

# Chemical Approaches to Discovery and Study of Sources and Targets of Hydrogen Peroxide Redox Signaling Through NADPH Oxidase Proteins

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## Keywords

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## Abstract

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a prime member of the reactive oxygen species (ROS) family of molecules produced during normal cell function and in response to various stimuli, but if left unchecked, it can inflict oxidative damage on all types of biological macromolecules and lead to cell death. In this context, a major source of H<sub>2</sub>O<sub>2</sub> for redox signaling purposes is the NADPH oxidase (Nox) family of enzymes, which were classically studied for their roles in phagocytic immune response but have now been found to exist in virtually all mammalian cell types in various isoforms with distinct tissue and subcellular localizations. Downstream of this tightly regulated ROS generation, site-specific, reversible covalent modification of proteins, particularly oxidation of cysteine thiols to sulfenic acids, represents a prominent posttranslational modification akin to phosphorylation as an emerging molecular mechanism for transforming an oxidant signal into a dynamic biological response. We review two complementary types of chemical tools that enable (*a*) specific detection of H<sub>2</sub>O<sub>2</sub> generated at its sources and (*b*) mapping of sulfenic acid posttranslational modification targets that mediate its signaling functions, which can be used to study this important chemical signal in biological systems.

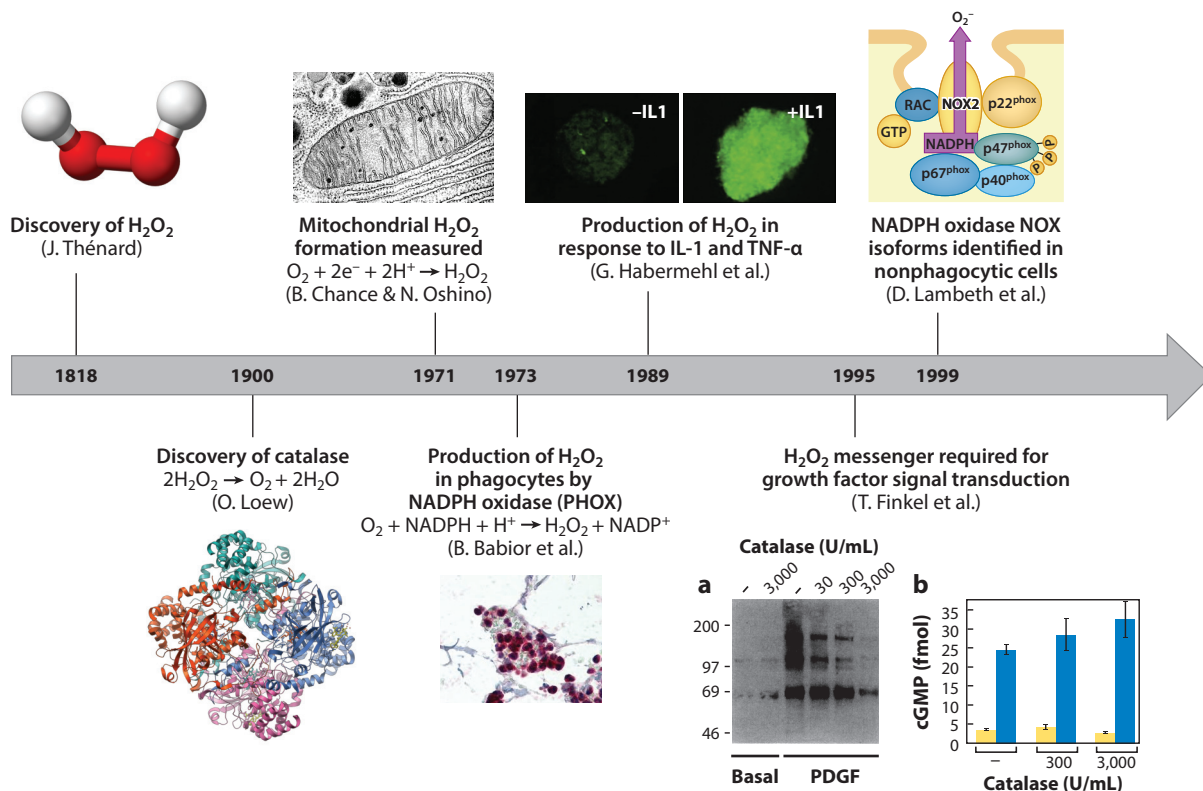
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## INTRODUCTION

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is widely perceived as a reactive and toxic chemical inimical to the processes of life. It was discovered by Thénard (1) in 1818, after which it was used as a bleaching agent and disinfectant. In 1900, Loew's (2) discovery of catalase, which efficiently decomposes and thus eliminates this molecule, reinforced the perception of  $\text{H}_2\text{O}_2$  as an unwanted toxin. The 1971 observation by Chance & Oshino (3) that the body itself generates  $\text{H}_2\text{O}_2$  in the mitochondria was interpreted as an inefficiency of aerobic metabolism, wherein  $\text{H}_2\text{O}_2$  was an accidental by-product (4). Even Babior et al.'s (5) discovery of the phagocytic oxidase in 1973, which deliberately produces superoxide ( $\text{O}_2^-$ ) and its dismutation product  $\text{H}_2\text{O}_2$ , did not alter this view. The enzyme seemed to be confined to specialized immune cells, in which it produced  $\text{H}_2\text{O}_2$  in the interior of the phagosome specifically for its toxic properties in order to destroy engulfed pathogens (5, 6). Indeed, subsequent documentation by Habermehl and colleagues (7) of the production of  $\text{H}_2\text{O}_2$  in response to the proinflammatory cytokines interleukin-1 and tumor necrosis factor  $\alpha$  reinforced the idea that the biological role of  $\text{H}_2\text{O}_2$  was restricted to the immune system. More recently, however,  $\text{H}_2\text{O}_2$  has become widely appreciated as a beneficial molecule with more subtle functions than bacterial killing. The Finkel group's (8) 1995 discovery of deliberate  $\text{H}_2\text{O}_2$  production by vascular smooth muscle cells, followed by the detection of widespread enzymatic production of reactive oxygen species (ROS, a term used to describe diverse small oxygen-containing molecules such as  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$ , hypochlorite, and hydroxyl radical) in 1999 by Lambeth and colleagues (9), has supported the concept that  $\text{H}_2\text{O}_2$  is a cellular signaling agent and challenged the idea that its presence is indicative solely of oxidative stress (10). From that point forward, the perceived role of  $\text{H}_2\text{O}_2$  in biology has thus progressed from a toxin to an agent of the immune system to a key

**ROS:** reactive oxygen species



**Figure 1**

The perceived role of  $\text{H}_2\text{O}_2$  in biology has evolved from toxin to agent of the immune system to a key component of signaling pathways. Abbreviations: cGMP, cyclic guanosine monophosphate; IL, interleukin; PDGF, platelet-derived growth factor; TNF, tumor necrosis factor.

component of such diverse biological phenomena as the circadian rhythm (11, 12), cell migration (13–15), and stem cell proliferation (**Figure 1**) (16).

Elucidating the signaling roles of  $\text{H}_2\text{O}_2$  in normal physiology and disease has been hampered by the difficulty of detecting  $\text{H}_2\text{O}_2$  itself, as well as  $\text{H}_2\text{O}_2$ -induced modifications, with chemical specificity in complex biological systems. After a brief introduction reprising the major ROS produced by cells and mechanisms of thiol oxidation, we focus on recent progress in chemical approaches for specific detection of  $\text{H}_2\text{O}_2$  and different oxidative posttranslational modifications (oxPTMs) of protein cysteine thiols; we emphasize those chemical properties that differentiate one ROS or cysteine modification from another. In keeping with this general theme, we restrict our attention to reagents that have enabled the discovery and understanding of new redox biology within native cellular environments. Along the way, we complement this discussion with examples from the literature that highlight ways in which redox signaling via cysteine oxidation can be used to control protein function and signal transduction pathways.

## HYDROGEN PEROXIDE AND REACTIVE OXYGEN SPECIES

Among the diverse collection of cellular ROS,  $\text{H}_2\text{O}_2$  stands out as a molecule with ideal signaling properties. It possesses sufficient reactivity to modify potential signaling targets, yet it is also

**oxPTM:** oxidative posttranslational modification

stable enough to diffuse appreciable distances within the cell, reacting with select targets (17). Furthermore, it can be rapidly produced and quickly eliminated, making it ideal for signaling bursts. Average  $\text{H}_2\text{O}_2$  concentrations in healthy mammalian cells can range from 1 to 700 nM; higher levels are associated with oxidative stress and cell death (18). However, local  $\text{H}_2\text{O}_2$  concentrations can be considerably higher than the average value. The floodgate model of oxidative signaling proposes that rapid increases in local  $\text{H}_2\text{O}_2$  can temporarily overwhelm antioxidant defenses, allowing reaction with targets for signaling purposes (19). Thus, whereas high global concentrations of  $\text{H}_2\text{O}_2$  cause oxidative stress, brief bursts can be beneficial to cellular physiology. Cellular sources of  $\text{H}_2\text{O}_2$  include primarily the mitochondria and the endoplasmic reticulum (ER) (20), as well as various oxidases, including the NADPH oxidase (Nox) proteins (4), amine oxidases (21), acyl-CoA oxidases (22), and cytochrome P450 enzymes (23). Among these sources, the Nox family of proteins appears to be critical for the production of  $\text{H}_2\text{O}_2$  for signaling purposes. Unlike other oxidases, the Nox proteins generate  $\text{O}_2^-$  as their primary product, which can then disproportionate to  $\text{H}_2\text{O}_2$ . The founding member of the Nox family is the phagocytic oxidase, which was characterized in the context of immune defense before the discovery of  $\text{H}_2\text{O}_2$  as a signaling agent. This enzyme generates ROS within phagocytes in order to damage and eliminate engulfed pathogens. More recently, however, Nox family members were found to be ubiquitously expressed in mammalian tissue types (10). Thus, the widespread expression and deliberate  $\text{H}_2\text{O}_2$  production of these enzymes are incompatible with the idea that  $\text{H}_2\text{O}_2$  is solely a toxic by-product. Indeed, these facts suggest that  $\text{H}_2\text{O}_2$  serves a beneficial function. Combined with the ideal chemical properties of  $\text{H}_2\text{O}_2$  as a messenger molecule, the aforementioned observations have motivated the study of Nox-derived  $\text{H}_2\text{O}_2$  signaling.

