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Discovering mechanisms of signaling-mediated cysteine oxidation

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Summary

Accumulating evidence reveals hydrogen peroxide as a key player both as a damaging agent and, from emerging evidence over the last decade, as a second messenger in intracellular signaling. This rather mild oxidant acts upon downstream targets within signaling cascades to modulate the activity of a host of enzymes (e.g. phosphatases and kinases) and transcriptional regulators through chemoselective oxidation of cysteine residues. With the recent development of specific detection reagents for hydrogen peroxide and new chemical tools to detect the generation of the initial oxidation product, sulfenic acid, on reactive cysteines within target proteins, the scene is set to gain a better understanding of the mechanisms through which hydrogen peroxide acts as a second messenger in cell signaling.

Introduction

With the emerging understanding that hydrogen peroxide generation is a critical component of numerous receptor-mediated cell signaling events, investigators are seeking to identify, within given signaling networks, the particular oxidation-sensitive proteins, residues, and molecular events that lead to the modulation of cell signaling networks by hydrogen peroxide. We discuss herein some of the rapidly evolving chemical tools and experimental approaches being developed both for detecting oxidant generation and for identifying the molecular targets modified by the oxidants. We also summarize recent data supporting cysteine sulfenic acid as a key intermediate in the functional modulation of enzymes and transcription factors involved in peroxide-mediated cell signaling.

Localization and control of hydrogen peroxide for signaling

Reactive oxygen species (ROS) are produced in cells as a result of the partial reduction of oxygen by the electron transport chain in mitochondria, by lipoxygenases in the cytoplasm, and by flavoprotein oxidases in peroxisomes. It is increasingly appreciated that cytokines, growth factors and integrins also activate multicomponent NADPH oxidase enzymes (Nox) as part of the cell's receptor-mediated signaling responses. Both superoxide and, hence, hydrogen peroxide are generated, with the latter serving a particularly significant role as a second messenger in signaling pathways. Targeting of Nox proteins to specific subcellular compartments, including lipid rafts within membranes, endosomal signaling vesicles and other

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cellular organelles, may serve to offset the highly diffusible nature of hydrogen peroxide and limit the oxidation signal to these microenvironments [1–3]. Consistent with this, evidence is accumulating that antioxidant enzymes that serve to control intracellular peroxide levels, including glutathione peroxidases, catalases and especially peroxiredoxins [4], may themselves be regulated by these oxidants, allowing a threshold concentration of peroxide to accumulate around the site of Nox localization while preventing damage to cellular components outside these regions (reviewed in [5]).

Transient generation of localized ROS is important in receptor-mediated cell signaling, yet the ability to detect ROS with a high degree of spatial and temporal resolution remains a challenge [6]. Oxidation of widely used 2′,7′-dichlorodihydrofluorescein (DCFH) and dihydrorhodamine 123 (DHR) reagents as detected by enhanced fluorescence gives a sense of the ROS burst, but these reagents exhibit a relative lack of specificity toward their oxidants, and are also subject to autoxidation and photo-oxidation. The recent design and synthesis of new, highly selective chemical sensors of hydrogen peroxide, particularly those which become fluorescent upon peroxide-mediated removal of a boronate-based protecting group, is a very exciting development in this regard [7]. These and other new ratiometric, protein-and/or nanoparticle-based reagents for peroxide sensing [8–10] are promising tools for continuously monitoring intracellular peroxide generation, yet will need to overcome limitations such as irreversibility and relatively slow rates of reaction to achieve the level of sensitivity required for “instantaneous” and nonperturbing detection of signaling-relevant hydrogen peroxide (for recent reviews, see [11,12]).

