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CHEMICAL REVIEWS

Review

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Cysteine-Mediated Redox Signaling: Chemistry, Biology, and Tools for Discovery

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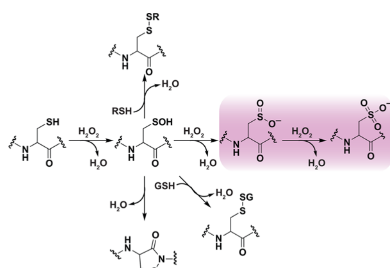

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1. INTRODUCTION

Reactive oxygen, nitrogen, and sulfur species, referred to as ROS, RNS, and RSS, respectively, are produced during normal cell function and in response to various stimuli. An imbalance in the metabolism of these reactive intermediates results in the phenomenon known as oxidative stress. If left unchecked, oxidative molecules can inflict damage on all classes of biological macromolecules and eventually lead to cell death. Indeed, sustained elevated levels of reactive species have been implicated in the etiology (e.g., atherosclerosis, hypertension, diabetes) or the progression (e.g., stroke, cancer, and neurodegenerative disorders) of a number of human diseases.[1] Over the past several decades, however, a new paradigm has emerged in which the aforementioned species have also been shown to function as targeted, intracellular second messengers with regulatory roles in an array of physiological processes.[2] Against this backdrop, it is not surprising that considerable ongoing efforts are aimed at elucidating the role that these reactive intermediates play in health and disease.

Site-specific, covalent modification of proteins represents a prominent molecular mechanism for transforming an oxidant signal into a biological response. Amino acids that are candidates for reversible modification include cysteines whose thiol (i.e., sulfhydryl) side chain is deprotonated at physiological pH, which is an important attribute for enhancing reactivity. While reactive species can modify other amino acids (e.g., histidine, methionine, tryptophan, and tyrosine), this Review will focus exclusively on cysteine, whose identity as cellular target or "sensor" of reactive intermediates is most prevalent and established.[3] Oxidation of thiols results in a range of sulfur-containing products, not just disulfide bridges, as typically presented in biochemistry textbooks. An overview of the most relevant forms of oxidized sulfur species found in vivo is presented in Chart 1.

Sulfur occupies a unique position in biology because of its ability to adopt a wide range of oxidation states (−2 to +6) and chemically unique forms or "chemotypes"[3a] each with distinct pathways of formation, physical and reactivity properties. Redox reactions of cysteine residues can lead to an array of post-translational modifications that are an important mechanism for the regulation of proteins from all major functional categories

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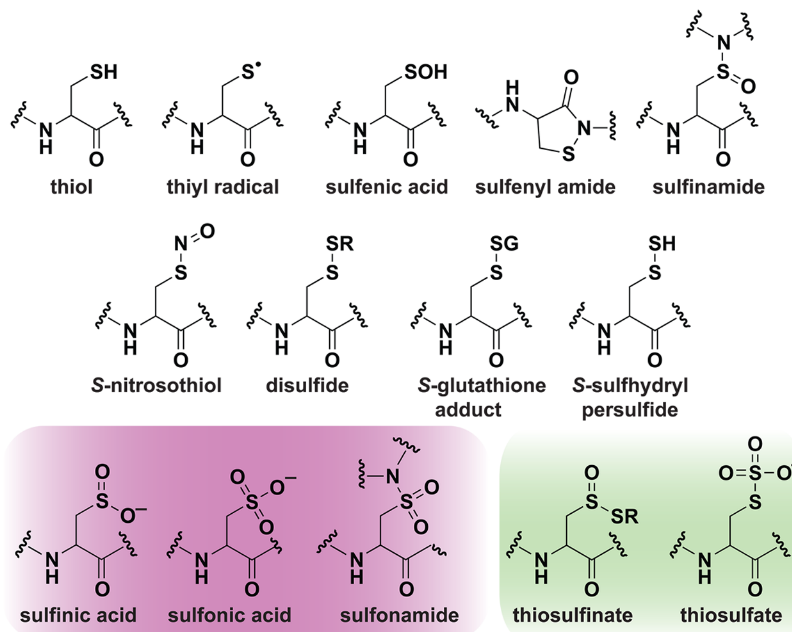
Chart 1. Biologically Relevant Cysteine Chemotypes[a]



[a]Red, irreversible modifications. Green, unique enzyme intermediates. Note: Additional modifications can form as enzyme intermediates including thiyl radicals, disulfides, and persulfides.

(e.g., enzymes, contractile, structural, storage, and transport proteins). Among these modifications are reversible, regulatory disulfides, thiosulfinates, *S*-glutathionylation, sulfenic acids, sulfenamides, sulfinamides, *S*-nitrosylation, and persulfides in conjunction with largely irreversible species, such as sulfinic acids, sulfonic acids, and sulfonamides that are often viewed as hallmarks of oxidative stress and disease.[4] In regards to terminology, we note that the "-yl-" particle in the terms above has gained widespread use in recent years[5] as an analogy to other post-translational modifications, such as phosphorylation or acetylation, and is not intended to indicate a specific mechanism of *S*-group attachment or a radical-associated process.

The reversibility of many oxidative post-translational modifications (oxPTMs) of cysteine thiols highlights their ability to function as a binary "switch", regulating protein function, interactions and localization, akin to phosphorylation. Given this analogy, and the discovery of biological RO/N/S-generating systems, it not surprising that investigation of cell signaling pathways involving oxidation of cysteine residues has emerged as an extremely active area of research. However, elucidating the functional role of cysteine oxPTMs in normal physiology and disease has been hampered, in part, because of the difficultly in detecting these modifications in complex biological systems with chemical specificity. After a brief introduction reprising major RO/N/S species produced by cells and mechanisms of thiol oxidation, we focus this review on different oxPTMs of protein cysteine thiols, with particular emphasis on those chemical properties that differentiate one modification from another. In keeping with this general theme, we review recent progress in using chemical approaches to develop probes that enable selective and direct detection of individual modifications within their native cellular environment. Along the way, we complement this discussion with examples from the literature that highlight ways in which cysteine oxidation can be used to control protein function and cell signaling pathways.

2. CYSTEINE REACTIVITY AND OXIDANT SENSITIVITY

Ionization constants (pK_a) for the low-molecular weight thiols, cysteine (Cys), and glutathione (GSH), are 8.3 and 8.8, respectively. However, pK_a values for cysteine residues in proteins can be strongly influenced by the local environment. For example, the two active-site cysteines in the DsbA disulfide oxidoreductase have pK_a values of 3.5 and 10.[6] Low pK_a protein thiols, particularly those ionized at physiological pH, are often referred to as "reactive cysteines".[7] Features of the protein environment that can facilitate thiol ionization include proximity to positively charged amino acids,[8] hydrogen bonding,[9] and location at the N-terminal end of an α-helix (N_{cap}).[10] For example, N_{cap} effects on cysteine reactivity have recently been noted in the thiol peroxidase, peroxiredoxin 1 (Prx1),[11] and the epidermal growth factor receptor (EGFR) kinase.[11b,12]

Although the molecular basis remains incompletely understood, empirical observations indicate that not all cysteine residues in an individual protein are equally sensitive to oxidation. Since thiolates are much stronger nucleophiles than thiol groups, one key factor in oxidization susceptibility is low pK_a. This fact is highlighted by the observation that many biological oxidants, such as hydrogen peroxide (H_2O_2), react exclusively with the thiolate anion.[13] On the other hand, as noted by Winterbourn and Hampton, low pK_a is not the only determinant of oxidant reactivity.[14] To illustrate this point, one need only to consider the 1 000 000-fold difference in reaction rate constants of H_2O_2 with the active site cysteine of peroxiredoxin 2 ($pK_a \approx 5-6$; 2×10^7 M^{-1} s^{-1})[15] and protein tyrosine phosphatases (PTPs), such as PTP1B ($pK_a \approx 5.4$; 20 M^{-1} s^{-1}).[16] Structural and functional studies suggest that the superior reactivity of Prx2 is due to a protein environment that is preorganized to activate both the peroxidatic cysteine and the

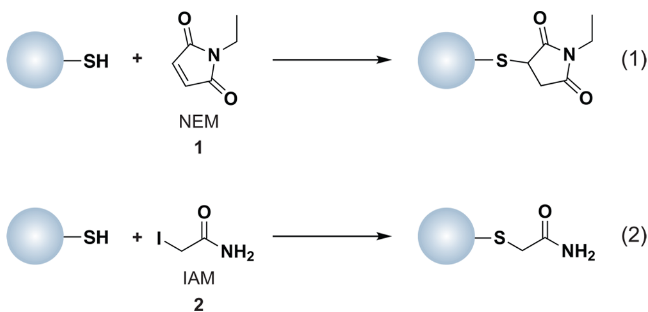
peroxide substrate, as well as to stabilize the transition state for the S_N2 substitution reaction.[11a,15] In short, low pK_a protein thiols are prime candidates for oxidation, but it is also important to recognize that "reactive cysteine" and "oxidant-sensitive cysteine" are not always synonymous with one another. A more extensive discussion of this topic has been presented by Winterbourn and colleagues.[2d,14]

2.1. Methods to Identify Low-pK_a Cysteine Residues

From first principles, we know that cysteine reactivity depends on features of the local protein microenvironment; however, there is still much to learn about sequence and structural motifs that are associated with lowering cysteine thiol pK_a.[9b] One approach to understand these features is to generate a comprehensive list of proteins that harbor low pK_a cysteines and collate this information with sequence and three-dimensional (3D) structural data. To this end, a number of methods have been developed to identify low pK_a cysteine residues in proteins.