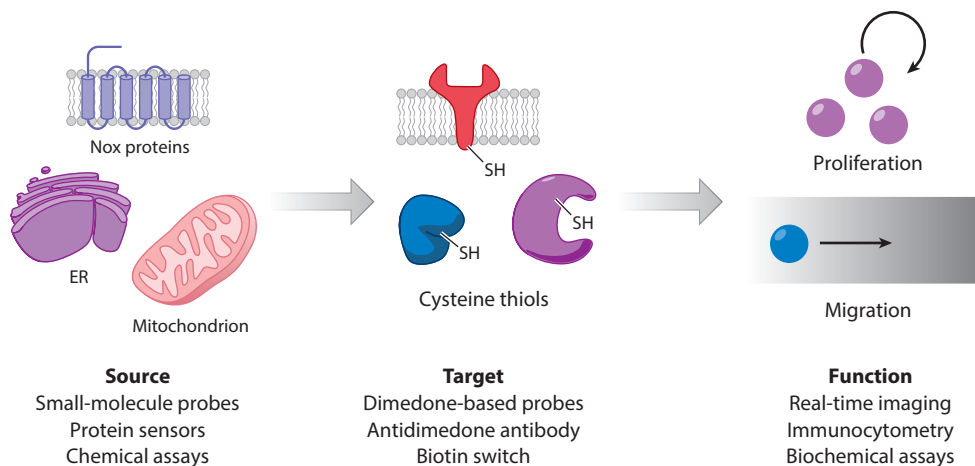
## CHEMICAL TOOLS FOR DETECTION OF HYDROGEN PEROXIDE SOURCES

### Criteria for Hydrogen Peroxide Probes

To gain a complete understanding of the function of  $\text{H}_2\text{O}_2$ , we must have the ability to detect its production at the source, the effects on its immediate target, and the downstream consequences (Figure 2). Novel probes must meet a high bar in terms of their selectivity, kinetics, and photo-physical properties. An ideal chemical probe for  $\text{H}_2\text{O}_2$  must be inert to the conditions present in the cell and should not require the addition of other reagents. Furthermore, the probe must be selective against similar analytes, and it should exhibit a turn-on, rather than turn-off, response in order to minimize background. Ideally, a family of probes with varying wavelengths should be available so that their use can be tailored to the specific application and made compatible with probes for different species.

### Types of Hydrogen Peroxide Detection Technologies

Numerous strategies have been implemented to address the issue of detecting  $\text{H}_2\text{O}_2$ , including approaches based on proteins (24–27), nanotubes (28), hyperpolarization (29), ultrasound (30), mass spectrometry (MS) (31), and chemiluminescence (32). Of particular interest is a recent innovative enzyme-based detection method utilizing a variant of ascorbate peroxidase (APX). APX expressed intracellularly catalyzes the  $\text{H}_2\text{O}_2$ -dependent conversion of Amplex Red into a detectable fluorescent product, and this method has been used successfully to detect  $\text{H}_2\text{O}_2$  produced as a result of antibiotic treatment in *Escherichia coli* (27). The potential of the chemiluminescence modality is best demonstrated by the firefly luciferin-based boronate probe PCL1 (Figure 3b), which can be



**Figure 2**

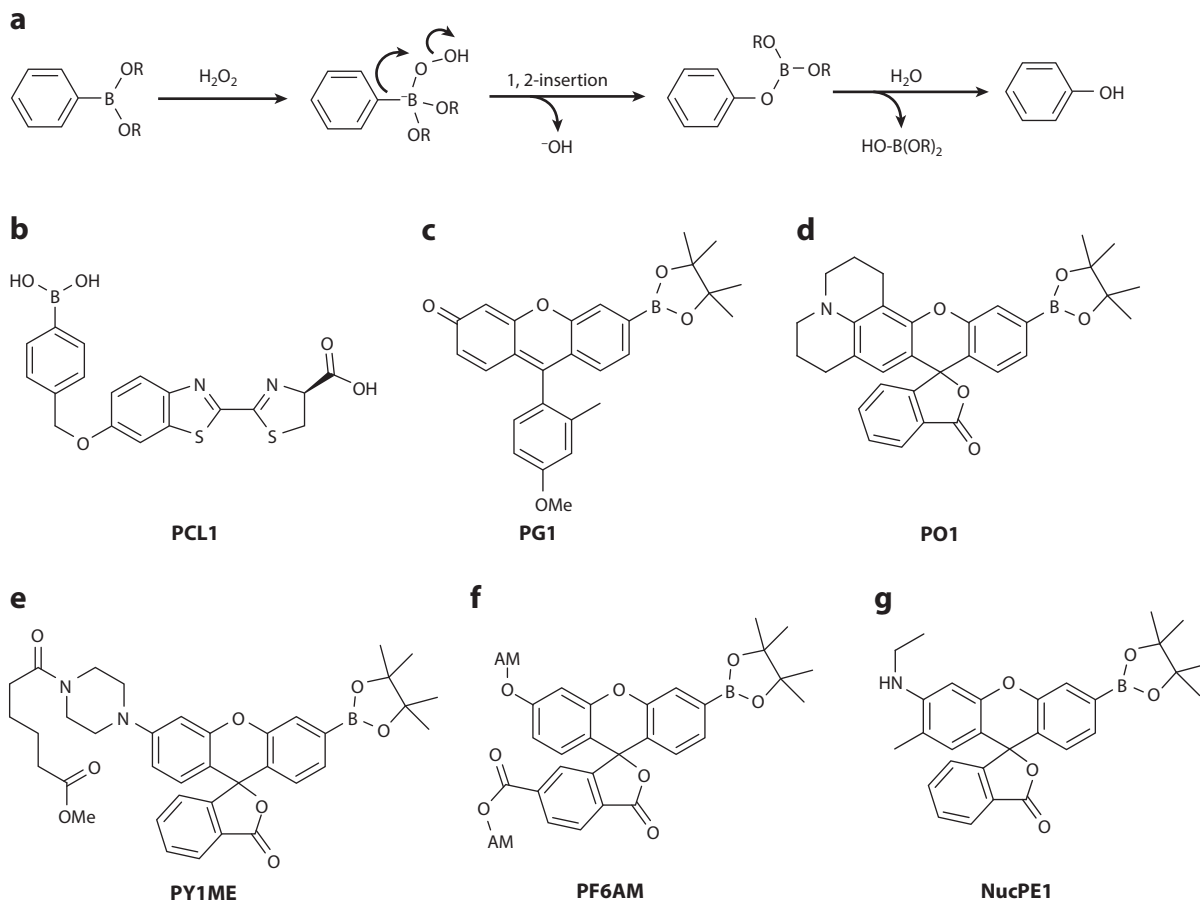
Strategies for detection of the sources, targets, and downstream consequences of  $\text{H}_2\text{O}_2$  signaling. Abbreviations: ER, endoplasmic reticulum; Nox, NADPH oxidase.

used in a luciferase-expressing mouse to measure  $\text{H}_2\text{O}_2$  bursts in live mice (**Figure 4a**) (33). The next-generation probe PCL2 contains a second reactive trigger to report on simultaneous  $\text{H}_2\text{O}_2$  generation and caspase activity in a model of acute inflammation (34). The most promising class of probes consists of small-molecule fluorescent sensors. These sensors do not require additives or particular enzymes, and a wide variety of analogs are accessible to synthetic chemists, permitting the development of a flexible array of tools with differing optical, photophysical, and chemical properties.