Cysteine redox chemistry

It is increasingly clear that a major component of the ROS-linked modulation of cell signaling pathways is the dynamic regulation of protein function by reversible thiol modification. For many proteins, the first product of cysteine oxidation by hydroperoxides, cysteine sulfenic acid (R-SOH) (Figure 1), undergoes rapid condensation either with another protein thiol (as an intra- or intermolecular interaction) or with a small molecule thiol like glutathione or cysteine to form a disulfide bond. Thiol-based reductants can then return this species to the fully reduced thiol state (Figure 1). Disulfide bonds can stabilize extracellular proteins, protect against irreversible inactivation, stabilize associations within protein complexes, modify structures to create, destroy or modulate functional sites, and ultimately regulate enzymatic or transcriptional activity of proteins. Under conditions where reactive nitrogen species (RNS) are generated, signaling through reversible S-nitrosation, perhaps in addition to the typical ROS-generated products, may also play an important role [13]. The features of the protein microenvironment that control thiol reactivity and stability of the resulting modifications are beginning to be analyzed [14].

In certain cases, the initial sulfenic acid may itself be stabilized within the protein microenvironment. This does, however, potentially leave the protein sulfenic acid vulnerable to hyperoxidation to the largely irreversible sulfinic or sulfonic acids (Figure 1), further modifications that could, in spite of their typically irreversible status, also provide a signaling role analogous to a “fire alarm” regarding cellular redox status [15].

Sulfenamide (sulfenyl-amide) has recently been recognized as another stabilized oxidation product generated through condensation of the sulfenic acid with a neighboring backbone amide nitrogen (Figure 1); this 5-membered ring structure appears to represent yet another form of oxidized cysteine resistant to hyperoxidation. The initial evidence for this species was crystallographic [16], leaving some doubt as to the relevance of this species in a biological setting. On the other hand, a functional role for the generation of such a species is suggested by the profound changes in structure imparted to the active site of protein tyrosine phosphatases

with this modification, changes which may enhance access by reductants and/or modulate interactions with other domains ([17]; reviewed in [18]). Studies using model chemistry have also better defined the parameters which promote this cyclization reaction in the constrained setting of a protein active site [19]. Furthermore, recent studies of the peroxide sensitive transcriptional regulator, OhrR from *Bacillus subtilis*, support stable formation of the sulfenamide species in solution and possibly *in vivo* for this protein [20].

Redox regulated peroxiredoxin function

All of these examples of cysteine chemical states, with the possible exception of sulfenamides, are observed in the highly expressed peroxiredoxin enzymes (including recently-reported S-nitrosation [21]). These enzymes, which catalyze the reduction of hydroperoxide substrates via a catalytic cysteine at the active site, have been shown to exhibit high sensitivity (k_{cat}/K_m of ~10^7 M^{-1} s^{-1}) and specificity toward H_2O_2 in at least some cases [22,23], and their activity is regulated by phosphorylation, oligomerization, and oxidation state [5]. During the normal catalytic cycle, the peroxidatic cysteine becomes oxidized to a sulfenic acid, then undergoes one or more steps of inter- or intrasubunit disulfide bond formation and thiol-disulfide exchange with other thiols in order to return these enzymes to their reduced, activated state [24,25]. In the mammalian peroxiredoxins, hyperoxidation competes successfully with disulfide bond formation under certain conditions, causing these enzymes to be “turned off” at their sulfenic acid redox switch when local peroxide levels rise (Figure 2). The physiological significance of this hyperoxidation is supported by several observations. (1) The susceptibility of eukaryotic peroxiredoxins toward hyperoxidation, a characteristic not observed in their prokaryotic counterparts, is linked to the presence of particular evolved structural features within these enzymes [4,25]. (2) For at least some peroxiredoxins, hyperoxidation to sulfinic acid is reversible as catalyzed, through highly specialized chemistry, by specific enzymes called sulfiredoxins (and perhaps sestrins), enzymes which appear to have co-evolved with this sensitivity [26,27]. (3) Hyperoxidation of Prxs has been detected as a result of tumor necrosis factor signaling, suggesting at least a role for this modification in apoptotic signaling processes [28]. While hyperoxidation of peroxiredoxin proteins on a global level has yet to be detected during growth factor signaling, such modifications, if generated in localized regions around signaling complexes, may be difficult to detect within the large pool of cytoplasmic peroxiredoxins. There is no doubt that it is early in our understanding of when and how this functional hyperoxidation switch contributes to biological processes. Still, there is a clear link between peroxide sensing and transcriptional activation through the modulation of the peroxiredoxin redox state in several yeast species (see [5] for a review), and both cell cycle arrest [15] and enhanced chaperone activity of the peroxiredoxin proteins [29–31] have been reported as functional outputs of peroxiredoxin hyperoxidation. Interestingly, recent studies in macrophages have demonstrated that nitric oxide affects expression of peroxiredoxins and sulfiredoxin, as well as sensitivity of peroxiredoxins toward hyperoxidation, highlighting the intricate interplay between RNS and ROS regulated signal transduction [32].

