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Chemical Biology Approaches to Study Protein Cysteine Sulfenylation¹

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Abstract

The oxidation of cysteine thiol side chains by hydrogen peroxide to afford protein sulfenyl modifications is an important mechanism in signal transduction. In addition, aberrant protein sulfenylation contributes to a range of human pathologies, including cancer. Efforts to elucidate the roles of protein sulfenylation in physiology and disease have been hampered by the lack of techniques to probe these modifications in native environments with molecular specificity. In this review, we trace the history of chemical and biological methods that have been developed to detect protein sulfenylation and illustrate how a recent cell-permeable chemical reporter, DYn-2, has been used to detect identify intracellular targets of endogenous H₂O₂ during growth factor signaling, including the EGF receptor. The array of new tools and methods discussed herein enables the discovery of new biological roles for cysteine sulfenylation in human health and disease.

Although cysteine is present at a low percentage in proteins, it is considered a critical amino acid with numerous biological functions. Due to its highly reactive thiol side chain (RSH), cysteine is susceptible to various posttranslational modifications, which regulate protein structure and function.^{1–4} In biological redox systems, cysteine can be oxidized to sulfenic acid (RSOH) by reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) or reactive nitrogen species (RNS) such as peroxynitrite (ONOO[−]). This reversible modification has emerged as central reaction pathway in biological redox systems.

Owing to its intrinsic reactivity, sulfenic acid can undergo numerous chemical transformations (**Figure 1**). For example, when another protein thiol is in close proximity to sulfenic acid, these two species can react to form a disulfide bridge, an important modification that regulates protein folding, oligomerization, and other cysteine modifications (such as *S*-glutathionylation). Nitrogen nucleophiles present in small molecules or the polypeptide backbone can also react with sulfenic acid to form a sulfenamide (RSNR₂). A prototypical example of sulfenamide formation has been demonstrated in the protein tyrosine phosphatase 1B (PTP1B). Indeed, x-ray crystallographic and cellular studies demonstrate that oxidation of active site cysteine of PTP1B to sulfenic acid is followed by conversion to an intramolecular cyclic sulfenamide,

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which can protect the phosphatase from irreversible oxidation.⁵ In the absence of proximal thiols or other nucleophiles, the sulfenic acid modification can be stabilized or, under oxidative stress conditions, be further oxidized to the largely irreversible sulfinic ($-\text{SO}_2\text{H}$) or sulfonic acid ($-\text{SO}_3\text{H}$). With such diverse reactivity, the level of protein sulfenic acid modifications not only serves as a biomarker of redox signaling in biological systems, but also has significant implications for probing pathologies associated with oxidative stress, such as cancer⁶ and cardiovascular disease⁷.

Owing to its biological importance, intensive efforts have been focused on detection of protein sulfonylation in cellular systems.⁸ In this mini review, we discuss methods for detecting protein sulfonylation, with particular emphasis on chemoselective bioorthogonal reaction-based probes as well as application of these reagents to study redox-mediated growth factor signal transduction.

Spectroscopic methods

Methods such as x-ray crystallography and NMR can afford substantial insight into the structure and local protein microenvironment of the sulfonylated cysteine. For example, the Cys42 redox center in the flavoprotein NADH peroxidase had been characterized in the sulfonic acid form, but the oxidation pathway was not clearly identified. Subsequent x-ray crystallography⁹ and ^{13}C NMR¹⁰ studies revealed that Cys42 forms a sulfenic acid in its oxidized state, supporting the proposed catalytic role of this residue. Another case is the active site Cys50 sulfenic acid in an archaeal peroxiredoxin (Prx) from *Aeropyrum pernix* K1 (ApTPx), which was crystallized and identified as the intermediate in the oxidation of Prx by H_2O_2 ¹¹. A more recent example is a global transcriptional regulator SarZ in *Staphylococcus aureus*, whose reduced, sulfenic acid and mixed disulfide forms of Cys13 have been resolved by crystallography.¹² Sulfenic acids have also been directly observed by mass spectrometry (MS) in a few proteins such as aldose reductase (AR),¹³ transcriptional factor OhrR,¹⁴ and methionine sulfoxide reductase (MsrA)¹⁵. Although spectroscopic methods provide important information about the existence and environment of the sulfenic acid modification, such methods are limited to recombinant protein samples and are not applicable to complex biological systems.