## Early Small-Molecule Fluorescent Probes

The fluorescent probes dihydrodichlorofluorescein ( $\text{H}_2\text{DCF}$ ) and dihydrodichlorofluorescein diacetate ( $\text{H}_2\text{DCFDA}$ ) have been used extensively to detect ROS (35). These probes have been fruitfully employed in biological studies, but their lack of ROS specificity and photoactivation of the unoxidized molecule can confound results (36). Another ROS detection technology of historical interest is the Amplex Red assay (37). This assay is sensitive, but the requirement of the enzyme additive makes it difficult to use within many cell types. In this context, the Chang laboratory (38–41) introduced a useful class of biocompatible small-molecule fluorescent probes that rely on the oxidation of an aryl boronate by  $\text{H}_2\text{O}_2$  to produce a phenol (**Figure 3a**). The boronate moiety masks a phenol, causing the unactivated probe to exhibit significantly lower fluorescence than the oxidized molecule. By appending this chemical modification to various fluorescent scaffolds, one can produce various probe molecules. The boronate oxidation takes advantage of the chemical characteristics of  $\text{H}_2\text{O}_2$ —specifically, its enhanced,  $\alpha$ -effect nucleophilicity and its weak O–O bond (42). The electrophilic boronate undergoes a nucleophilic addition by  $\text{H}_2\text{O}_2$ , forming a charged tetrahedral boronate complex. The complex then undergoes a 1,2 insertion in which the C–B bond migrates onto a peroxide oxygen molecule, breaking the weak O–O bond and releasing water. Hydrolysis of this intermediate produces boric acid, concomitantly freeing the activated probe.

This oxidation is highly selective for  $\text{H}_2\text{O}_2$  over other biologically relevant species, including  $\text{O}_2^-$ , hypochlorite, hydroxyl radical, alkyl hydroperoxides, and nitric oxide (43). Although more

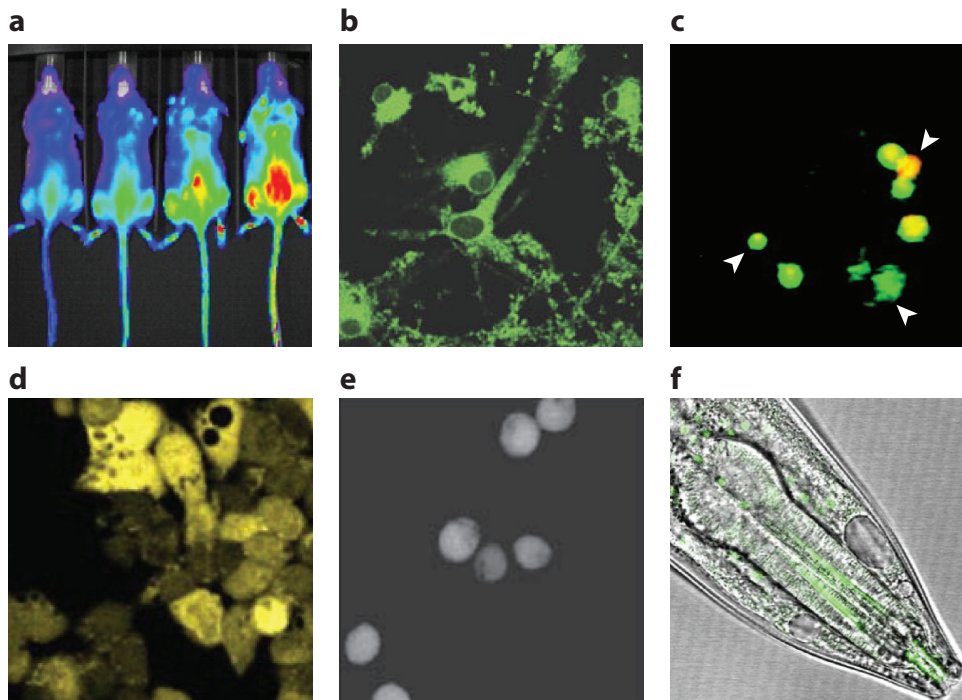


**Figure 3**

Aryl boronates are a selective platform for  $\text{H}_2\text{O}_2$  detection. (a) General oxidation of an aryl boronate by  $\text{H}_2\text{O}_2$ . (b–g) Selected small-molecule boronate-based probes for  $\text{H}_2\text{O}_2$ . Abbreviations: PG1, Peroxy Green 1; PO1, Peroxy Orange 1; NucPE1, Nuclear Peroxy Emerald 1; PF6AM, Peroxyfluor-6 acetoxymethyl ester; PY1ME, Peroxy Yellow 1 Methyl Ester.

recent findings have shown that certain boronates also react toward highly reactive species such as peroxynitrite, these molecules are generally produced in much lower quantities and exhibit short biological half-lives compared with that of  $\text{H}_2\text{O}_2$  (44–46). In experimental settings in which peroxynitrite or similar highly reactive species are a concern, boronate-based probes must be used with caution in concert with appropriate controls, generally either reducing  $\text{H}_2\text{O}_2$  levels with antioxidants or inhibiting its enzymatic production. The first-generation probe Peroxyfluor 1 (PF1) featured dual aryl boronates, which produced a large turn-on response. However, the turn-on kinetics were relatively slow because the probe required two equivalents of  $\text{H}_2\text{O}_2$  to produce the maximal turn-on (40). Nevertheless, this probe marked the innovation of a water- and cell-compatible chemistry that was subsequently improved and has allowed the discovery of novel  $\text{H}_2\text{O}_2$  signaling processes relating to neuronal, stem, and immune cells (41). In this context, note that this  $\text{H}_2\text{O}_2$ -sensitive aryl boronate chemistry has been applied toward other goals, such as the use of masked metal chelators (47, 48) and enzyme inhibitors (49) as prodrugs.





**Figure 4**

Applications of small-molecule boronate probes. (a) Response of PCL1 to increasing amounts of  $\text{H}_2\text{O}_2$  in live mice (33). (b) PG1 detects  $\text{H}_2\text{O}_2$  production by neurons stimulated with epidermal growth factor (51). (c) PO1 and APF detect three distinct types of phagosomes that produce primarily  $\text{H}_2\text{O}_2$  (orange), primarily hROS (green), and both (yellow) (43). (d) PF6AM detects  $\text{H}_2\text{O}_2$  production by adult hippocampal progenitor cells stimulated with fibroblast growth factor 2 (55). (e) PY1ME detects increased  $\text{H}_2\text{O}_2$  uptake by cells expressing aquaporin-3 (16). (f) NucPE1 detects nuclear  $\text{H}_2\text{O}_2$  fluxes in *Caenorhabditis elegans* (64). Abbreviations: APF, aminophenyl fluorescein; hROS, highly reactive oxygen species; NucPE1, Nuclear Peroxy Emerald 1; PF6AM, Peroxyfluor-6 acetoxymethyl ester; PG1, Peroxy Green 1; PO1, Peroxy Orange 1; PY1ME, Peroxy Yellow 1 Methyl Ester. Modified from References 33, 51, 43, 55, 16, and 64.

## Monoboronate Probes for Detecting Endogenous Hydrogen Peroxide Production

The monoboronate probe Peroxy Green 1 (PG1) (**Figure 3c**) marked a key advance over the first-generation PF1. This asymmetric probe, based on a Tokyo Green platform (50), features a single reactive boronate handle, allowing for much more rapid signal accumulation (51). This probe has been used to directly image the endogenous  $\text{H}_2\text{O}_2$  burst of A431 cells upon epidermal growth factor (EGF) stimulation. PG1 has also been validated in primary neuronal culture, a more technically challenging model system, enabling the first direct fluorescence imaging of the neuronal  $\text{H}_2\text{O}_2$  burst in response to EGF (**Figure 4b**). PG1 demonstrates the utility of real-time fluorescence imaging in mapping out cellular signaling pathways. Specifically, owing to the rapid and reliable response of this probe, investigators determined that the neuronal  $\text{H}_2\text{O}_2$  burst in response to EGF depends on the EGF receptor (EGFR) kinase domain, phosphatidylinositol 3-kinase (PI3K), Rac1, and Nox. Furthermore,  $\text{H}_2\text{O}_2$  production is independent of NO synthase and, by extension, peroxynitrite, as shown by inhibitor controls. This study (51) established these conclusions by sequentially applying pharmacological inhibitors of these various enzymes and

**EGF:** epidermal growth factor

**EGFR:** epidermal growth factor receptor

monitoring the response of the probe to the  $\text{H}_2\text{O}_2$  produced, if any. Thus, it is evident that the boronate probe platform opens up a host of possibilities for elucidating fundamental biological processes, particularly in the realm of cellular signal propagation.

Further derivatives exploit the modularity of the monoboronate platform to expand the available color palette. Access to probes with sensitivity to varying wavelengths of light allows users to monitor two or more analytes concurrently, taking advantage of the power of multichannel imaging experiments. Additional green monoboronate probes include Peroxyfluor 2 (PF2) and Peroxyfluor 3 (PF3), based on the fluorescein scaffold, and the slightly redshifted Peroxy Emerald 1 (PE1), a rhodol derivative. Peroxy Yellow 1 (PY1) is an additional rhodol-based probe with an excitation maximum at 514 nm, and Peroxy Orange 1 (PO1), a rhodol constructed from a julolidine building block (**Figure 3d**), can be excited at 540 nm (43). The latter probe was used successfully in a dual-channel imaging experiment in RAW264.7 macrophages in combination with aminophenyl fluorescein (APF), a green probe that reacts with highly reactive oxygen species (hROS), which include a subset of ROS, such as peroxynitrite, hypochlorite, and hydroxyl radical, that are more oxidizing relative to  $\text{H}_2\text{O}_2$  (52). PO1 exhibits sufficient spectral separation from APF to allow simultaneous imaging of both  $\text{H}_2\text{O}_2$  and hROS. With the ability to visualize these two ROS fluxes in the same space at the same time, investigators found that RAW264.7 macrophages contain three types of phagosomes when activated: phagosomes that produce primarily  $\text{H}_2\text{O}_2$ , those that produce primarily hROS, and those that produce both (**Figure 4c**) (43). Perhaps more importantly, direct treatment with HOCl did not generate a fluorescence response from the PO1 boronate, demonstrating that these types of probes show selectivity for  $\text{H}_2\text{O}_2$  over this ROS in cellular contexts.