Detection of reversibly oxidized cysteine residues in proteins

As a category of regulatory posttranslational modifications (PTMs) acting as switches for modulating protein function, oxidation is a newcomer to the field relative to phosphorylation, glycosylation, ubiquitination and acetylation. Given the rapidly expanding interest in this field, a number of methods for the proteome-wide detection of reversible thiol oxidation have been developed, with most relying on the use of thiol modifying agents and detection of either thiols lost during oxidation (which would include those undergoing irreversible oxidation), or those regained (after blocking of free thiols initially present) upon reversal of specific or general oxidized species by reductants (for reviews, see [33–35]). Such approaches have been the basis for “biotin switch” methods to detect either S-nitrosation based on selective ascorbate reduction

of these PTMs, or “sulfenation” (generation of sulfenic acid) based on selective arsenite reduction [36]. The successful use of such approaches requires not only comprehensive blocking of all free thiols at the beginning of the procedure, but also an absolute specificity of the reductant used for the targeted PTM, and a stability of that PTM through a multi-step procedure, all aspects which may limit the utility of such methods.

Alternative methods have been developed very recently which allow for the detection of sulfenic acid modifications on proteins based on the distinct chemical attributes of this species. Detection of sulfenic acids in proteins has the advantage of targeting the direct protein product of cysteine modification by hydrogen peroxide, lipid hydroperoxides and peroxynitrite; a potential disadvantage is that the lifetime of the sulfenic acid moiety may be quite short for many proteins, requiring a rapid chemical trapping procedure to detect such species. Another important advantage to the detection of the sites of sulfenic acid formation as one readout of protein oxidation is the identification via this method of the actual reactive site where the oxidation chemistry is initiated, whereas detection of downstream products may involve a great deal more complexity and would not necessarily identify the initial site of attack.

Although most of the new methods described here rely on small molecule probes for detecting sulfenic acid formation, one very recent article also reported the utility of using an engineered, sulfenic acid-sensing module of a yeast transcription factor, Yap-1, to detect protein sulfenic acid formation [37]. NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) has been used in the past to distinguish sulfenic acids from thiols in pure proteins through UV-visible spectroscopy or mass spectrometry, but this labeling agent reacts with both thiols and sulfenic acids and is readily removed with dithiothreitol, thus limiting its utility for proteomics-based analyses [38]. On the other hand, dimedone irreversibly alkylates oxidized cysteine residues and has been used as a sulfenic acid specific modifying agent for years. Consequently, several newly developed methods have enabled the linkage of fluorescent [39,40] or affinity-based probes [39,41] to a dimedone-like core reagent to allow for the incorporation of detectable labels into proteins at their sulfenic acid sites (Figure 3). Initial results regarding the specificity of the reagents [40], as well as their detectability, rates of labeling and utility in cells and tissues [39,41,42], suggest that these will be highly useful chemical tools for addressing the biological relevance of cysteine oxidation in many systems in the future.

