH) is another method that avoids using basic condition reactions. MSH can selectively react at Cys and this selectivity allowed conversion of Cys to DHA under a combination of factors such as high nucleophilicity of Cys, the pH, and differential accessibility of side chains. However, use of MSH can generate side reactions with other amino acids (nucleophilic amino acid residues, Asp, Glu, Met, Lys, and His). To avoid side reactions, bis-alkylation-elimination of Cys procedure was employed.

Bis-alkylation-elimination is complicated by some variables: leaving group identity, nature of the intermediate (cyclic sulfonium including ring size) and solubility in water. After several experiments, it was found that 1,4-dialkylating reagent is the best to lead to the formation of the cyclic tetrahydrothiophenium intermediate as precursor to DHA. This method was successfully used with three model proteins, including an enzyme and antibody, using reagent 1,4-dihlaobutane derivatives from adipic acid, on the other side, above three methods have problems using high pH condition.

Detection of DHA at Cys residue ($\Delta m = -34$ Da) by MS/MS often is accompanied by appearance of Ser at Cys residue (Jeong et al., 2011; Jeong, Lee, & Lee, 2012). Initially, it was believed that the Ser is produced from DHA by the Michael addition of a hydroxide nucleophile (Scheme 15). However, the addition reaction, contrary to the conventional understanding, was not facile enough to form Ser. Therefore, a mechanism other than using DHA as the precursor to Ser was required and one plausible pathway was proposed (Jeong et al., 2011). One of the oxidative modification products of Cys, thiosulfinate could undergo intramolecular substitution reaction to produce Ser through the formation of an oxazolidine ring (Okamoto, Soumura, & Xian, 2009).

V. CONCLUSIONS

ROS plays key roles in various signaling pathways related to health and disease; its homeostasis inside cells is well balanced by controlled production and elimination of ROS and regulation of the signaling pathways. Disturbances in balancing of ROS homeostasis induce disease states. However, target proteins of ROS, depending on their location, amount and timing of ROS generation, and its regulation mechanisms are not well understood. In this review, currently available proteomic technologies including MS/MS analysis, searching algorithms to identify the PTMs, newly identified Cys modifications, and fundamental chemistry of Cys residues are illustrated. Further identification of ROS target proteins, target Cys sites, and modified species and the quantitative analysis of Cys employing new proteomic technologies, is unveiling the biological functions of these modifications. This should help understand the modes of action of ROS at molecular levels. We proposed the term "ROSics" for the science describing chemistry and proteomics of Cys modifications in redox biology and the principles of mode of action of ROS at molecular levels. We hope "ROSics" will be accepted and further validated by establishing -omics technologies that help identify the molecular changes inside cells that occur in response to ROS, leading to biological validation of target proteins as a key switch proteins and to studies of the regulation dynamics and basic principles of cellular responses.

ACKNOWLEDGEMENTS

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