### Trappable Hydrogen Peroxide Probes

The boronate-based scaffold is also flexible enough to accommodate trapping and targeting groups, allowing for the modulation of the spatial localization of these chemical tools. A trappable probe is one that can freely diffuse across the cellular membrane to enter the cell but then tends to remain inside the cell, enhancing the sensitivity of the probe by increasing its intracellular concentration (53). In practice, this goal can be achieved by masking a carboxylic acid or phenol as an ester. Trappable probes can be removed from the extracellular media, allowing confirmation that the observed signal corresponds to an increase in  $\text{H}_2\text{O}_2$  flux within the cell rather than a result of extracellular oxidation.

A first-generation trappable yellow  $\text{H}_2\text{O}_2$  probe, Peroxy Yellow 1 Methyl Ester (PY1ME) (**Figure 3e**), has been used to address an apparent paradox in Nox-derived  $\text{H}_2\text{O}_2$  signaling. Nox proteins produce extracellular  $\text{H}_2\text{O}_2$ ; therefore, to play an intracellular signaling role, this Nox-derived  $\text{H}_2\text{O}_2$  must cross the plasma membrane. PY1ME was used to test the hypothesis that  $\text{H}_2\text{O}_2$  can travel through mammalian cell aquaporins (54). PY1ME features good cellular retention upon washing, and importantly, washing guarantees that the probe signal originates only from within the cells.  $\text{H}_2\text{O}_2$  treatment of probe-loaded human embryonic kidney (HEK) 293T cells overexpressing aquaporin-1 (AQP1), AQP3, AQP8, or a control plasmid demonstrates that  $\text{H}_2\text{O}_2$  flux into the cell is enhanced over control by AQP3 and AQP8, but not AQP1 (**Figure 4d**) (55). This result is reasonable given that AQP1 is a classical aquaporin known to transport only water, whereas AQP3 and AQP8 are exemplars of aquaglyceroporins and unorthodox aquaporins, respectively, which are known to transport small solutes in addition to water (54).

The green boronate probe Peroxyfluor-6 acetoxymethyl ester (PF6AM) features two acetoxymethyl (AM) groups, one masking a carboxylic acid and the other masking a phenol (**Figure 3f**), which are well-known trapping groups (53). These AM groups greatly enhance the accumulation of PF6AM in cells, as well as its sensitivity. This probe has been used to elucidate



a beneficial role of  $\text{H}_2\text{O}_2$  in the brain. Specifically, exposing adult hippocampal progenitor cells to fibroblast growth factor 2 (FGF-2), which is known to regulate proliferation, results in  $\text{H}_2\text{O}_2$  production that is detectable by PF6AM (**Figure 4e**) (16). The  $\text{H}_2\text{O}_2$  produced oxidizes the active-site Cys124 of the protein phosphatase PTEN, converting it into a disulfide with Cys71. The oxidized PTEN is unable to catalyze dephosphorylation (56), most importantly of the kinase Akt, which propagates the FGF-2 signal (57). This  $\text{H}_2\text{O}_2$ -dependent cascade ultimately maintains normal neuronal stem cell growth and proliferation. Use of PF6AM has also demonstrated that the  $\text{H}_2\text{O}_2$  burst in neural stem cells is Nox dependent, as the Nox inhibitor diphenyleneiodonium chloride (DPI) abrogates both the probe's fluorescence turn-on and the downstream dephosphorylation of Akt. Furthermore, RNA interference knockdown of the specific isoform Nox2 also abolishes the signaling phenotype, which conclusively implicates this protein in the pathway (16). PF6AM has also been fruitfully employed to detect endogenously generated  $\text{H}_2\text{O}_2$  in the developing chick lens (58), Nox-dependent  $\text{H}_2\text{O}_2$  in stimulated neutrophils (59), and H-RAS<sup>V12</sup>-induced  $\text{H}_2\text{O}_2$  production in human fibroblasts (60).

### Targetable Hydrogen Peroxide Probes

In addition to trapping strategies, targeting  $\text{H}_2\text{O}_2$  probes to specific subcellular regions can enhance their sensitivity and spatial resolution. The mitochondrion is a natural organelle to target with such a strategy, owing to its known  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  production profile. The boronate-based probe MitoPY1 features a triphenylphosphonium moiety for mitochondrial targeting (61). This lipophilic, cationic group allows MitoPY1 to travel down the cellular charge gradient into the mitochondrion (62). MitoPY1 is a yellow probe, and it is sensitive enough to detect paraquat-induced  $\text{H}_2\text{O}_2$  in the mitochondria, which presages its utility in elucidating oxidative stress in models of neurodegeneration, such as in Parkinson's disease. MitoPY1, in conjunction with PF6AM, has also been used to detect  $\text{Na}^+$ -dependent  $\text{H}_2\text{O}_2$  increases in mitochondria-rich epithelial cells of the renal medullary thick ascending limb (63). The nucleus is another chemically targetable organelle of interest with respect to  $\text{H}_2\text{O}_2$  signaling, and the  $\text{H}_2\text{O}_2$ -responsive probe Nuclear Peroxy Emerald 1 (NucPE1) (**Figure 3g**) was determined to be localized to the nucleus. This probe has been used to demonstrate a decrease in nuclear  $\text{H}_2\text{O}_2$  flux in *Caenorhabditis elegans* overexpressing the *Sir-2.1* gene, which is implicated in extending the life span of model organisms (**Figure 4f**) (64). One can target further subcellular locales with  $\text{H}_2\text{O}_2$ -selective probes by taking advantage of SNAP labeling technology (65). In this paradigm, the enzyme *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT) is fused to a protein of interest or a localizing sequence and expressed in a relevant biological system. The fluorescent probes SNAP–Peroxy Green 1 (SPG1) and SNAP–Peroxy Green 2 (SPG2) can then be introduced into the cells to tag the arbitrarily localized AGT protein. This strategy has been demonstrated for localization to the plasma membrane, nucleus, mitochondria, and ER (66).

## CHEMICAL TOOLS FOR ELUCIDATING HYDROGEN PEROXIDE OXIDATIVE POSTTRANSLATIONAL MODIFICATION TARGETS

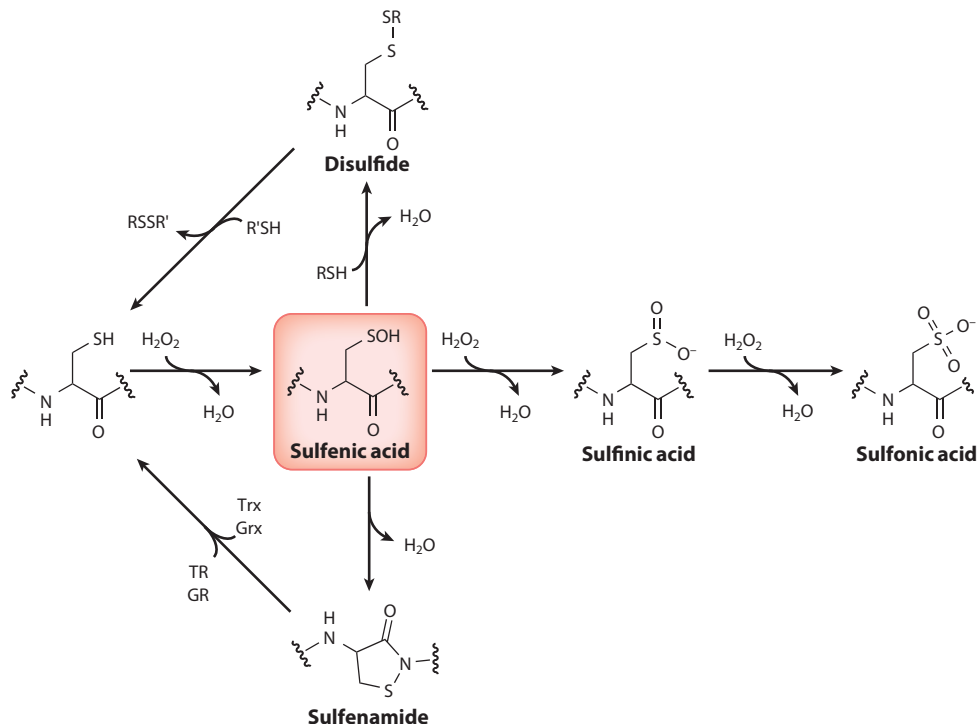
Cysteine is the most sensitive protein side chain to  $\text{H}_2\text{O}_2$ -mediated oxidation. Cysteine is oxidized via a two-electron nucleophilic substitution that yields a sulfenic acid (a process known as sulfenylation), and this oxidation is implicated in several important biochemical transformations (**Figure 5**). The propensity of cysteine residues toward oxidation is influenced mainly by three general factors: (a) thiol nucleophilicity, (b) protein microenvironment, and (c) proximity to ROS (67). Second-order rate constants for cysteine oxidation can vary dramatically within proteins, ranging from 20 M/s for protein tyrosine phosphatases (PTPs) to  $10^7$  M/s for peroxiredoxin