Computational methods to identify reactive cysteines in the proteome are often based on the conservation of redox-active cysteine residues, particularly those required for catalysis.[17] Chemical methods typically employ reagents such as *N*-ethylmaleimide (NEM, **1**) or iodoacetamide (IAM, **2**), which form covalent adducts with sulfhydryl groups by Michael addition or nucleophilic substitution (S_N2), respectively (Chart 2). The reaction of NEM with thiols is faster than IAM and less

Chart 2. Protein Thiols React with *N*-Ethylmaleimide (NEM, 1, Equation 1) and Iodoacetamide (IAM, 2, Equation 2) by Michael Addition or S_N2 Displacement, Respectively

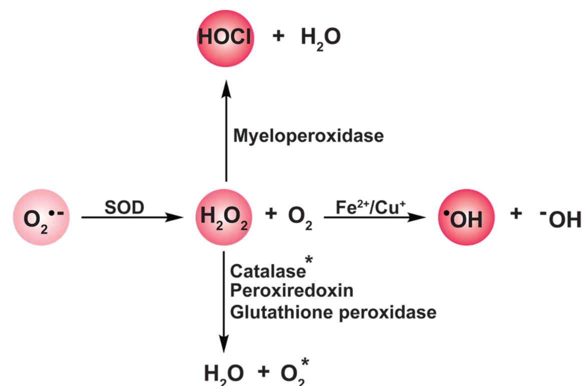


dependent on pH.[18] However, IAM is more specific for thiols than NEM, which can modify side chain amines, such as histidine and lysine, when used in large excess or at basic pH.[19] Since the thiol primarily reacts with IAM as the unprotonated thiolate anion, this reagent is most frequently used to identify low pK_a cysteines, also referred to as the "reactive thiol proteome".[18,20] Both NEM and IAM can be conjugated to biotin or fluorophores to facilitate enrichment of labeled proteins, followed by one or two-dimensional (1 or 2D) gel electrophoresis with subsequent identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In one recent example, *N*-(biotinoyl)-*N'*-(iodoacetyl)-ethylenediamine, commonly referred to as biotinylated iodoacetamide (BIAM), was used to identify surface-exposed reactive cysteine residues in *Saccharomyces cerevisiae*.[21] In yet earlier examples, BIAM and 5-iodoacetamido-fluorescein were used at low micromolar concentrations and mildly acidic pH to label reactive thiols.[22] The majority of methods for profiling reactive cysteine residues use the alkylating reagent at a single concentration; however, a recent study by Weerapana et al. employed a range of IAM concentrations and differential isotopic labeling to identify reactive cysteines.[23] Identifying low pK_a cysteine thiols affords a list of proteins that are candidates for redox-mediated modification, but additional studies are required to evaluate oxidant sensitivity.

3. REACTIVE OXYGEN SPECIES (ROS) IN BIOLOGICAL SYSTEMS

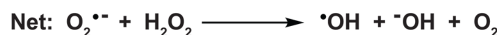
Among biologically relevant and abundant ROS (Chart 3), superoxide ($O_2^{\bullet-}$) and H_2O_2 appear most important in

Chart 3. Formation and Transformation of Biologically Relevant Reactive Oxygen Species (ROS)[a]



Haber-Weiss Reaction

Fenton Reaction



[a]Superoxide ($O_2^{\bullet-}$), formed predominantly from the mitochondrial electron transport chain and NADPH oxidase enzyme complexes (not shown), is dismutated to hydrogen peroxide (H_2O_2) and oxygen by superoxide dismutases (SOD). H_2O_2 is in turn metabolized by catalases, peroxiredoxins, and glutathione peroxidases. Additionally, H_2O_2, alone or in concert with $O_2^{\bullet-}$, can react with trace metal ions (Fe^{2+} or Cu^{+}) to generate hydroxyl radical ($^{\bullet}OH$) via Fenton or Haber−Weiss chemistry, respectively. In phagosomes, H_2O_2 serves as a substrate for myeloid peroxidase to produce hypochlorous acid (HOCl) and water. Color intensity correlates to relative ROS reactivity.

receptor-mediated signaling. Although rates of cellular $O_2^{\bullet-}$ production can be high, in most mammalian cells the steady-state concentration is estimated to be in the low picomolar range (note that cellular concentrations and half-lives for ROS are approximate and can vary considerably depending on the cell type, nutritional and environmental conditions, as well as the stage of the cell-cycle).[24] This is due to the rapid rate constant for spontaneous dismutation of $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen ($\sim10^5$ M^{-1} s^{-1}) or as catalyzed by the superoxide dismutase (SOD) enzyme family, which is 10^4 times as fast ($\sim10^9$ M^{-1} s^{-1}).[25] In turn, antioxidant enzymes, such as peroxiredoxin (Prx), catalase (CAT), and glutathione peroxidase (GPx), maintain steady-state intracellular H_2O_2 levels in the nanomolar to low micromolar range.[24b,26] Compared to other ROS in Chart 3, H_2O_2 is a mild oxidant and has the longest cellular half-life (~1 ms).[2a,24b,26,27] Owing to its relative