Selected new examples of functional protein oxidation

The activities of many proteins in signaling pathways have been shown to be upregulated or downregulated in reponse to ROS, but many such reports lack clarity as to which protein, and especially which cysteine residue(s) within that protein, is/are actually sensitive to oxidants. We compile here a limited selection of recent reports describing new data for transcription factors and enzymes for which cysteine oxidation has been either proven or likely to occur through sulfenic acid formation and has recently been shown to have a functional effect on protein function (for other examples, see reference [43]).

Mechanistic details of the distinct molecular pathways for sensing organic hydroperoxides by a transcriptional repressor, OhrR, from two different organisms, *Xanthomonas campestris* **(***Xc*OhrR) and *Bacillus subtilis* (*Bc*OhrR), have recently been elucidated. Although both proteins initially generate sulfenic acid at their reactive cysteine (Cys22 in *Xc*OhrR and Cys15 in *Bs*OhrR), *Xc*OhrR quickly forms an intersubunit disulfide bond with Cys127 in that protein [44], whereas *Bs*OhrR has no other cysteine [20]. Instead, formation in the latter protein of either a mixed disulfide with low molecular weight thiols (S-thiolation) or a protein sulfenamide (Figure 1) is required to abrogate DNA binding activity and allow transcription at the target sites.

Protein tyrosine phosphatases were the first signaling-relevant enzymatic activities clearly shown to undergo, and be inhibited by, sulfenic acid formation through oxidation at the nucleophilic cysteine that forms the phosphoenzyme intermediate of PTP1B during catalysis [45] (recent reviews include references [18,46]). Recently, Rinna *et al.* showed that PTP-1B becomes glutathionylated in a rat alveolar macrophage cell line (NR8383) after either exposure to 100 μM exogenous hydrogen peroxide or ADP-stimulation of the respiratory burst [47]. Recent strong evidence for sulfenic acid formation and subsequent disulfide bond formation has been presented for MAP kinase phosphatase 3, a dual-specficity phosphatase which dephosphorylates Jun N-terminal kinases (JNK) [48]. Finally, in naïve T-cells stimulated with anti-CD3 and anti-CD28 antibodies, both SHP-1 and SHP-2 were shown to form sulfenic acid by trapping oxidized cysteines with DCP-Bio1 (Figure 3), immunoprecipitation of the protein of interest, and blotting with streptavidin to detect incorporation of biotin [42].

Another category of oxidation targets important in signaling are regulatory protein kinases which are typically activated through phosphorylation cascades. While sulfenic acid formation has yet to be proven, the activity of many kinases appear to be regulated by hydrogen peroxide-mediated oxidation of non-catalytic cysteines (reviewed in [49]). New mechanistic details of the effects of oxidation are also emerging in this area. For example, when the catalytic subunit of cAMP-dependent protein kinase (PKA) was treated with hydrogen peroxide, Cys199 formed a disulfide bond with either glutathione or another cysteine residue within the protein, leading to dephosphorylation of a critical proximal Thr residue (Thr197) and inactivation of the protein [50]. cGMP-dependent protein kinase (PKGIα) was shown to form an interpro tein disulfide bond between Cys46 on two subunits after treatment of isolated perfused rat hearts with hydrogen peroxide that correlated with an increase in kinase activity and cellular relocalization of PKGIα; neither response was observed in the C46S mutant. On the other hand, the non-receptor tyrosine kinase c-Abl was shown to be inactivated by modification of cysteine residues by thiol alkylating agents and S-glutathionylation [51]. Other kinases that have been shown to be regulated by cysteine modification include IKKβ and MEKK1 (reviewed in [49,52]).