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**PTP:** protein tyrosine phosphatase

**Prx:** peroxiredoxin

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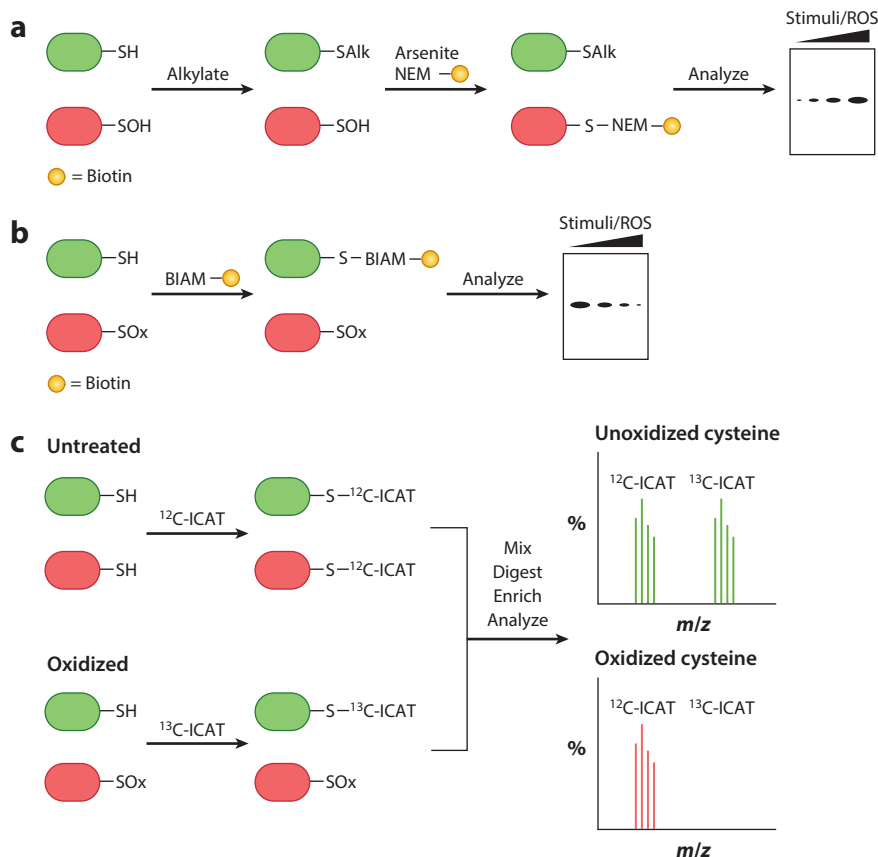


**Figure 5**

Oxidative modifications of cysteine residues by  $\text{H}_2\text{O}_2$ . Abbreviations: GR, glutaredoxin; Grx, glutaredoxin reductase; TR, thioredoxin; Trx, thioredoxin reductase.

(Prx), and suggest that proximity to their Nox oxidant source may be an important determinant of protein oxidation (68). Oxidized cysteine residues can exist as stable sulfenic acids if they are inaccessible to low-molecular-weight thiols, such as glutathione (GSH), and such stable modifications have been observed in more than 40 proteins (69–71). Depending on the surrounding protein microenvironment and redox conditions, the sulfenic acid may also undergo secondary reactions to yield more stable cysteine oxoforms, such as disulfides, cyclic sulfenamides (72–75), sulfinic acids ( $\text{RSO}_2\text{H}$ ), and sulfonic acids ( $\text{RSO}_3\text{H}$ ) (76–78). The condensation of a sulfenic acid to a disulfide or sulfenamide protects against irreversible overoxidation (73, 74), as S–S and S–N bonds can be reduced through the activity of thioredoxin/thioredoxin reductase (Trx/TR) or glutaredoxin/glutaredoxin reductase (Grx/GR) systems (68, 79).

Cysteine sulfenylation has emerged as an important oxPTM in the spatial and temporal regulation of protein activity during redox signaling events (80). Sulfenic acids have been identified in the catalytic cycle of multiple enzymes, and the formation of sulfenic acids has also been linked to oxidative stress–induced transcriptional changes in bacteria (81, 82). Less is known about the mechanisms that underlie sulfenic acid–mediated regulation of mammalian protein function and signaling pathways; however, the oxidation of cysteine residues has been associated with an expanding range of biological activities and has been implicated in the regulation of metabolism, immune responses, stem cell biology, pathogenesis of cancers, neurodegeneration, and growth factor signaling pathways (67, 83, 84).



**Figure 6**

Indirect approaches to detect protein sulfenic acids. (a) Modified biotin switch technique adapted to indirectly detect sulfenic acid-modified proteins (85, 86). (b) Loss of reactivity with thiol-modifying reagents, such as BIAM, indirectly monitors cysteine oxidation. (c) ICAT reagents determine the ratio of oxidized cysteine residues (87, 88). Abbreviations: BIAM, biotinylated iodoacetamide; ICAT, isotope-coded affinity tag; NEM, *N*-ethylmaleimide; ROS, reactive oxygen species.

## Indirect Methods to Detect Sulfenic Acids

Indirect approaches monitor the reactivity of unoxidized cysteines as a proxy for changes in redox homeostasis. Oxidized cysteines are identified by either the loss of labeling by thiol-modifying reagents or the restoration of labeling after reduction. These indirect approaches block all free thiols with alkylating agents and therefore are typically restricted to analyses of cell lysates or purified proteins. The main indirect chemical method is derived from the biotin switch assay and involves (a) alkylation of free thiols, (b) reduction of sulfenylated cysteines with arsenite, and (c) labeling of nascent thiols with biotinylated *N*-ethylmaleimide (NEM) or other tagged alkylating agents (**Figure 6a**). The biotinylated proteins can be enriched and digested for proteomic analysis or imaged by Western blot analysis to assess changes in cysteine oxidation (85, 86). Alternatively, unoxidized cysteines can be alkylated using biotinylated iodoacetamide (BIAM), in which loss of labeling correlates with sulfenylation (**Figure 6b**). A variation of this approach led to the development of an acid-cleavable BIAM-based isotope-coded affinity tag (ICAT) that allows for

the simultaneous identification and quantification of redox-sensitive cysteine thiols (**Figure 6c**) (87, 88).

## Nucleophilic Methods to Directly Detect Sulfenic Acids

Several methods that take advantage of the electrophilic nature of sulfenic acids have been developed for their direct detection. The selective reaction between dimedone and protein sulfenic acids was first reported in 1974 by Benitez & Allison (89) (**Figure 7a**). Under aqueous conditions, cyclic 1,3-diketones do not react with thiols, sulfinic acids, or other functional groups commonly found in biomolecules (90). Indeed, dimedone itself can be a powerful tool to identify sulfenic acid modifications through whole-proteome shotgun proteomics (91), and the chemically selective reaction of dimedone has been exploited to detect sulfenylated proteins by MS, fluorescence, and avidin blotting approaches (92).

Dimedone lacks spectral or affinity tags to aid in subsequent analysis of sulfenylated proteins. The first dimedone-based probes consisted of 1,3-cyclohexadione nucleophiles directly conjugated to fluorophores such as isatoic acid and 7-methoxycoumarin (DCP-MCC) (**Figure 7b**) (90). A stable protein sulfenic acid derived from  $\text{H}_2\text{O}_2$  treatment of a mutant form of the bacterial peroxidase AhpC (C165S) selectively reacted with the fluorophore-functionalized 1,3-cyclohexadione derivatives. Additionally, the compounds did not react toward protein thiols, disulfides, nitrosothiols, sulfinic acids, or sulfonic acids.