Electrophilic Probes

Although the sulfur atom in sulfenic acid has significant electrophilic character, it also functions as a weak nucleophile. For example, sulfenic acid can react with the electrophilic compound 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl)¹⁶ to give the sulfoxide product. In addition, NBD-Cl reacts with amines, thiols and the tyrosine phenol group to yield fluorescent conjugates. For this reason, NBD-Cl is not a chemically selective reagent for sulfenic acid detection. On the other hand, the difference of the absorption wavelengths for the products (maximum: -NH-NBD 480 nm; -O-NBD 382 nm; -S-NBD 420 nm; -S(O)-NBD 347 nm) observed in some proteins may permit positive identification of sulfenic acids relative to other amino acid adducts (**Figure 2**).¹⁷ In addition, the sulfoxide product formed upon reaction of NBD-Cl with sulfenic acid can be monitored by MS. Using this approach, sulfenic acid modifications in AhpC peroxidase, NADH peroxidase,¹⁷ OhrR repressor,¹⁴

PTPs,¹⁸ recombinant human alpha 1-antitrypsin,¹⁹ and human serum albumin²⁰ have been successfully identified. However, the application of this method is limited to relatively pure or isolated proteins.

Genetically-Encoded Probes

The transcription factor Yap1 in *Saccharomyces cerevisiae* activates expression of antioxidant genes in response to oxidative stress and functions in a two-component system with its partner protein, the thiol peroxidase Gpx3. Upon H₂O₂ exposure, the active site Gpx3 cysteine (Cys36) is oxidized to sulfenic acid, which reacts with Cys598 of Yap1's C-terminal cysteine-rich domain (cCRD) to form a mixed disulfide intermediate. Resolution of this Gpx3-Yap1 disulfide by intramolecular thiol-disulfide exchange with Cys303 of Yap1 then leads to the formation of an inter-domain disulfide bond between Cys303-Cys598 in Yap1 and regeneration of reduced Gpx3. As shown in **Figure 3**, a Yap1-cCRD probe has been constructed that consists of the C-terminal cysteine rich domain of Yap1, containing the Cys620Ala and Cys629Thr mutations flanked by both an N- and C-terminal His₆ tag. In this Yap1-cCRD variant, Cys598 can form mixed disulfides with sulfenic acid-modified proteins in heterologous expression systems such as *Escherichia coli*, that are detected by SDS-PAGE or enriched and identified by LC-MS/MS.²¹ More recently, this method was used to identify 42 proteins that undergo sulfenylation in *Saccharomyces cerevisiae* after treatment with exogenous H₂O₂.²² Although some interesting features are noted in this system, the Yap1-cCRD variant must be expressed in host cells and, since it is protein-based, it may exhibit substrate bias when compared to chemical-based probes.

Indirect Detection Methods

Indirect detection methods require protection of all thiols by thiol-specific reagents, such as iodoacetamide or *N*-ethylmaleimide, in the first step. These methods vary in the steps after thiol-protection, where different reagents and reactions are employed for transformation of the sulfenic acid to a thiol or sulfonic acid. For example, after thiol blocking with maleimide, sodium arsenite (NaAsO₂) was used to reduce sulfenic acid in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to the thiol form²³, which could be tagged with biotin (**Figure 4A**).^{24,25} Levels of protein sulfenylation may also be estimated by the change in enzyme activity before and after arsenite reduction (**Figure 4B**).^{26,27} Using this strategy, protein sulfenic acid modifications have been observed in tissues treated with exogenous H₂O₂.²⁴ Another strategy using “hyper”oxidation has been applied in to detected sulfenylated protein tyrosine phosphatases (PTPs). As shown in **Figure 4C**, protein thiols are blocked in lysates with iodoacetic acid (IAA) followed by immunoprecipitation, and oxidation of any surviving sulfenic acid to sulfonic acid with pervanadate. An antibody that detects the sulfonic acid form of the highly conserved PTP active site can then be applied to detect oxidized PTPs by immunoblot and/or enrichment and LS-MS/MS analysis.^{28,29} However, the instability of many protein sulfenic acids during the lengthy series of chemical manipulations required by indirect detection methods often precludes robust detection.

Bioorthogonal strategy using nucleophilic probes

Compared to the approaches discussed above, mild carbon nucleophiles such as 1,3-diones present many advantages for detecting protein sulfenylation, including direct and selective detection. Nucleophiles such as 5,5-dimethyl-1,3-cyclohexanedione (dimedone) react with sulfenic acid to form stable thioether conjugates (**Figure 5**),³⁰ but show no reactivity toward other electrophilic sulfur species such as disulfides and *S*-nitrosothiols.

To date, dimedone derivatives have been modified to develop various probes for detecting sulfenic acids *in vitro* and cells. Based on their chemical structure and detection mechanism, these reagents and related strategies are classified and discussed below.