Other enzymes whose activity has recently been shown to be modified through the initial formation of sulfenic acid formation include Cathepsin B and L, two cysteine-based proteases that lose 50% to 75% of their activity after treatment with 10 μM hydrogen peroxide or protein/peptide hydroperoxides [53]. Rat phospholipase A_2 ($iPLA_2\beta$) exhibited a H_2O_2-dependent loss of activity correlated with loss of free thiol groups; cysteine sulfenic acid formation was proposed based on mass spectrometric evidence, dithiothreitol reversibility and a reduced sensitivity to oxidative inhibition upon mutagenesis of Cys651 [54]. Reversible redox regulation was also observed for SUMO proteases from human (SENP1) and yeast (Ulp1) through oxidation at the active site cysteine [55]. Cysteine sulfenic acid, as well as sulfinic and sulfonic acids, were identified at the active site of yeast Ulp1 through crystallographic studies. Finally, a comprehensive study of sulfenic acid formation and reactivity toward a panel of thiol-containing small molecules and potential reductants was conducted recently for human serum albumin, the highly abundant blood protein known to be reactive toward hydrogen peroxide [56].

Conclusions

Progress in and continued development of new chemical tools to evaluate, with high spatial and temporal resolution, the generation of hydrogen peroxide in response to cell signaling events, as well as to identify sites and *in vivo* relevance of peroxide-mediated oxidation of enzymes and transcription factors, bodes well for expanding our understanding of molecular events in ROS-dependent signal transduction. This will have important implications in better defining both normal physiological and pathological processes, and will allow for the identification of new therapeutic targets based on better defined oxidative mechanisms.

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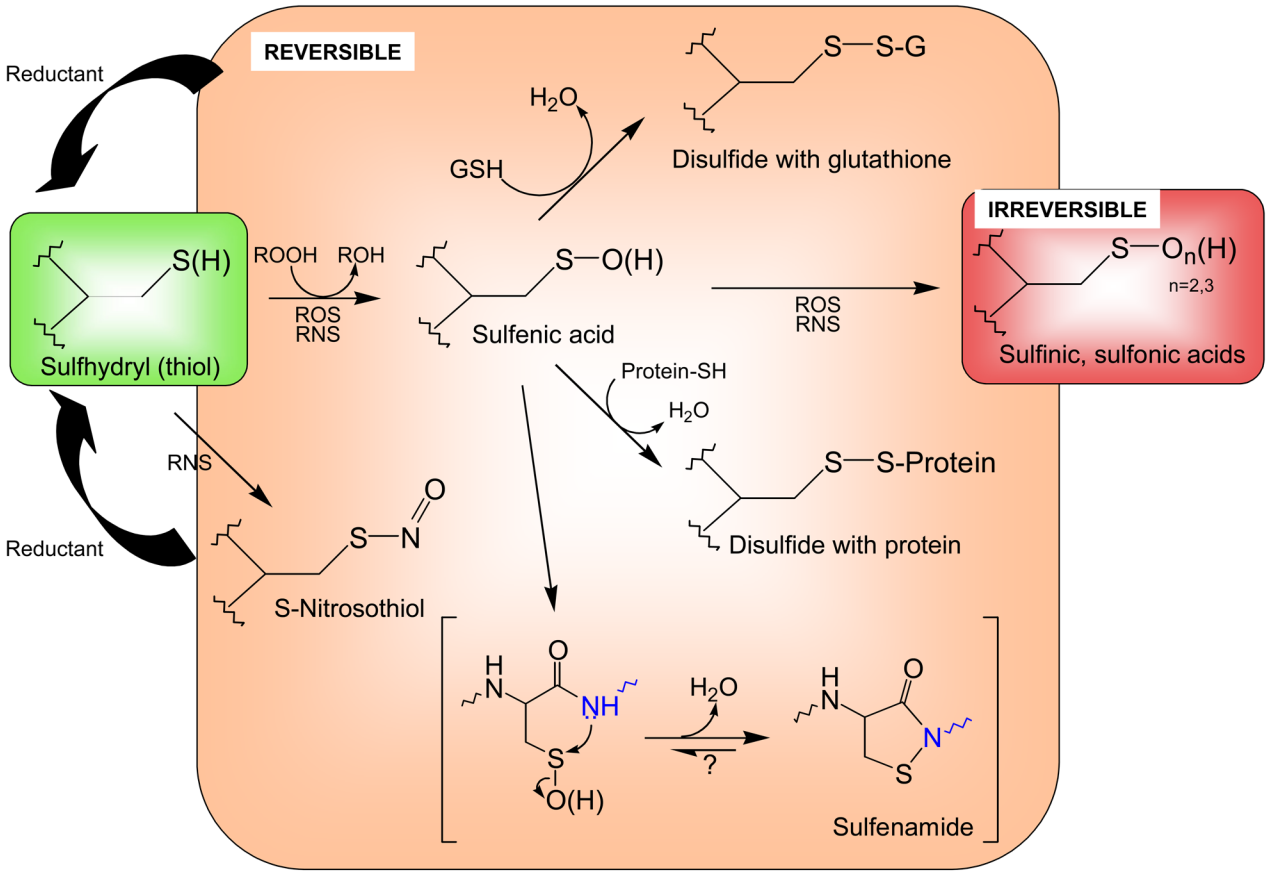