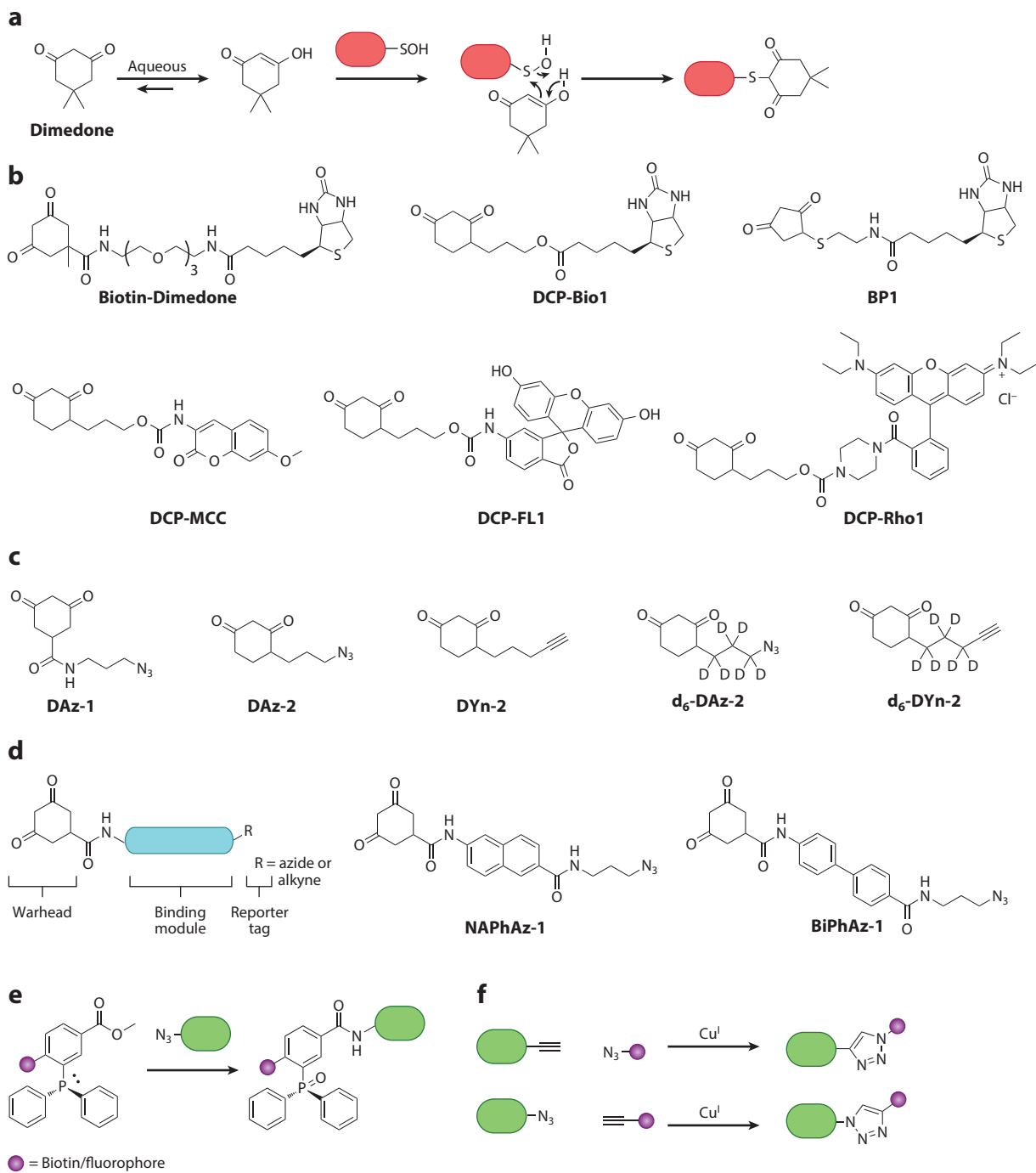
Probes that incorporate biotin handles (Biotin-Dimedone and DCP-Bio1) for affinity tag isolation, or fluorophore tags such as fluorescein (DCP-FL1) and rhodamine (DCP-Rho1) for visualization, have also been synthesized (**Figure 7b**) (93, 94). Uses of these probes to study sulfenylated proteins in cells and in cell lysates include a proteomic analysis of isolated  $\text{H}_2\text{O}_2$ -perfused rat hearts to understand oxidative stress in cardiac tissues (94); detection of vascular endothelial growth factor (VEGF)-stimulated oxidation of proteins to elucidate VEGF receptor signaling and angiogenesis (95, 96); and detection of sulfenylated PTPs and protein tyrosine kinases (PTKs) during cell signaling (97–99). Furthermore, 1,3-diketone nucleophiles that deviate from the quintessential dimedone scaffold, such as 4-(ethylthio)cyclopentane-1,3-dione (BP1) (100) and  $\beta$ -ketoesters (101), have also been reported to act as chemoselective nucleophiles toward sulfenylated proteins (**Figure 7b**).

Direct conjugation of a probe to biotin or a fluorophore can have detrimental effects on the efficiency of labeling. Commonly, tagged derivatives suffer from diminished cell uptake and trafficking properties (102–105). As a consequence, sulfenylated proteins often need to be labeled from cell lysate. However, the redox balance of the cell is disrupted by lysis (106), leading to artificial oxidation, which can both increase the probability of observing false positives and overoxidize sulfenylated proteins. Additionally, biotin- or fluorophore-tagged probes display decreased binding efficiency and biased protein labeling due to the increased steric bulk of the probe (104, 107).

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### Figure 7

Dimedone-based probes for chemoselective detection of protein sulfenic acids. (a) Chemoselective reaction to yield a stable thioether adduct. (b) Dimedone-based probes directly conjugated with biotin or fluorescent tags (90, 93, 94, 100, 101). (c) Dimedone-based probes with bioorthogonal handles for subsequent enrichment or detection of sulfenic acid-modified proteins (71, 101, 108, 114, 116, 118). (d) Redox-based probes that target the redox-sensitive catalytic cysteine in protein tyrosine phosphatases through incorporation of a chemical scaffold with high affinity for the enzyme's active site (107, 124). (e,f) Bioorthogonal reactions for appending tags to dimedone-based probes with handles (109, 110). Abbreviations: BP1, 4-(ethylthio)cyclopentane-1,3-dione; DCP-FL1, fluoresceinamine-5'-N-[3-(2,4-dioxocyclohexyl)propyl]carbamate; DCP-MCC, 3-(2,4-dioxocyclohexyl)propyl 7-methoxy-2-oxo-2H-chromen-3-ylcarbamate; DCP-Rho1, rhodamine B [4-[3-(2,4-dioxocyclohexyl)propyl]carbamate]piperazine amide.



An alternative approach for the detection of sulfenylated proteins within cells utilizes small, membrane-permeable dimedone derivatives bearing either an alkyne or azide handle (**Figure 7c**). These smaller probes alleviate issues surrounding diffusion across the cell membrane (**Figure 7c**). DAz-1 consists of a 1,3-cyclohexanedione nucleophile functionalized with an azide handle through an amide bond at the 5 position (108). Following covalent modification of sulfenylated proteins, biotin or fluorophore tags can be conjugated onto the azide via Staudinger ligation (**Figure 7e**) or Huisgen [3 + 2] cycloaddition (click chemistry) (**Figure 7f**) (109, 110). DAz-1 is selective for sulfenylated proteins in their purified form and in living cells (71). Initial studies with DAz-1 revealed differences in protein labeling patterns between cell lysates and live cells, highlighting the importance of probing intact cells to investigate the redox regulation of proteins. DAz-1 was also used to study the importance of sulfenylation of a resolving cysteine in the thiol peroxidase (Gpx3), which senses oxidative stress in yeast (111), and to identify a unique reducing system in the bacterial periplasm that protects single cysteine residues from oxidation (112).

Subsequent dimedone-based probes, DAz-2 and DYn-2, directly incorporate an azide or alkyne handle at the 4 position of the warhead and show enhanced nucleophilicity over DAz-1 (**Figure 7c**) (113). DAz-2 was used to profile the global sulfenome in human tumor cell lines and uncovered more than 175 new sulfenylated proteins, which are distributed throughout the cell and function in a diverse array of biological processes (114). DYn-2 incorporates an alkyne handle because several studies indicate that alkynyl-chemical reporters, in combination with azido-detection tags, offer superior sensitivity relative to the reverse combination (105, 115). Recently, DYn-2 was used in global profiling studies to reveal dynamic protein sulfenylation during EGF signaling in human epidermal A431 cells and determined that a key residue (Cys797) of the EGF receptor kinase is susceptible to sulfenylation and that it regulates receptor kinase activity. Additionally, several PTPs were found to be sulfenylated in response to EGF stimulation, and the sensitivity of each protein toward oxidation correlated with its proximity to the oxidant source (Nox). EGFR is the most sensitive to oxidation because it forms a complex with Nox2 (116). Therefore, it is surmised that the extent of sulfenylation of redox-sensitive proteins depends on their proximity to the Nox oxidant source and is likely to be distinct for varying cell signaling pathways and cell type-specific phenomena.

## Immunological Methods to Detect Sulfenic Acids

Immunological methodologies may offer enhanced selectivity and sensitivity for detecting sulfenylated proteins from a complex proteome. To this end, an antibody that is specific for the dimedone-cysteine thioether adduct epitope has been developed and applied to a protein microarray to monitor differences in sulfenic acid modification across various cancer cell lines (103). Additionally, the dimedone antibody has been used to demonstrate cysteine sulfenylation during EGF signaling and colocalization of the oxidized proteins with Nox2 (116). A similar antibody has been employed to detect dimedone-modified glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and to study GAPDH sulfenylation in cardiac myocytes exposed to exogenous H<sub>2</sub>O<sub>2</sub> (117).