1) Probes Directly Conjugated to Biotin or Fluorescent Tags

Poole and colleagues have developed dimedone probes directly linked to reporter tags such as biotin or fluorophores (**Figure 6**).³¹ These probes have been applied to trap sulfenylated proteins in lysates, and depending on the permeability of the reporter tag, in cells. For example, using the biotinylated probe, DCP-Bio1, sulfenylation of protein tyrosine phosphatases SHP-1 and SHP-2 was demonstrated in CD8⁺ T cell lysates, which is critical for ERK1/2 phosphorylation, calcium flux, cell growth, and proliferation of naive CD8⁺ and CD4⁺ T cells.³² This probe has also been applied to identify sulfenic acid modification of Akt2 Cys124, and revealed the inhibition of Akt2 kinase activity by PDGF-induced ROS,³³ as well as in the study of vascular endothelial growth factor (VEGF) stimulated protein sulfenylation in human umbilical vein endothelial cells (HUVECs).³⁴ Recently, 1,3-cyclopentadione derived probe **2** has also been developed and demonstrates moderately enhanced labeling of sulfenylated C165S AhpC protein.³⁵ A dimedone probe **5** linked to a biotin tag has also been developed in our laboratory; however, chemical probes conjugated to biotin suffer from poor cell-permeability and restrict their use to cell lysates.³⁶

2) Azide- or Alkyne-Functionalized Probes

Such probes are dimedone derivatives modified by either an azide or alkyne chemical handle. After trapping sulfenic acid, the handles are reacted with alkynyl- or azido-functionalized biotin or fluorophore reporters *via* Staudinger ligation or Huisgen cycloaddition (also known as click chemistry) to form stable conjugates for further study (**Figure 7A**). This general strategy has proven more facile than using probes directly linked to reporters in numerous ways. For example, the azide-functionalized probe DAZ-1 showed much higher efficiency in trapping sulfenylated proteins in cells, as compared to probe **5**.³⁶ Another azide-modified probe, DAZ-2, has been applied in a global-proteome wide study in which more than 175 new proteins from human tumor cells were identified as targets for protein sulfenylation.³⁷ Modification of these probes with an affinity-based binding module has also provided a new way to study sulfenylation in specific classes of signaling proteins.³⁸ As shown in **Figure 7B**, probes **10** and **11** with hydrophobic aryl binding modules showed excellent sensitivity and selectivity for detecting sulfenic acid modification of the active site cysteine in PTPs.

Efforts have also been made towards the development of probes with cleavable biotin tags, which can facilitate the proteomic study of the targeted proteins. As shown in **Figure 7C**, after modification with probe **7** or **12**, the proteins were conjugated with compound **13** with a carbamate linker that can be cleaved by treatment with TFA; the released proteins were subsequently subjected to MS/MS analysis.³⁹ Alkyne-functionalized β -ketoester probe **14** has also been developed to trap protein sulfenic acids, followed by click-chemistry with biotin derivative **15**. The protein conjugate is subsequently cleaved from the biotin tag *via* a hydroxylamine-mediated cyclization reaction for LC-MS/MS analysis (**Figure 7D**).⁴⁰ On the other hand, multiple protein adducts with a single cysteine-containing protein has been observed with the reported β -ketoester unit (P. Martinez and K. Carroll, unpublished results). Hence, the selectivity of the β -ketoester chemical group for sulfenic acid requires further evaluation.

3) Immunochemical Detection

The cysteine-S-dimmedone thioether moiety represents a unique chemical epitope, which has been exploited to elicit antibodies that recognize this protein adduct (**Figure 8A**).⁶ The resulting antibody was found exquisitely specific, context-independent and capable of visualizing sulfenic acid formation in cells. Using this strategy, differences in thiol redox status between normal and cancer cell lines were evaluated and revealed a diverse pattern of sulfenylation across different subtypes of breast tumors.⁶ Recently, another dimedone-based antibody was developed to study the role of GAPDH in H₂O₂-stimulated oxidant signaling in isolated rat ventricular myocytes.⁴¹

4) Isotope-Coded Probes

These probes are classified into two different strategies for quantifying redox-dependent changes in the extent of protein sulfenylation. One method, as shown in **Figure 8B**, used a class of reagents termed, isotope-coded dimedone and iododimmedone (ICDID) to label thiols and sulfenic acids, respectively.⁴² The degree or extent of protein sulfenylation can then be analyzed by LC-MS/MS. The other strategy employs *d*₀-DAz-2 and *d*₆-DAz-2 probes to label sulfenylated proteins in response to changing H₂O₂ concentrations. The labeled proteins were then ligated with a biotin tag *via* click chemistry, followed by avidin enrichment and analysis by LC-MS/MS (**Figure 7C**).³⁹ This probe set allows for ratiometric quantification of sulfenylation between different cellular redox states.