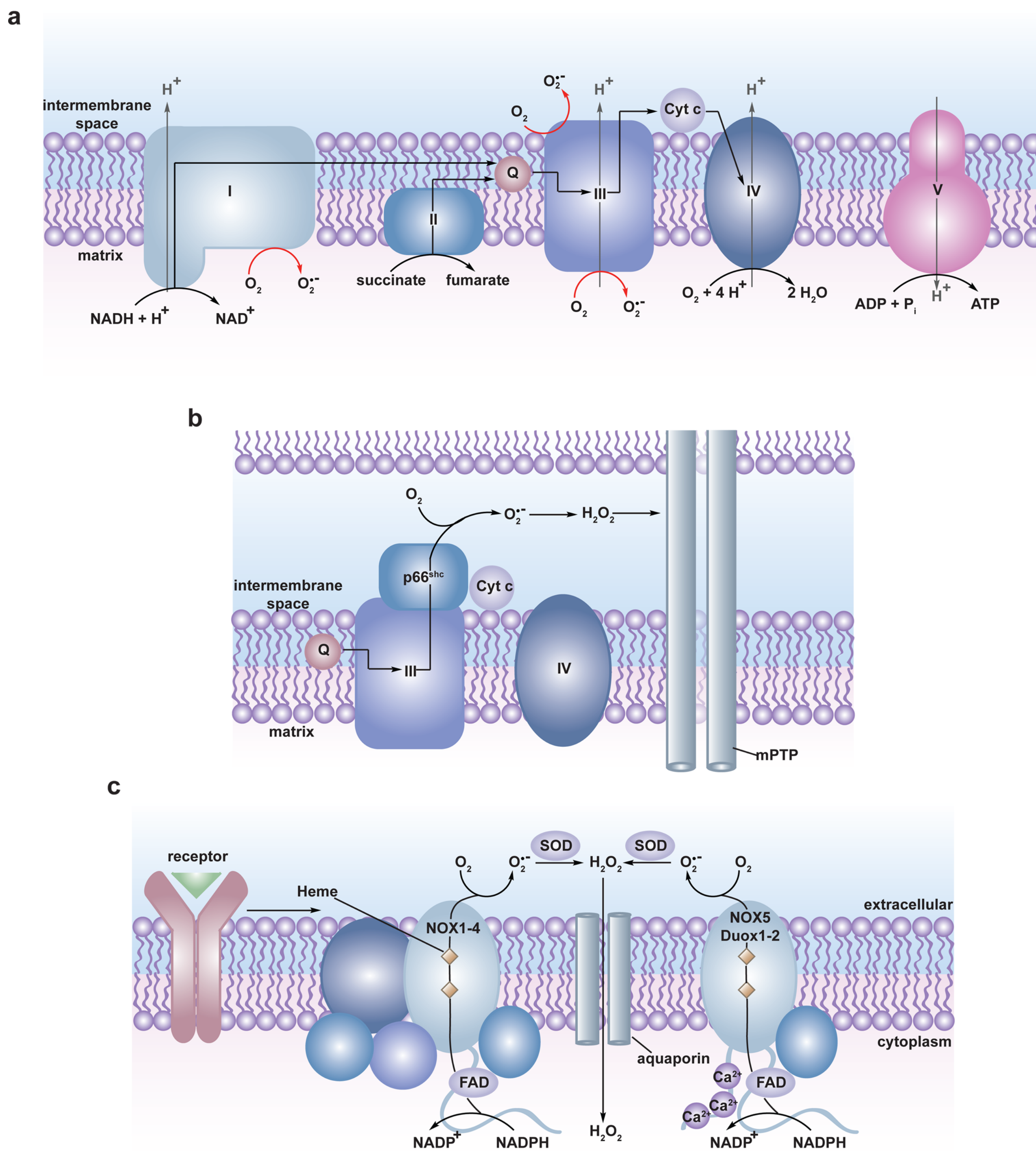


Figure 1. Biological sources of reactive oxygen species (ROS). (a) The mitochondrial electron transport chain (ETC). Four protein complexes (I–IV) funnel electrons (black arrows) from NADH and succinate in the matrix to ultimately reduce molecular oxygen to water and establish a proton gradient (gray arrows) that is harnessed by complex V to generate ATP. Electrons can leak prematurely from the ETC at complexes I and III (red arrows) to generate superoxide ($O_2^{\bullet-}$) in either the matrix or intermembrane space. (b) p66 (Shc) facilitates pro-apoptotic $O_2^{\bullet-}$ or H_2O_2 production in the mitochondria. In response to UV irradiation or growth factor deprivation, p66 (Shc) localizes to the mitochondria where it interacts with complex III to divert electrons from cytochrome c directly to molecular oxygen to generate $O_2^{\bullet-}$ or H_2O_2. This H_2O_2 can translocate to the cytoplasm (not shown) where it can influence signaling, and can regulate opening of the mitochondrial permeability transition pore (mPTP), which initiates mitochondrial swelling and apoptosis. (c) NOX enzyme complexes assemble at distinct regions of the plasma membrane or intracellular membranes to regulate localized ROS production in response to diverse signals. Receptor stimulation initiates the recruitment of specific coactivating proteins or calcium to one of seven NOX catalytic cores. Once activated, NOX enzymes funnel electrons from NADPH in the cytoplasm through FAD and heme cofactors across the membrane to generate $O_2^{\bullet-}$ (NOX1-2) or H_2O_2 (Duox1-2) on the extracellular/lumenal face. $O_2^{\bullet-}$ is dismutated to H_2O_2 and oxygen either spontaneously or as enhanced by SOD, which can translocate across the membrane by diffusion or, more likely, through aquaporin channels to regulate protein activity and signaling in the cytoplasm.

stability and selective reactivity, H_2O_2 appears well suited for a second messenger role.

The relative stability and uncharged nature of H_2O_2 may permit its diffusion through membranes, though this diffusion would be less rapid than that of gases, such as nitric oxide ($^{\bullet}NO$) and hydrogen sulfide (H_2S). Recent studies indicate that aquaporins, a family of small (24−30 kDa) pore-forming integral membrane proteins, can also mediate H_2O_2 transport.[28] Underscoring its diffusible nature and relative stability, H_2O_2 is known to function as a mobile paracrine signal to regulate plant cell differentiation[29] as well as recruitment of immune cells for wound healing in eukaryotes.[30] By contrast, the negatively charged $O_2^{\bullet-}$ does not freely diffuse across membranes (though evidence for its translocation via anion channels has been reported[31]). The protonated form of $O_2^{\bullet-}$ ($HO_2^{\bullet}$ p$K_a \approx$ 4.9) is membrane permeable but is only present in low amounts at physiological pH (<0.2% at pH 7.4). Nonetheless, $HO_2^{\bullet}$ may be relevant in phagocytes where $O_2^{\bullet-}$ may reach a steady-state concentration of ∼25 μM.[32]

H_2O_2 alone, or in concert with $O_2^{\bullet-}$, can also react with trace metal ions (Fe^{2+} or Cu^{+}) to generate the hydroxyl radical ($^{\bullet}OH$) via Fenton or Haber−Weiss chemistry, respectively (Chart 3).[33] Unlike $O_2^{\bullet-}$ and H_2O_2, whose production and metabolism are regulated processes, there are no known enzyme antioxidants for $^{\bullet}OH$ neutralization. The $^{\bullet}OH$ is a strong oxidant and reacts indiscriminately at diffusion-limited rates with protein, DNA, and lipid biomolecules,[24b,34] which contributes to its short cellular half-life (∼1 ns).[24b] In healthy cells, $^{\bullet}OH$ formation is low since H_2O_2 metabolism and metal ion concentrations are both tightly regulated to avoid toxicity. Conversely, pathologies that are associated with aberrant H_2O_2 metabolism or the presence of adventitious uncomplexed metal ions are often associated with increased $^{\bullet}OH$ production and oxidative damage. For instance, mutations in Cu,Zn-SOD linked to familial amyotrophic lateral sclerosis (FALS) enhance $^{\bullet}OH$ formation by Fenton and Haber−Weiss reactions and contribute to motor neuron degeneration.[35]

3.1. ROS Production and Metabolism

The subsections below outline important biological sources of ROS, which are formed as byproducts of respiration or by the action of enzymes. Although our discussion is focused primarily on the initial species generated by reduction of oxygen ($O_2^{\bullet-}$ and H_2O_2) important secondary products, such as hypohalous acids (HOX) are also briefly covered. The interested reader is also directed to these sources for more information about the regulation of ROS metabolism[26,36] and methods for ROS detection.[37]

3.1.1. Mitochondrial Sources of ROS. The mitochondrial electron transport chain (ETC) funnels electrons from reduced metabolic components (NADH and $FADH_2$) in the mitochondrial matrix through four protein complexes (I−IV) in which molecular oxygen serves as the terminal electron acceptor and is reduced to water (Figure 1a). The energy released during electron transfer is used to establish a proton gradient across the inner mitochondrial membrane that is harnessed to drive the production of the primary cellular energy source, adenosine-5′-triphosphate (ATP) via ATP synthase (complex V). This is an imperfect system, however, and electrons can leak prematurely from the ETC at complexes I and III resulting in the univalent reduction of molecular oxygen to $O_2^{\bullet-}$ in either the matrix (complex I and III) or the intermembrane space (complex III) (Figure 1a).[26,38] It is estimated that 0.15−2% of molecular oxygen consumed is converted to $O_2^{\bullet-}$ by the mammalian ETC.[38b,39] While this figure may seem low, mammals consume a large amount of oxygen resulting in the constitutive production of a significant amount of $O_2^{\bullet-}$ (and H_2O_2 through $O_2^{\bullet-}$ dismutation). For example, mutant mice lacking mitochondrial manganese-SOD (Mn-SOD) exhibit neonatal lethality resulting from neurodegeneration and cardiomyopathy, which may be rescued by small-molecule scavengers of $O_2^{\bullet-}$.[40] Deletion of individual SOD genes is also detrimental to bacteria[41] and yeast[42] survival further highlighting the impact of $O_2^{\bullet-}$ production in the ETC. Clearly, mitochondria are significant contributors to cellular H_2O_2 generation by dismutation of $O_2^{\bullet-}$ from the ETC.

The amount of mitochondrial-derived $O_2^{\bullet-}$ is variable[43] and regulated by a number of factors, such as oxygen concentration, proton motive force,[44] ETC efficiency,[45] and the availability of electron donors. Pathologies that include neurodegenerative disorders, cancer, and diabetes are associated with mitochondrial dysfunction and enhanced ROS production.[46] Mitochondrial stress and ROS-dependent AMP kinase activation have also been implicated in maternally inherited hearing loss.[47] Recent studies in mice and yeast have revealed an evolutionarily conserved mechanism that cells use to control mitochondrial $O_2^{\bullet-}$ production.[48] This is accomplished by adjusting the flux through metabolic pathways that regulate the flow of electrons into the ETC. Interestingly, these studies show that ROS-dependent inactivation of pyruvate kinase or a switch in isoform expression can redirect metabolic flow through the pentose phosphate pathway, which makes the reduced nicotinamide adenine dinucleotide phosphate (NADPH) required to maintain cellular redox homeostasis.