## Methods to Quantify Sulfenic Acids

In addition to detection, identification, and mapping of sulfenylated proteins, dimedone-based probes can be used to quantify redox-dependent changes associated with physiological and pathological processes. The isotope-coded d<sub>6</sub>-dimedone and 2-iododimedone (ICDID) strategy was developed for this purpose. The key feature of the ICDID strategy is that the pair of probes generate nearly chemically identical proteins that differ only in a 6-deuterium label. The ICDID method consists of the following steps: (a) Sulfenylated proteins are derivatized with d<sub>6</sub>-dimedone, (b) excess reagent is removed and free thiols are labeled with iododimedone, (c) protein samples



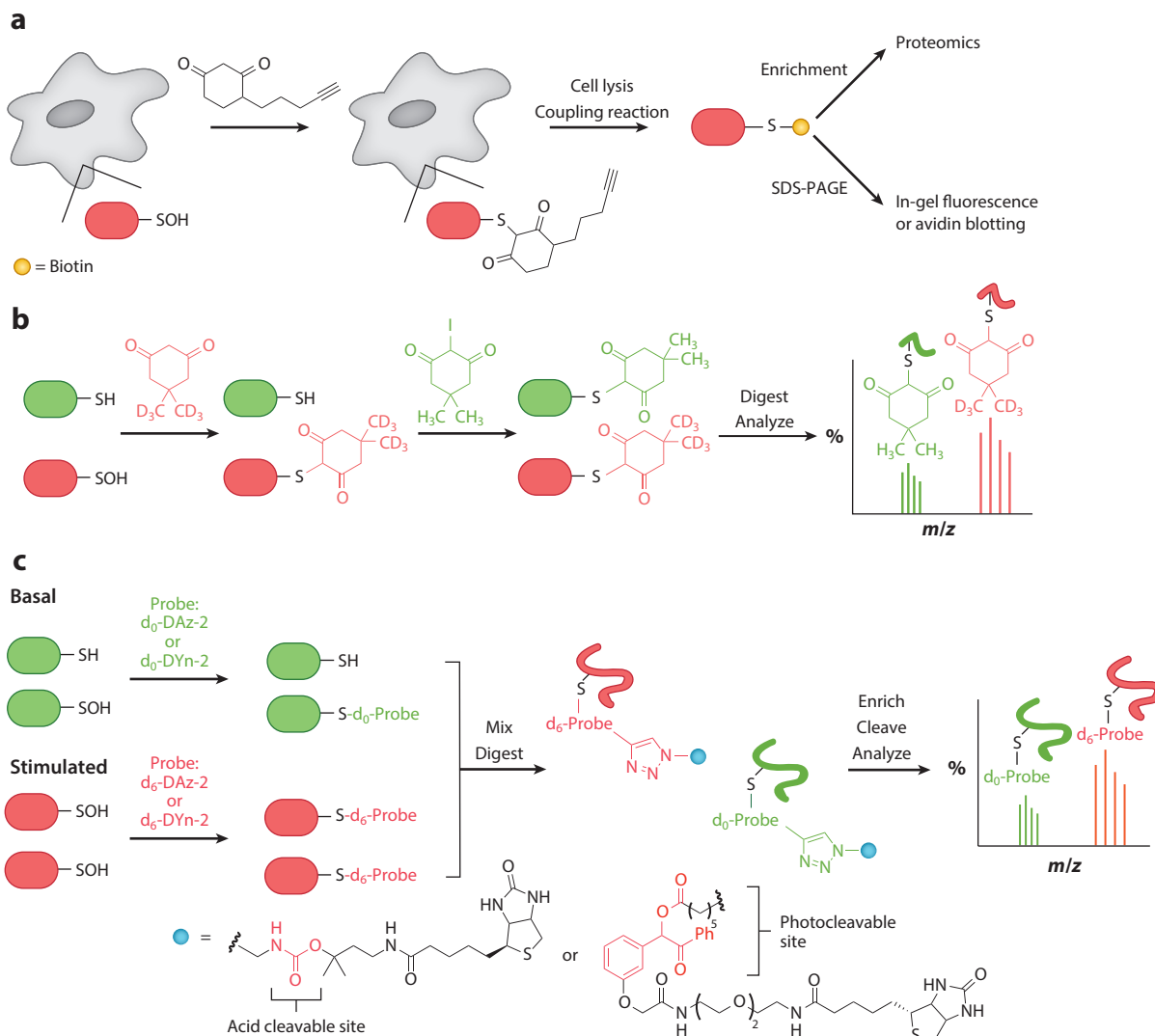
are digested, and (*d*) the resulting peptides are separated and analyzed by liquid chromatography–mass spectrometry (LC-MS) (**Figure 8b**). The extent of sulfenylation of a particular cysteine residue can be determined by dividing the heavy-isotope-labeled peak by the sum of the heavy- and light-isotope-labeled peaks (118).

Alternatively, a set of isotopically light and heavy versions of DAZ-2 were developed to monitor relative changes in protein sulfenylation (119). A key feature of the isotopically labeled DAZ-2 is that it facilitates mapping and quantifying the specific site of cysteine oxidation. The use of biotin tags for enrichment and purification of tagged protein sulfenic acids can give rise to complicated tandem mass spectrometry (MS/MS) spectra due to fragmentation of the biotin handle, making database searching challenging. Therefore, it would be ideal to remove the biotin tag after enrichment of the labeled proteins or peptides, and various cleavable linkers have been reported for this purpose. The isotopically labeled DAZ-2 derivatives can be used in combination with a trifluoroacetic acid–cleavable linker to facilitate enrichment, elution, detection, and quantification of sulfenylated proteins (**Figure 8c**) (119). Additionally,  $\beta$ -ketoesters functionalized with an alkyne handle have been reported and evaluated as  $\text{NH}_2\text{OH}$ -cleavable probes for the detection of sulfenylated proteins (101), although we note that linear diketones have been reported to cross-react with amines such as lysine (120). Recently, a highly efficient chemoproteomics work flow that utilizes isotopically light and heavy versions of DYn-2 (**Figure 7c**) was used to identify  $\sim 1,000$  sulfenylated sites on more than 700 different proteins in cells (121). Moreover, the work flow allowed for quantification of changes in cellular sulfenylation during growth factor stimulation and identified a novel site of oxidation on SIRT6, a histone deacetylase, revealing an unexpected role of this protein as a redox sensor and transducer.

### Protein-Specific Methods to Detect Sulfenic Acids

Although dimedone-based probes are broadly useful in investigations of protein cysteine oxidation, the low abundance of specific signaling proteins has hindered their detection in traditional dimedone-based approaches. Reversible PTP oxidation is an important cellular regulatory mechanism (122), and due to the lack of sensitive and robust methods for detecting oxidized PTPs (123), investigators have developed a direct method to detect oxidized phosphatases in cells to facilitate investigations of PTP redox cycling during cell signaling events. These redox-based probes (RBPs) consist of three components: (*a*) a cyclic 1,3-diketone nucleophile that reacts with oxidized active-site cysteine; (*b*) a module that directs binding to the PTP target; and (*c*) a reporter tag used for the identification, purification, or direct visualization of the probe-labeled proteins (**Figure 7d**) (107). The RBPs exhibited enhanced binding and detection of the sulfenylated PTPs compared with the parent compounds lacking the binding module. More recently, RBPs bearing an alkyne handle were used to investigate insulin-induced  $\text{H}_2\text{O}_2$  production and ROS-mediated oxidative inactivation of PTP1B (124), revealing a physiological role of redox regulation during insulin signaling. It seems likely that the RBP methodology can be applied to other phosphatases to facilitate a better understanding of their role in redox biology.

Immunochemical approaches have also been developed to detect specific proteins prone to oxidation. One approach utilizes an antibody that recognizes the conserved catalytic motif found in all PTPs in conjunction with an antibody that recognizes the terminally hyperoxidized catalytic cysteine (125). This indirect method has been used for the global proteomic assessment of the PTP “redoxome” (126). Importantly, this method relies on an alkylate–reduce–oxidize approach, similar to the alkylate–reduce–alkylate modified biotin switch approach mentioned above (**Figure 6a**), so it cannot differentiate phosphatases regulated by reversible oxidation from those that are inherently hyperoxidized. Another approach utilizes an antibody consisting of a single-chain variable



**Figure 8**

Methods to directly detect protein sulfenic acids. (a) Direct in situ labeling of sulfenylated proteins using cell-permeable chemoselective reagents. (b) Isotope-coded dimedone 2-iododimedone allows for quantification of sulfenylated proteins (118). (c) Use of ACLs and isotopically labeled DAZ-2 to facilitate enrichment and quantification of sulfenylated proteins (119). Abbreviations: ACL, acid-cleavable linker; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

fragment (ScFv) that has been generated to directly and selectively detect the unique structural conformation adopted by the oxidized PTP1B once the oxidized active-site cysteine condenses to the cyclic sulfenamide (127). These conformation-sensing antibodies trapped PTP1B in the inactive conformation, permitting sustained insulin signaling in HEK cells.

As phosphatases are viable therapeutic targets, in the broader sense the development of protein-targeted probes has unveiled the possibility of developing a new class of lead compounds for the development of alternative therapeutics to ameliorate diseases associated with aberrant phosphatase

activity. Indeed, trapping of an oxidized phosphatase by way of small molecules bearing a nucleophilic center may be a practical means to inhibit further catalytic activity and offers an out-of-the-box approach for the design and development of a new class of therapeutics. This concept can be further adapted to other classes of proteins that contain a redox-sensitive cysteine, such as EGFR (128–130).