Figure 1. Biological modifications of cysteine thiols

Reactive cysteine thiols (green), typically in their ionized, thiolate form ($R\text{-}S^{-}$), are oxidized by such oxidants as hydrogen peroxide, organic hydroperoxides, hypochlorous acid and peroxynitrite to form sulfenic acids, which may be stabilized or go on to form other reversible (disulfides or sulfenamides, orange) or irreversible (sufinic and sulfonic acid, red) species. Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) promote these oxidations. Note that the generation of sulfenamide (bottom) involves attack of a neighboring amino acid's amide nitrogen (blue) on the sulfenic acid sulfur. Although sulfinic and sulfonic acids are shown here as irreversible modifications, recent discoveries show that some peroxiredoxins in this state can be recovered through action of specialized sulfinic acid reductases (sulfiredoxins).

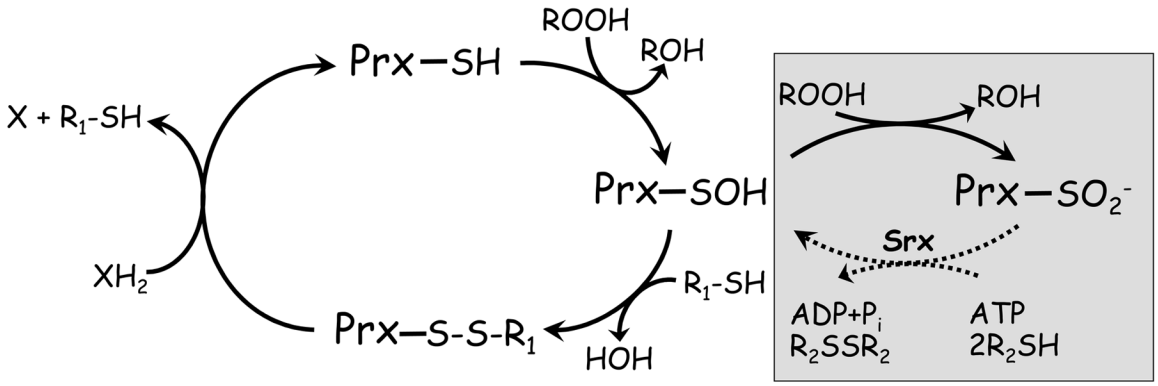

Figure 2. Peroxiredoxin catalytic and regulatory redox cycles

Peroxiredoxins (Prx) have in common the first step of catalysis whereby the active site cysteine thiol (in its thiolate form) attacks the hydroperoxide substrate, releasing the corresponding alcohol and the enzyme in its sulfenic acid (SOH) form. For catalytic recycling, a resolving cysteine (R_1-SH) in the same or another subunit typically forms a disulfide bond with the peroxidatic cysteine, and the enzyme is regenerated by small molecule or protein electron donors. The sulfenic acid can also act as a redox-sensitive switch, converting the enzyme to an inactive sulfinic acid form in the presence of excess hydroperoxide substrate (gray box). Enzymes called sulfiredoxins (and perhaps sestrins) can regenerate activity in some Prx through an ATP-dependent reduction (dotted line).