Extrinsic and intrinsic signals can also regulate mitochondrial $O_2^{\bullet-}$ production. This process is strictly dependent on the adaptor protein p66(Shc), which regulates the level of ROS, apoptosis induction, and lifespan in mammals.[49] Cell signals including growth factor deprivation, oxidative stress, or UV irradiation induce translocation of p66(Shc) into the mitochondria where it promotes electron transfer from Complex III directly to oxygen, enhancing $O_2^{\bullet-}$ production (Figure 1b).[50] After conversion to H_2O_2 through dismutation, this ROS diffuses into the cytoplasm where it decreases the activity of FoxO3, a transcription factor that regulates the expression of mitochondrial antioxidant enzymes, including Mn-SOD and catalase.[51] The reduction in antioxidant capacity further increases mitochondrial oxidative stress and enhances the pro-apoptotic function of p66(Shc).[52] Of note, mutant Mn-SOD heterozygous knockout mice exhibit marked sensitization of the mitochondrial permeability transition pore (mPTP) and premature induction of apoptosis.[53] Mice lacking p66(Shc) live ∼30% longer and show increased resistance to oxidative stress and age-related pathologies, marking it as a potential therapeutic target for diseases that are associated with oxidative damage.[26,49,50,54] Several studies suggest an additional role for mitochondrial ROS in immune system function.[55] For instance, a recent report demonstrated recruitment of mitochondria to phagosomes in infected activated murine macrophages and that mitochondrial-derived ROS was required for microbial killing.[56] Mice lacking p66(Shc) also exhibit decreased $O_2^{\bullet-}$ production in macrophages, highlighting another potential role for p66(Shc)-regulated mitochondrial ROS production.[57]

Although a thorough review of the plant literature in this area is beyond the scope of this review, we would be remiss if we did not note that in plant cells $O_2^{\bullet-}$ is also produced in the

mitochondria by the ETC, as well as other subcellular compartments, such as chloroplasts and peroxisomes through photorespiration.[58] The amount of ROS generated via photorespiration can increase in response to environmental constraints, including biotic and abiotic stresses. The interested reader is referred to the following extensive reviews for additional information on this topic.[59]

3.1.2. Enzymatic Generation of ROS. In addition to mitochondrial sources of $O_2^{\bullet-}$, this reactive intermediate can be generated as a byproduct during the catalytic cycle of numerous enzymes, such as "nonspecific" peroxidases (i.e., haem-containing peroxidases capable of using H_2O_2 to oxidize a range of substrates), as well as xanthine and aldehyde oxidases.[3a,60] Electron leakage from NADPH cytochrome P450 reductases present in the endoplasmic reticulum (ER) can also generate $O_2^{\bullet-}$ during hormone and drug metabolism.[61] The autoxidation of glyceraldehydes, reduced flavin mononucleotide ($FMNH_2$), and reduced flavin adenine dinucleotide ($FADH_2$) can also produce $O_2^{\bullet-}$, albeit with slow reaction kinetics.[24b,c] As noted above, the dismutation of $O_2^{\bullet-}$ provides a major source of H_2O_2 in cells. In addition, there are numerous enzymes that produce H_2O_2 without the intermediacy of $O_2^{\bullet-}$, including xanthine, glucose, lysyl, monoamine, and D-amino acid oxidases, as well as the peroxisomal pathway for beta-oxidation of fatty acids.[62] The contribution of these sources of $O_2^{\bullet-}$ and H_2O_2 to redox signaling remains to be determined.

In activated phagocytes of the immune system, myeloperoxidase- and eosinophil peroxidase-catalyzed oxidation of halide (Cl^-, Br^-, I^-) and pseudohalide (SCN^-) ions converts H_2O_2 to the corresponding hypohalous acid (HOX), such as hypochlorous acid (HOCl) (Chart 3).[2d,32e,63] HOXs react preferentially with thiols and methionine residues and these potent oxidants are generally believed to be responsible for much of the bactericidal activity of neutrophils. The reaction of HOCl with $O_2^{\bullet-}$ is also known to generate $^{\bullet}OH$ and is proposed to serve as the primary source of $^{\bullet}OH$ in neutrophils.[64] The interested reader is referred to the following sources for additional information on this unique class of oxidants.[2d,32e]

A variety of extracellular signals including peptide growth factors, cytokines, and G-protein-coupled receptor (GPCR) agonists and, more recently, mechanical distortion in cardiomyocytes[65] trigger deliberate production of ROS through activation of NADPH oxidase (NOX) complexes.[66] NOX-derived ROS is required for propagation of many pathways[12,65,67] and the maintenance of essential stem cell populations in the brain.[68] NOX complexes produce ROS with one of seven enzymatic cores (NOX1-5, Duox1, and Duox2) that exhibit differential cell- and tissue-specific expression patterns. As illustrated in Figure 1c, activation of NOX requires association of a flavin adenine dinucleotide (FAD) cofactor, distinct membrane and cytoplasmic coactivator proteins (Nox1-4, Duox1, and Duox2) or binding of calcium to the intracellular domain (Nox5, Duox1 and Duox2).[36a,b,d] As follows, NOX activation can be tightly controlled by signal-mediated recruitment of these coactivating proteins[69] or cofactors,[69c,70] which are likely to be pathway- and isoform-specific.

The activated NOX transports an electron from cytoplasmic NADPH through FAD and heme cofactors across plasma and intracellular membranes to produce $O_2^{\bullet-}$ on the extracellular/lumenal face (Figure 1c).[36a,b,d,71] $O_2^{\bullet-}$ is then dismutated to H_2O_2 and molecular oxygen, either spontaneously or via extracellular SOD,[72] though some NOX isoforms (Duox1 and Duox2) are equipped with an extracellular peroxidase domain that is believed to directly mediate two-electron reduction of molecular oxygen to H_2O_2.[73] Translocation of electrons from the cytoplasm across biological membranes with the concomitant release of protons from NADPH results in local acidification proportional to oxidant production. In neutrophil phagosomes, where NOX2 is estimated to produce $O_2^{\bullet-}$ at steady-state levels of 25 μM,[32d] sustained NOX2 activity is coupled to voltage-gated proton channels to mitigate local acidification.[74] A similar dependence on a voltage-gated proton channel has been demonstrated for prolonged NOX activation in active B cells.[75] The efflux of electrons also results in net positive charge accumulation on the ROS-producing face, which may promote electron transfer through NOX. Recently, a nonselective cation (Ca^{2+}, Na^+, K^+) channel called, TRPM2, was shown to be activated by NOX-derived ROS.[76] TRPM2 activation depolarized the plasma membrane, which dampened NOX-mediated ROS production in phagosomes. This finding presents a novel mechanism by which cells can regulate the amplitude and duration of NOX activity.

Within a given signaling pathway, identifying which NOX isoform is acting as the primary ROS source is usually accomplished by determining the relative expression level of each isoform using isoform-specific antibodies[12] or by over-expressing the isoform of interest.[77] However, inherent differences in antibody affinity and specificity issues can complicate these determinations, and protein overexpression does not reflect native conditions. Many cell types express multiple NOX isoforms, making it difficult to discern isoform-specific roles in a given signaling pathway, as knockout or siRNA knockdown studies are not always feasible. The participation of NOX in a given signaling pathway is commonly assessed using a number of small molecule inhibitors, including apocynin or the flavin analog, diphenyleneiodonium (DPI). These results should be interpreted with caution, as both compounds have been shown to have off-target effects in some cell types.[78] Isoform-specific NOX inhibitors would greatly assist in dissecting the role of individual NOX family members in signaling pathways.[79] For example, a peptide inhibitor that is highly specific for NOX2 has been used to study its role in vascular $O_2^{\bullet-}$ production in mice[80] and during mechanical distortion in cardiomyocytes.[65] High-throughput screens have also identified small-molecule inhibitors of NOX1[81] and NOX2.[82]

H_2O_2 that results from NOX activation can enter the cytoplasm through diffusion, or as recently shown, by transport through aquaporin channels where it can mediate distinct physiological responses, such as proliferation, differentiation, and apoptosis.[26,83] Since H_2O_2 that is produced extracellularly or in the luminal space must enter the cytoplasm to modulate intracellular signaling pathways, one key question is how can its effects be localized? Much remains to be understood about this important aspect of redox signaling, however, one possible answer is that aquaporins are directed to lipid raft membrane microdomains[84] that are also enriched for NOX. Indeed, NOX isoforms are both temporally and spatially localized to distinct membrane regions via lipid rafts,[36b] activated receptors,[12,70] and focal adhesions.[85] Depending on the stimuli and cell type, NOX family members also localize to distinct subcellular compartments, such as the ER[86] and nucleus.[87] As will be discussed in more detail below, the localized activities of NOX, as well as

antioxidant enzymes that metabolize ROS may also help restrict H_2O_2 to regions where signaling proteins are similarly localized.