Redox Regulation of EGFR Signaling

In the last section, we discuss our recent study of protein sulfenylation during growth factor signaling in cells.⁴³ Epidermal growth factor (EGF) is known to bind the extracellular domain of the EGF receptor (EGFR) and leads to the assembly and activation of NADPH oxidase (Nox) complexes, which generate endogenous H₂O₂. To investigate cysteine oxidation events after the interaction of EGF with its receptor, we generated the alkyne-functionalized DYn-2 probe. As with other alkyne-functionalized small-molecules, DYn-2 demonstrated higher sensitivity for detecting sulfenylated proteins in A431 and HeLa cells. Since direct evidence of PTP oxidation in their cellular environment had not yet been reported, we used DYn-2 to study sulfenylation of signaling phosphatases such as PTEN,

PTP1B and SHP2. These PTPs were found to undergo EGF-dependent sulfenylation in A431 cells, each with a unique oxidation profile. In addition, we identified Cys797 in the ATP binding site of EGFR as a major sulfenylation target in cells, and further demonstrated that this modification enhances its intrinsic tyrosine kinase activity. Taken together, these and other data reveal protein sulfenylation as a signaling mechanism akin to phosphorylation during EGFR and other receptor-mediated signaling events (**Figure 9**). This study may also suggest that sulfenylation may serve as a general mechanism to regulate kinase activity, as numerous kinases possess a cysteine residue at the structural position corresponding to Cys797. Furthermore, as EGFR has been found overexpressed in carcinomas, including breast and lung cancers, and is an important therapeutic target in clinical trials, the finding that Cys797 undergoes sulfenylation in cells may have implications for covalent inhibitors that target this residue.

In summary, we have discussed the detection methods of protein sulfenylation up to date, their applications, and limitations. Methods using bioorthogonal chemical reporters have been successfully applied to trap and tag protein sulfenic acids in biological samples including cells without toxicity or interfering with other biological activities. With expanded structural diversity of chemical probes, those methods can target cysteine residues in specific proteins as well. It also opens a door for broader application when coupled with other techniques, such as MS for proteomics analysis and high-throughput screening for inhibitor identification. Eventually, these studies will lead to increased understanding of the physiological role of protein cysteine oxidation and its functional consequences in disease.

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Figure 1.

Sulfenic acid is at the center of redox cysteine modifications. A cysteine thiol can be reversibly oxidized by reactive oxygen or nitrogen species (Ox) to form sulfenic acid, which may be stabilized by the protein microenvironment or react with adjacent thiols to form intra- or inter-protein disulfides, or peptide backbone amides to form a cyclic sulfenamide (this modification has primarily been observed in PTP1B). Elevated cellular concentrations of ROS and RNS, can lead to further oxidation of sulfenic acid to sulfinic acid and/or sulfonic acid, the latter of which is considered to form irreversibly.

Figure 2.

Reaction of NBD-Cl with protein nucleophiles. NBD-Cl can react with various nucleophiles in biological systems and the conjugation products have different absorption wavelength. In some cases, the NBD-sulfenic acid conjugate can be identified by absorption at 347 nm.

Figure 3.

Detection of sulfenylated proteins in cells with a genetically encoded Yap1-cCRD probe. Cells expressing Yap1-cCRD are exposed to H_2O_2 and protein conjugates are extracted with trichloroacetic acid (TCA). Yap1-cCRD-captured proteins are affinity enriched by virtue of the histidine tag, cleaved and eluted with DTT or TCEP reducing agents. After sample enrichment, thiol alkylation and protease digestion, the resulting peptides are analyzed in LC-MS/MS.

Figure 4. Indirect detection of sulfenic acid. (A) Enzyme activity assay using arsenite-mediated reduction of sulfenic acids. (B) Indirect chemical detection using arsenite reduction. (C) Indirect chemical detection using pervanadate-mediated hyperoxidation.

Figure 5.
The reaction between a protein sulfenic acid and dimerone.

Figure 6.
1,3-Dione probes directly conjugated to biotin or fluorescent tags.

Figure 7.

Direct detection of sulfenic acid with cell-permeable 1,3-dione-based chemical probes. (A) Probes with azido or alkynyl tags and corresponding ligation reactions. (B) Probes with affinity-based binding modules that enhance the affinity of the probe to PTP active sites. (C) Direct detection of sulfenylated proteins using biotin modified with a TFA-cleavable carbamate linker. (D) Direct detection of sulfenylated proteins with a β -ketoester probe that can be cleaved by the reaction with hydroxylamine.

Figure 8.

Immunochemical and ratiometric detection of protein sulfenylation. (A) Antibody-based detection of the cysteine-dimedone protein adduct. (B) Quantification of protein sulfenylation using isotope-coded dimedone and iododimedone (ICDID).

Figure 9.

Model for redox regulation of EGFR signaling. The mitogen EGF binds to EGFR and induces the production of endogenous H_2O_2 in A431 cells through the enzyme, Nox2. A critical active site cysteine (Cys797) in EGFR is sulfenylated by H_2O_2 , which enhances its tyrosine kinase activity and regulates downstream signaling events.