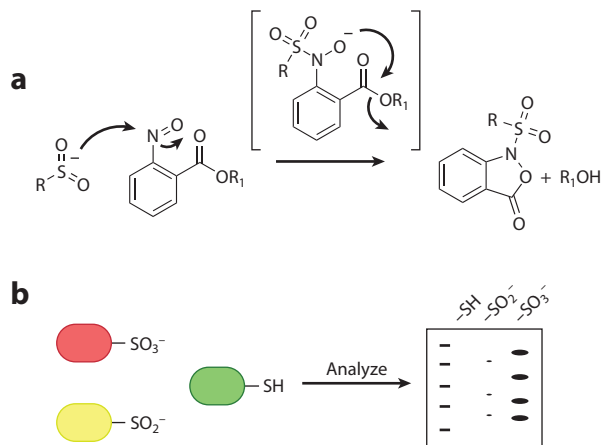
### Electrophilic Probes for Detecting Sulfenic Acids

The sulfur atom of the sulfenic acid acts as both an electrophile and a weak nucleophile. Due to this duality of cysteine sulfenic acids, electrophilic compounds can react with the oxidized cysteine residue. 7-Chloro-4-nitrobenzo-2-oxa-1,3-dizole (NBD-Cl) (131) has been used to detect sulfonylation of several proteins, including AhpC peroxidase, NADH peroxidase, OhrR repressor, PTPs, recombinant human  $\alpha_1$ -antitrypsin, and human serum albumin (HSA) (70, 131–134). Strained cycloalkynes such as bicyclo[6.1.0]nonyne (BCN) can detect sulfonylated proteins in vitro and may be used to detect sulfonylated proteins in vivo, although alternative reaction pathways may interfere because cysteine thiols are highly susceptible to thiol-yne addition and, in the presence of ROS, may lead to formation of a doubly conjugated by-product (135–139). Additionally, arylboronic acids and cyclic benzoxaboroles have been implemented for the reversible detection of sulfonylated proteins (140). Because boronic acids are susceptible to oxidation and can react with  $H_2O_2$ , these probes may not be suitable for the detection of sulfonylated proteins during redox signaling events.

### Direct Methods to Detect Sulfenic and Sulfonic Acids

Sulfenic acids can be oxidized to sulfinic acids in the presence of high concentrations of ROS, a process termed sulfinylation. Recent studies have implicated sulfinylation as a regulator of biological function. The formation and function of the cysteine sulfinic acid have been extensively characterized in Prx and have been proposed to regulate signaling events (19). The discovery of the sulfinic acid reductase sulfiredoxin (Srx), an ATP-dependent protein that specifically reduces the sulfinic acid in Prx, led to a paradigm shift in the understanding of protein regulation by  $H_2O_2$ , whereby sulfinic acids can serve a regulatory function analogous to that of a disulfide or sulfenic acid (141). Sulfinic acid has also been observed in several other proteins, including nitrile hydratase, matrilysin, and the Parkinson's disease protein DJ-1 (142–144). Current methods to detect protein sulfinic acids are relatively few, but include (a) a mass increase of 32 Da, (b) acidic electrophoretic gel shifts, and (c) antibodies that recognize a sulfinic or sulfonic acid peptide from a specific protein (145–147). Such approaches facilitate studies of sulfinic acids in individual proteins but have limited utility in global analyses.

An alternative approach is to design a probe in which the product of the reaction with the sulfinic acid is uniquely stable. Along these lines, researchers have studied aryl nitroso compounds for their unique reaction with sulfinic acids (148). The initial reaction to form a sulfinic acid-derived *N*-sulfonyl hydroxylamine product is reversible, but the intermediate can be trapped by ester-functionalized aryl nitroso compounds to yield an irreversible *N*-sulfonylbenziosoxazolone adduct with excellent first-order reaction rates ( $7.86 \text{ min}^{-1}$ ) in the formation of cyclic product (**Figure 9a**). The aryl nitroso compounds are chemoselective because reaction with a thiol yields a sulfonamide species that can be cleaved with nucleophiles. Additionally, the aryl nitroso compounds do not cross-react with other sulfur- and non-sulfur-containing biological functional groups. This novel selective ligation reaction may enable the detection of protein sulfinylation in biological systems.



**Figure 9**

Detection of sulfenic acid- and sulfonic acid-modified proteins. (a) Reaction of a sulfenic acid with an aryl nitroso compound to yield a stable cyclic sulfonamide analog (148). (b) Antibodies that recognize the sulfonic acid-modified proteins (146).

In the presence of strong oxidizing reagents, sulfenic acids can be further oxidized to sulfonic acids, which represent the highest oxidation state of the cysteine sulfur atom. The sulfonic acid modification has been characterized in a small group of proteins, including mammalian copper/zinc superoxide dismutase (Cu/Zn-SOD), in which damage resulting from hyperoxidation may play a role in diseases such as familial amyotrophic lateral sclerosis (149). Also, the sulfonic acid modification may be used to target proteins for degradation (150). Another study proposed that sulfonic acid modification of Prx can enhance its proposed chaperone activity (151). Elucidation of the sulfonic acid modification in biological and pathological states has been hindered by a lack of tools that can trap and tag the sulfonic acid modification on proteins. The use of antibodies to detect sulfonic acid is the most common technique for detection in specific proteins (**Figure 9b**) (146). However, the antibodies cannot be used to detect sulfonic acids in a whole proteome, and they cannot clearly differentiate the sulfenic from the sulfonic acids (152). Polyarginine (PA)-coated nanodiamonds have been used to selectively enrich sulfonic acid-modified peptides. BSA, used as a model system in this study, was oxidized with performic acid and digested; then sulfonic acid-containing peptides were enriched and eluted from PA-coated nanodiamonds with phosphoric acid, enabling the identification of oxidized peptides by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (152).

## OUTLOOK

As the perceived role of  $H_2O_2$  in biology has evolved from toxin to immune agent to signaling agent, so too has the chemical toolbox for studying ROS expanded in breadth and depth. Fluorescence and bioluminescence imaging offer attractive modalities for visualizing the sources and generation of  $H_2O_2$  and for exploring the role of this potentially harmful molecule in both pathology and physiology. Dimedone-based probes have shown great utility in the identification of the cysteine targets of  $H_2O_2$ -derived oxPTMs. However, the understanding of the importance of cysteine oxidation and of the roles it plays in redox biology continues to expand, and so must the chemical toolbox for elucidation of oxidative modifications in biological settings. The cellular lifetime of sulfenic acids has been hypothesized to be relatively short, and recent studies from the

Carroll group (113) have shown that the reaction between dimedone and a sulfenic acid is relatively slow. Thus, it is likely that many sulfenylated proteins remain undetected due to incomplete trapping of this oxPTM by dimedone-based probes. We need to expand our search toward novel nucleophiles that show enhanced reactivity and selectivity toward sulfenic acid. Moreover, the emergence of protein sulfinylation as a cell signaling mechanism motivates the development of robust tools and new approaches that can identify protein targets that are susceptible to sulfinylation. The widespread ability of different tissues to produce  $\text{H}_2\text{O}_2$ , and its relevance to numerous biological pathways and systems, presages further integration of this enigmatic ROS in the processes of life.

### SUMMARY POINTS

1. Nox-derived  $\text{H}_2\text{O}_2$  is an important signaling agent in biological systems, and  $\text{H}_2\text{O}_2$ -mediated oxidation of cysteines to cysteine sulfenic acids appears to play a key role in many signaling pathways.
2. The selective reaction between  $\text{H}_2\text{O}_2$  and aryl boronates has allowed the design of many aryl boronate-based probes, which offer a means to visualize  $\text{H}_2\text{O}_2$  fluxes with high spatial and temporal resolution in living systems when used with appropriate controls.
3. Based on the specific application, aryl boronate probes with differing excitation/emission wavelengths and subcellular localization can be selected for  $\text{H}_2\text{O}_2$  visualization.
4. Dimedone-based probes offer a way to visualize and identify the cysteine targets of  $\text{H}_2\text{O}_2$  oxidation. Many variants allow cysteine sulfenic acid detection by fluorescence or Western blot.
5. Dimedone-based probes have been developed for proteomics applications, in which sulfenic acid-containing proteins can be identified and the extent of sulfinylation can be quantified.

### FUTURE ISSUES

1. Whereas the sensitivity of current aryl boronate-based probes can detect endogenous  $\text{H}_2\text{O}_2$ , it is likely that many physiological  $\text{H}_2\text{O}_2$  fluxes remain below their detection limit. Detecting very small amounts of  $\text{H}_2\text{O}_2$  is an important goal for future methodologies.
2. Quantification of  $\text{H}_2\text{O}_2$  fluxes remains a difficult task with currently available small-molecule methodologies. This challenge will require the development of reversible, ratiometric sensors based on different chemical reactions than the ones currently employed.
3. Although dimedone-based probes have demonstrated broad utility in identifying sulfenylated proteins, their relatively slow reactivity may hinder the detection of low-abundance or transiently sulfenylated proteins. The development of new probes with faster reaction kinetics will be an important goal for the future.
4. Current methodologies for the detection of cysteine sulfinic acids and sulfonic acids have significant limitations. The development of new, broadly applicable detection methodologies will enhance our understanding of the scope of these oxPTMs, as well as the role(s) they play.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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