3.1.3. ROS-Metabolizing Enzymes. As stated above, dismutation of $O_2^{\bullet -}$ by SOD produces H_2O_2. The peroxiredoxin (Prx) and glutathione peroxidase (GPx) families are primarily responsible for the metabolism of H_2O_2 in cells. These enzymes decompose H_2O_2 to form water and molecular oxygen in a mechanism involving the oxidation of an active site cysteine (or selenocysteine in GPxs from higher eukaryotes).[88] The enzymes are recycled back to their active, reduced form by thioredoxin/thioredoxin reductase (Trx/TrxR) or glutathione/glutathione reductase (GSH/GR) systems using reducing equivalents from NADPH. Another H_2O_2-metabolizing enzyme, known as catalase, is present mainly in peroxisomes. Plants synthesize high concentrations of ascorbate,[59b] which is used as a substrate by ascorbate peroxidases to regulate H_2O_2 bioavailability in these systems.[89] Ascorbate peroxidases are subsequently reduced by a complex metabolic pathway, known as the glutathione-ascorbate cycle.[90] A growing list of antioxidant enzymes, including Prxs, are themselves subject to redox regulation, which could permit localized accumulation of H_2O_2 for signaling while simultaneously limiting the range of H_2O_2 diffusion.[11b,91]

3.2. Modification of Protein Cysteine Thiols by ROS

The reaction of ROS with protein thiols provides a mechanism by which cells can "sense" changes in the redox balance. Though H_2O_2 is most often associated with a second messenger role, there is also evidence to suggest that $O_2^{\bullet -}$ functions in this capacity. For instance, a recent study demonstrated that disparate gradients of $O_2^{\bullet -}$ and H_2O_2 differentially regulated plant root proliferation and differentiation, respectively implicating distinct activities for these ROS.[92] $O_2^{\bullet -}$ is a relatively unreactive radical and its primary cellular targets appear to be other radical species, such as nitric oxide ($^\bullet$NO) or metals. In proteins, $O_2^{\bullet -}$ can react with iron−sulfur clusters and heme centers leading to release and/or oxidation of iron.[13] Numerous iron−sulfur cluster- and heme-containing proteins are sensitive to $O_2^{\bullet -}$, including aconitase,[93] the bacterial transcription factor SoxR,[94] guanylate cyclase,[95] and myeloperoxidase.[96] Reactivity at protein metal centers is not unique to $O_2^{\bullet -}$, however, as metal-dependent peroxide sensors like *Bacillus subtilis* PerR have also been reported.[2a,83c,97] In contrast to redox switches based on peroxide-sensitive cysteine residues, PerR senses H_2O_2 by metal-catalyzed oxidation of histidine residues involved in coordinating Fe^{2+} (note that the mechanism involves reduction of H_2O_2 by Fe^{2+} to generate $^\bullet$OH, which then reacts rapidly with histidine). H_2O_2 may also modify tryptophan and tyrosine residues through a radical-based mechanism, but such reactions are much less favored and may not be physiologically relevant.[98]

H_2O_2 can directly oxidize the thioether group of methionine to yield two diastereomeric methionine sulfoxide products;[99] however, a large body of evidence identifies cysteine as the most sensitive amino acid residue to H_2O_2-mediated oxidation. The two-electron oxidation of a thiolate by H_2O_2 yields sulfenic acid, which is increasingly implicated in a number of important biochemical transformations. Second-order rate constants for this reaction can vary dramatically in proteins (e.g., 20−10^7 M^{-1} s^{-1}).[14] Once formed, the sulfenic acid is subject to several alternative fates (Figure 2). Depending on the microenvironment, the sulfenic acid modification can be stabilized as

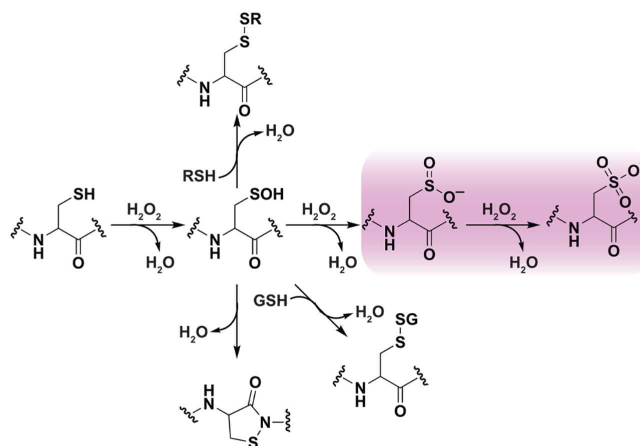


Figure 2. Oxidative modification of cysteine residues by hydrogen peroxide (H_2O_2). The initial reaction product of a low pK_a protein thiolate with H_2O_2 yields a sulfenic acid, whose stability is determined, in part, by its accessibility to additional thiols. Reaction with a second cysteine in the same or neighboring protein yields a disulfide. Alternatively, reaction with the low molecular weight thiol, glutathione (GSH) affords a specialized mixed disulfide called a glutathione disulfide. In some proteins in which a neighboring cysteine is not present, nucleophilic attack of a backbone amide on the sulfenic acid yields a cyclic sulfenyl amide. Each of these oxoforms can be reduced by the GSH/glutaredoxin or thioredoxin/thioredoxin reductase systems to regenerate the reduced thiolate (not shown). In the presence of excess H_2O_2, such as under conditions of oxidative stress, the sulfenic acid can be hyperoxidized to the largely irreversible sulfinic and sulfonic acid forms (red box).

observed in human serum albumin (HSA)[100] and more than 40 protein crystal structures.[9b,101] In this regard, there are several factors that appear to stabilize protein sulfenic acids, including the absence of thiols proximal to the site of formation or inaccessibility to low-molecular-weight thiols, such as GSH (γ-L-Glu-L-Cys-Gly).[3b] Reaction of sulfenic acid with a protein thiol or GSH yields an inter/intramolecular disulfide bridge or protein-*S*-GSH disulfide, respectively. Alternatively, in some proteins lacking a neighboring cysteine, a nitrogen atom of a backbone amide can react with sulfenic acid, forming a cyclic sulfenamide.[102] The formation of disulfide and sulfenamide states protects against irreversible overoxidation, as S−S and S−N bonds can be reduced through the activity of Trx/TrxR or GSH/glutaredoxin (Grx)/GR systems.[103] Sulfenic acid can also be reduced directly by the Trx system, through hydride transfer (H^-) from $FADH_2$ in a reaction catalyzed by NADH oxidase and NADH peroxidase enzymes from *Streptococcus faecalis*,[104] or through the DsbD/DsbG system in the bacterial periplasm.[105] In the presence of excess H_2O_2, sulfenic acid can be further oxidized to sulfinic (RSO_2H) and sulfonic (RSO_3H) oxyacids, though the observed rate constants for such reactions are generally slower (0.1−100 M^{-1} s^{-1}) than the initial thiolate oxidation event (Figure 2).[15,104b,106]

HOXs, such as HOCl, also mediate two-electron oxidation of cysteine. These reactions proceed through X^+ transfer to give an unstable sulfenyl halide, which rapidly hydrolyzes to sulfenic acid (>10^7 M^{-1} s^{-1} for HOBr and HOCl).[107] HOXs are aggressive oxidants and halogenating agents, which react with a wide range of cellular targets, including methionine, histidine, tryptophan, lysine, tyrosine, the protein backbone, nucleic acids and fatty acids. On the whole, the modifications of biomolecules that are mediated by HOX are numerous and