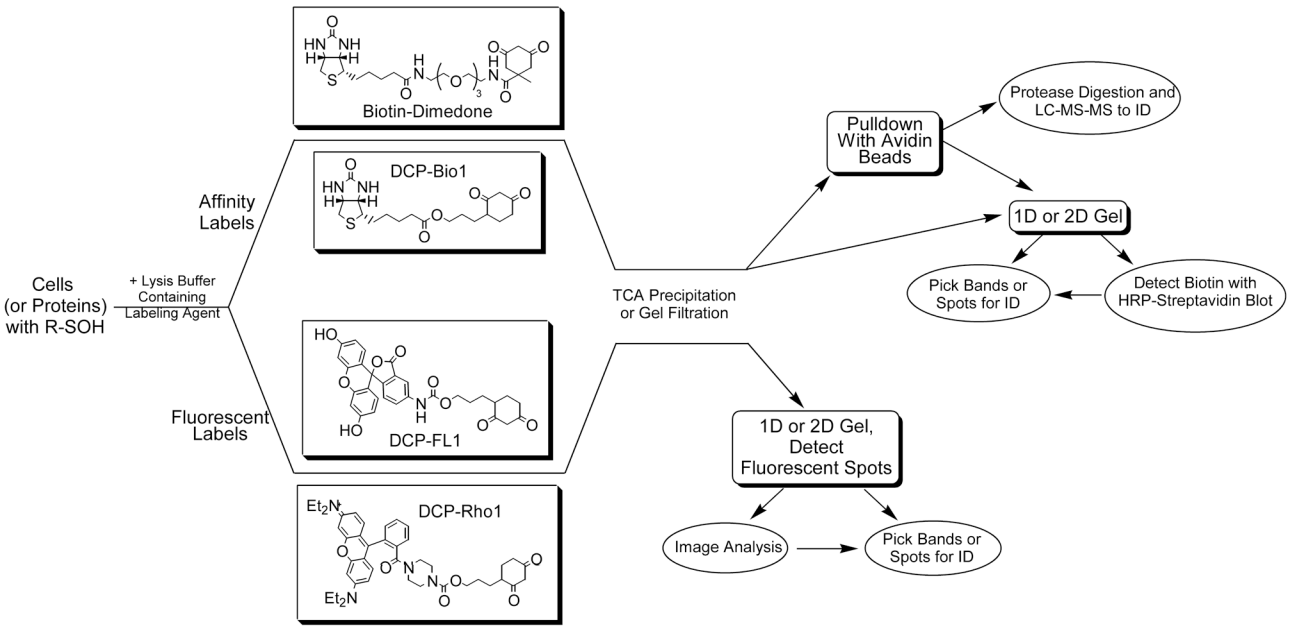

Figure 3. Strategies for detecting and isolating sulfenic acids in proteins

Cells or proteins with cysteine sulfenic acid modifications are incubated with either affinity (biotin-dimedone or DCP-Bio1, or two other affinity probes [39,41]) or fluorescently-tagged reagents (DCP-FL1 or DCP-Rho1, or two other fluorescein- and rhodamine-based probes [39]). The chemically reactive probes based on dimedone include a nucleophilic carbon between the two carbonyls of the cyclohexane ring that exhibits specificity toward sulfenic acids. After incubation, unreacted probes are removed from the protein samples by trichloroacetic acid (TCA) precipitation or gel filtration chromatography. Subsequent analytical procedures can include one-dimensional or two-dimensional gels in both cases, from which bands or spots can be excised, digested and analyzed by mass spectrometry (MS) for identification of the labeled protein. For the biotinylated samples, “pulldown” of the affinity-labeled proteins with avidin-linked beads can be carried out either before or after proteolytic digestion of the proteins in the samples, enriching in either labeled proteins or labeled peptides. Subsequent LC-MS-MS analysis can then be used to identify labeled proteins/peptides.