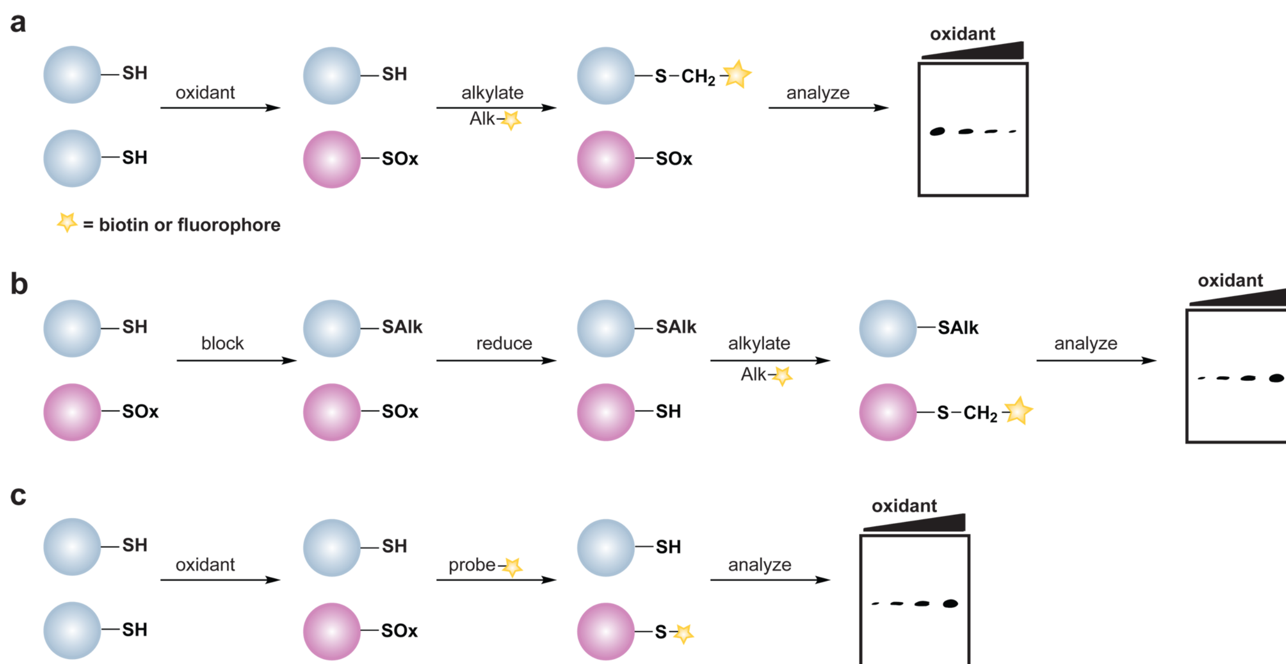


Figure 3. General overview of indirect and direct chemical methods to study protein oxidation. (a) Loss of labeling of oxidized thiols by an alkylating agent indirectly monitors protein oxidation. In response to oxidant treatment, susceptible cysteines are oxidized (purple) and thus are less reactive with alkylating agents such as NEM or IAM. Use of a biotinylated or fluorophore-conjugated alkylating agent permits detection by avidin blot or in-gel fluorescence, in which oxidized proteins exhibit a loss of signal. (b) Differential alkylation of reduced and oxidized thiols indirectly monitors protein oxidation. Free thiols (blue) are blocked with an alkylating agent such as NEM or IAM, reversibly oxidized thiols (purple) are reduced with a reducing agent such as DTT or TCEP, and nascent thiols are labeled with a second alkylating agent conjugated to biotin or a fluorophore. Oxidized proteins exhibit enhanced signal by avidin blot or in-gel fluorescence. (c) Direct chemical method to detect specific cysteine oxoforms. Samples are treated with a biotin or fluorophore-conjugated probe that selectively reacts with a distinct cysteine chemotype (purple) in which signal by avidin blot or in-gel fluorescence increases with increased protein oxidation.

highly damaging, which makes these oxidants highly effective toxic defense molecules that can be exploited by the human immune system to fight off microbial infection. As a final comment in this section, we note that the oxidation of cysteine thiols can also occur by one-electron redox pathways to give thiyl radicals, which undergo distinct sets of reactions. These transformations are briefly discussed in section 5 below (Reactive Sulfur Species (RSS) in Biological Systems) and we also refer the interested reader to the following sources for additional information.[3a,14,108]

3.3. Methods for Detecting ROS-Modified Cysteines

The reversible nature of cysteine sulfenic acid, disulfide and *S*-glutathionylation makes them well suited to control protein function during cell signaling. With the discovery of Sulfiredoxin (Srx) proteins,[109] which can convert the sulfinic acid modification back to the thiol form, cysteine sulfinic acids have also emerged as a potential regulatory mechanism. Consequently, there has been considerable effort to develop methods to study changes in protein cysteine oxPTM. These techniques include indirect and direct methods for detection. The majority of indirect methods to detect cysteine oxidation rely upon the loss of reactivity with thiol-modifying reagents (Figure 3a) or restoration of labeling by reducing agents such as dithiothreitol (DTT) (Figure 3b). The latter method requires a complete blocking of free thiols with alkylating agents prior to the reduction step and is therefore limited to studies in cell lysates or with purified proteins.

More recently, chemical biology approaches have facilitated the development of small molecule- and protein-based methods for direct detection of distinct oxidative cysteine modifications (Figure 3c). In the event that these small molecules are cell permeable, specific cysteine modifications can be detected directly in their native environment without cell disruption (i.e., lysis). This is an attractive approach since it preserves labile cysteine modifications and maintains the integrity of subcellular organelles. The latter is especially important as organelles like the nucleus, mitochondria, and cytoplasm have more reduced redox potentials whereas the secretory system and the extracellular space are more oxidizing environments.[110] Not surprisingly, cell lysis disrupts these individual redox environments and can result in substantial protein oxidation artifacts. The net result is to increase the challenges related to detecting low abundance modifications and in deciphering their biological significance. Likewise, cell disruption can hamper the detection of labile or transient cysteine modifications.

Methods to decrease oxidation artifacts in lysates have been reported, but these are often dependent upon the addition of trichloroacetic acid (which denatures proteins and can lead to acid-catalyzed overoxidation of labile modifications such as sulfenic acid) or on the addition of ROS-metabolizing enzymes to the lysis buffer.[111] Even with these considerations, lysis buffers can never accurately mimic the intracellular redox potential, thereby exposing redox-sensitive proteins to oxygen and a different redox environment. Direct detection methods may also be associated with their own limitations as the addition of a small-molecule probe to cells could alter the biological function under investigation. This issue can be addressed, at least in part, by adding the probe to cells after signal pathway activation and/or by monitoring the effect of probe addition on relevant downstream biological markers.[12]

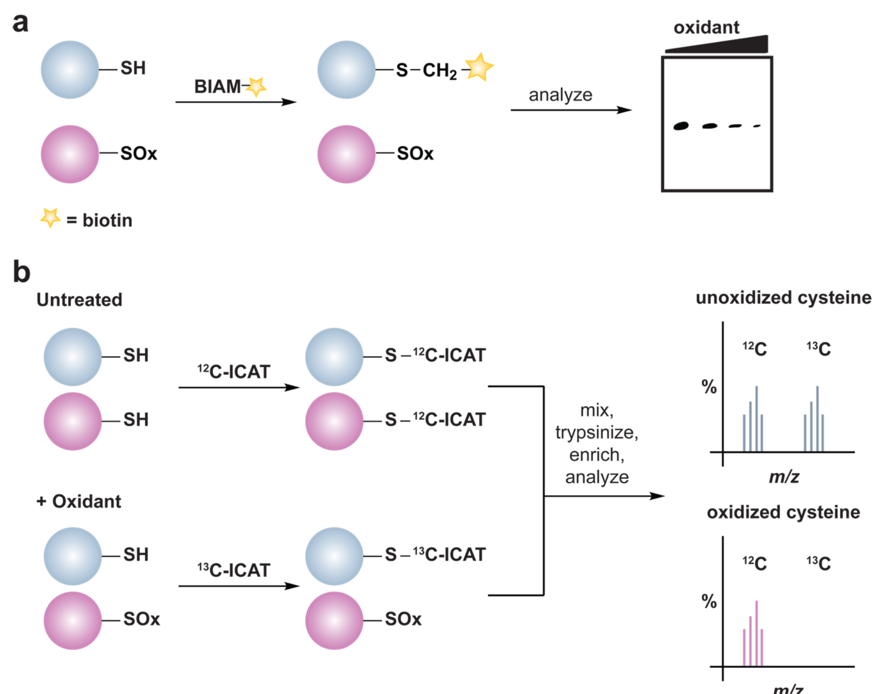


Figure 4. Indirect chemical methods to study general cysteine oxidation. (a) Loss of labeling of oxidized thiols by biotinylated-iodoacetamide (BIAM) indirectly monitors protein oxidation. Oxidized cysteines (purple) exhibit decreased reactivity with BIAM than reduced thiols, and are observed as a loss of signal by avidin blot. (b) Isotope-coded affinity tag (ICAT) reagents determine the ratio of oxidized thiols. Samples are untreated or subjected to oxidant. Free thiols are subsequently labeled with a light (^{12}C) ICAT reagent in the untreated sample and with a heavy (^{13}C) ICAT reagent in the oxidant-treated sample. As in panel a, reactive thiols (purple) exhibit decreased labeling upon oxidation. The samples are mixed, trypsinized, and enriched via the biotin affinity tag on the ICAT reagent. Eluted peptides are analyzed by LC-MS and heavy and light ICAT-labeled peptides are chemically identical, but differ in mass by 9 Da. The fraction of a thiol oxidized in the sample is determined by the ratio of heavy (^{13}C) to light (^{12}C) signal intensity, whereby thiols that are susceptible to oxidation (purple) will exhibit decreased signal intensity with the heavy ICAT reagent.

Another important consideration with direct detection methods is the rate at which probes react with the modified cysteine residue. If the reaction is slow, transient cysteine oxidation events may be missed. Conversely, if the reaction is too fast it could diminish the chemical selectivity of the probe or disrupt the biological process under study. In this way, moderately reactive probes for detecting individual oxidative cysteine modifications may be viewed as "spectators", which sample the redox-signaling environment with minimal biological impact. Increasing the concentration of probe can also compensate for modest rates of reaction, but appropriate controls must be performed to ensure that the underlying biology is not disturbed.

Collectively, indirect and direct methods to monitor cysteine oxidation have enabled the discovery of many proteins that can undergo redox modification in a wide range of organisms and different cell types. To highlight the progress made over the past few years in the redox biology field, the following subsections will independently address the chemical properties of ROS-mediated cysteine modifications and methods for their detection. We also discuss selected examples from the recent literature that highlight the ways in which distinct cysteine modifications can mediate critical biological events.

3.3.1. Indirect Approaches for Detecting ROS-Sensitive Cysteines. Several methods have been developed to monitor global changes in cysteine oxidation, but do not reveal the chemical nature of the modification. One of the most commonly used reagents for this purpose is the BIAM alkylating reagent. In these experiments, the diminished nucleophilicity of the oxidized cysteine residue results in lower reactivity with BIAM and correlates with a loss of protein labeling (Figure 4a). An adaptation of this methodology that permits simultaneous identification and quantification of oxidant-sensitive cysteine thiols employs an acid-cleavable BIAM-based isotope-coded affinity tag (ICAT).[112] In this method, free thiols are differentially alkylated with isotopic versions of the ICAT reagent and the extent of cysteine oxidation is determined by the ratio of light (^{12}C) and heavy (^{13}C) ICAT label by LC-MS/MS (Figure 4b).

A subsequent alternative approach incorporates treatment with a reducing agent into the workflow (Figure 3b). Such protocols require free thiol alkylation, a reduction step with DTT or tris(2-carboxyethyl)phosphine (TCEP), and labeling of nascent thiols with a tagged alkylating agent, such as BIAM. In this approach, changes in cysteine oxidation are detected as differences in sample BIAM alkylation as assessed by avidin blot and oxidized proteins can be identified by enrichment and LC-MS/MS analysis (Figure 3b). In addition to BIAM, alternative biotinylated or fluorophore-modified alkylating reagents can be used to differentially alkylate thiols and these methodologies have been used to monitor protein oxidation in response to exogenous oxidants (e.g., H_2O_2 or diamide)[21,113] or to ROS-promoting stimuli (e.g., peptide growth factors).[114] A similar workflow has also been used to identify substrates of the Trx/TrxR and GSH/Grx/GR systems.[113] Alternatively, protein substrates of the aforementioned reducing systems can be identified through their inclusion in the reduction step.[21] For instance, BIAM-alkylated nascent thiols will represent oxidized

proteins that were selectively reduced by the Trx/TrxR or GSH/Grx/GR systems. Together with the ICAT technology, this method has been used to identify protein disulfide targets of the Trx/TrxR system in plant extracts.[115] In addition to studying the oxidized proteome, changes in total thiol content in protein and low molecular weight thiols, including GSH and homocysteine, can be indicative of fluctuations in biological redox balance and, in some cases, serves as a diagnostic function for disease. In this vein, an active area of research is the development of sensitive probes to monitor fluctuations in total thiol content.[116]

3.3.2. Direct and Selective Approaches for Detecting ROS-Sensitive Cysteines.

3.3.2.1. Disulfides. Disulfide bond formation in proteins is a widely recognized cysteine modification that has important roles in protein folding and stability. Under normal cellular conditions, disulfide bond formation occurs largely in the extracellular space or the endoplasmic reticulum (ER). In this organelle, a class of enzymes called protein disulfide isomerases (PDI) inserts disulfides into nascent proteins that are destined for export to the extracellular milieu.[117] By comparison, disulfide bonds are rare and generally transiently formed in the cytoplasm, mitochondria, or nucleus where thiol-dependent reductases maintain a reducing environment. Exceptions exist, however, as the sulfhydryl oxidase Erv1 and oxidoreductase Mia40 form a relay system that introduces disulfide bonds in substrate proteins in the mitochondrial inner membrane.[118] Under oxidative stress conditions the intracellular redox balance can shift to support disulfide bond formation in reducing compartments until redox homeostasis is restored.

A major route of disulfide formation is by thiol condensation with sulfenic acid (Figure 2). These processes can occur either intra- or intermolecularly, and the rate of disulfide bond formation is dependent, in part, upon the distance between the two cysteine residues. Estimated rate constants for intra- and intermolecular disulfide bond formation are 10 s^{-1} and 10^5 M^{-1} s^{-1}, respectively.[119] Once formed, disulfides are relatively stable to most physiological nucleophiles and are generally cleaved by other thiols as in thiol-disulfide exchange (nucleophilic substitution) reactions (Figure 5).[120] The thiol in a disulfide with the lower pK_a will be the better leaving group and often dictates which cysteine is released in thiol-disulfide exchange. Indeed, this strategy is employed by the thiol-disulfide exchange catalysts in the cell, such as protein disulfide isomerases (PDI).[121] Disulfides can also be oxidized to generate a thiosulfinate, which can subsequently react with a thiol to give disulfide and sulfenic acid products (Figure 5). The prevalence or biological significance of the thiosulfinate is unknown, however, it is interesting to note that this species forms as an intermediate during Srx-catalyzed sulfinic acid reduction of Prxs.[122] Although the intermediate thiosulfinate is formed via a mechanism distinct from disulfide oxidation, its formation implies that the thiosulfinate may be a physiologically relevant, yet understudied modification. Further oxidation of a disulfide yields a thiosulfonate (Figure 5), which releases a disulfide and sulfinic acid subsequent to reaction with a thiol. Thiosulfonates have not been detected in cells, but could possibly be formed as an enzyme intermediate in sulfonic acid reduction akin to sulfinic acid reduction via sulfiredoxin, though an enzyme capable of catalyzing such a reaction is currently unknown.[3a]

Global studies to identify proteins that undergo disulfide bond formation implicate this modification in the regulation of,

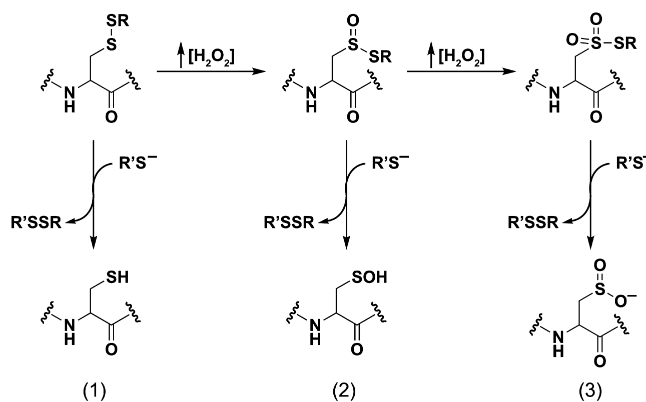


Figure 5. Possible fates of protein disulfides. Once formed, a protein disulfide (inter- or intramolecular) can undergo thiol-disulfide exchange with a third cysteine within the same or neighboring protein (eq 1). Herein, pK_a of the disulfide thiols and thiol accessibility influence which cysteine is expelled. In the presence of high concentrations of H_2O_2, disulfides can additionally be oxidized to the thiosulfinate and thiosulfonate forms, though these reactions are very slow. Because of the potential for resonance stabilization or decreased pK_a, subsequent reaction of these intermediates with a third cysteine affords a disulfide and a sulfenic acid (eq 2) or sulfinic acid (eq 3). The biological relevance of the thiosulfinate and thiosulfonate modifications is unknown due to a lack of means to study these oxoforms, however, a thiosulfinate forms as an intermediate during the sulfiredoxin catalytic cycle.

among others, redox homeostasis, chaperone activity, metabolism, transcriptional regulation, and protein translation.[111b,113] Once formed, disulfides can impact enzyme activity, subcellular localization, as well as protein−protein interactions.[71] For example, the activity of certain PTPs is inhibited by disulfide bond formation involving the active site cysteine and the so-called backdoor cysteine.[106b,123] This regulatory mechanism is also observed in certain members of the caspase family of cysteine proteases.[124] Numerous studies have demonstrated an increase in protein phosphorylation in response to receptor activation that is dependent upon endogenous H_2O_2 production.[12,65,67,68] Owing to this observation and their conserved catalytic cysteine residue, PTPs were initially proposed as the major cellular targets of signaling-derived H_2O_2.[125] Kinases are now also believed to be redox regulated, though in many cases the molecular details are much less well characterized. Nonetheless, it has been established that serine/threonine kinases PKG1α[126] and ATM[127] are activated by intermolecular disulfide formation between homodimers that, in the case of PKG1α, enhances its affinity for target proteins. By contrast, intermolecular disulfide formation between Src tyrosine kinase monomers appears to inhibit kinase activity,[128] though Src has also been shown to be activated by H_2O_2.[129] Differential regulation by H_2O_2 may be explained, in part, by modification of multiple cysteine residues. For example, oxidative inhibition of Src involves Cys277, which is not conserved in all Src family kinases.[128] The Src-family kinase Lyn, which encodes a glutamine at the site corresponding to Cys277, is activated by ROS in neutrophils suggesting that oxidative activation of this enzyme involves a different cysteine residue.[30e] Additional proteins whose activity have recently been shown to be modulated by disulfide bond formation include the bacterial chaperone Hsp33,[130] the nonspecific cation channel TRPA1,[131] and the glycolytic enzyme pyruvate kinase M2 (PKM2).[48a]

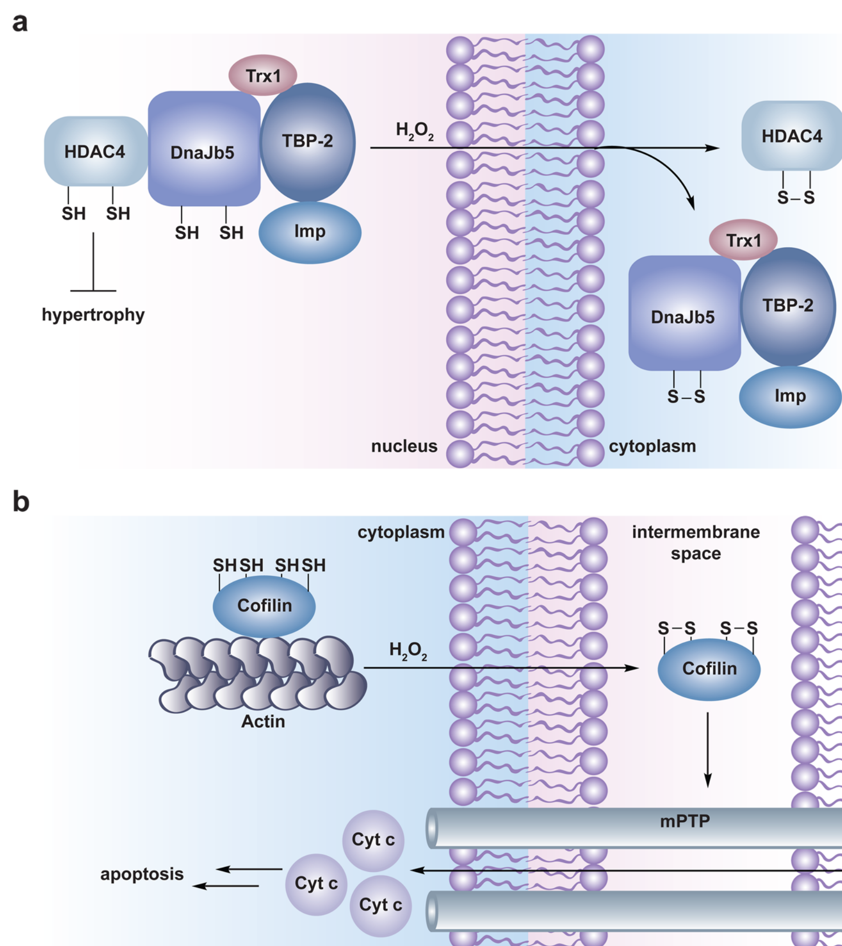


Figure 6. Disulfide-mediated redox regulation of subcellular localization and protein–protein interactions. (a) Model for redox regulation of cardiac hypertrophy by HDAC4. The class II histone deacetylase HDAC4 normally deacetylates histones to suppress expression of genes involved in cardiac hypertrophy. Nuclear localization of HDAC4 is mediated by its association with importin α (Imp) via a multiprotein complex including the small molecular chaperone DnaJb5, the thioredoxin binding protein TBP-2, and thioredoxin (Trx1). In response to oxidant, HDAC4 and DnaJb5 undergo intramolecular disulfide bond formation, which causes dissociation and nuclear export of the complex permitting derepression of genes involved in hypertrophy. Upon removal of H_2O_2, Trx1 is believed to reduce the disulfides in HDAC4 and DnaJb5 to restore assembly and nuclear localization of the complex (not shown). (b) Model for redox regulation of apoptosis by cofilin. Cofilin associates with actin in the cytoplasm to disassemble actin filaments for cytoskeletal reorganization. In the presence of H_2O_2, two intramolecular disulfides form in cofilin permitting its relocation to the mitochondria by an unresolved mechanism. In the mitochondria, cofilin interacts with the mPTP to stimulate pore opening, mitochondrial swelling, cytochrome c release, and ultimately induction of apoptosis.

Disulfide bond formation can also influence the subcellular localization of a protein and/or protein–protein interactions. For example, intramolecular disulfide formation in the *Saccharomyces cerevisiae* transcription factor Yap1 induces a conformational change that masks the nuclear export signal (NES) and precludes interaction with the nuclear export receptor, Crm1. This results in nuclear accumulation of Yap1 and active transcription of genes involved in the oxidative stress response.[132] Intramolecular disulfide formation in the small molecular chaperone, DnaJb5 and the class II histone deacetylase, HDAC4 results in sequential dissociation of the DnaJb5-HDAC4 complex, unmasking of the HDCA4 NES to mediate its cytoplasmic localization and derepression of target genes involved in hypertrophy (Figure 6a).[71,133] A recent study by Shacter and colleagues indicates that oxidative stress-induced formation of two intramolecular disulfides in the actin-regulatory protein, cofilin leads to dissociation of the actin-cofilin complex. Additionally, oxidation of cofilin enables its mitochondrial accumulation (by an unresolved mechanism) where it can interact with the mPTP to promote mitochondrial swelling, cytochrome c release, and ultimately induction of apoptosis (Figure 6b).[134]

Methods to detect protein disulfide formation often use reducing and nonreducing SDS-PAGE gel electrophoresis (Figure 7a). Intermolecular disulfides are detected as reducing agent-sensitive protein complexes that migrate at a molecular mass equal to the that of the two oxidized proteins, as seen for PKG1α,[126] Src,[128] and ATM[127] dimers (Figure 7a, right). Intramolecular disulfide bond formation can also lead to altered migration on gels, as observed for *S. cerevisiae* thiol peroxidase Gpx3,[66c,135] PKM2,[48a] or PTEN (Figure 7a, left).[123b] Cysteine residues involved in disulfide bond formation can also be identified by the differential alkylation-type approach mentioned above. In this method, thiols are alkylated prior to sample separation by nonreducing SDS-PAGE; the protein band corresponding to the oxidized proteins of interest is then reduced in-gel with DTT or TCEP, and nascent thiols are labeled with a second alkylating agent. The protein is then digested in-gel and the differentially alkylated cysteine residues are identified by LC-MS/MS analysis.[127,134]

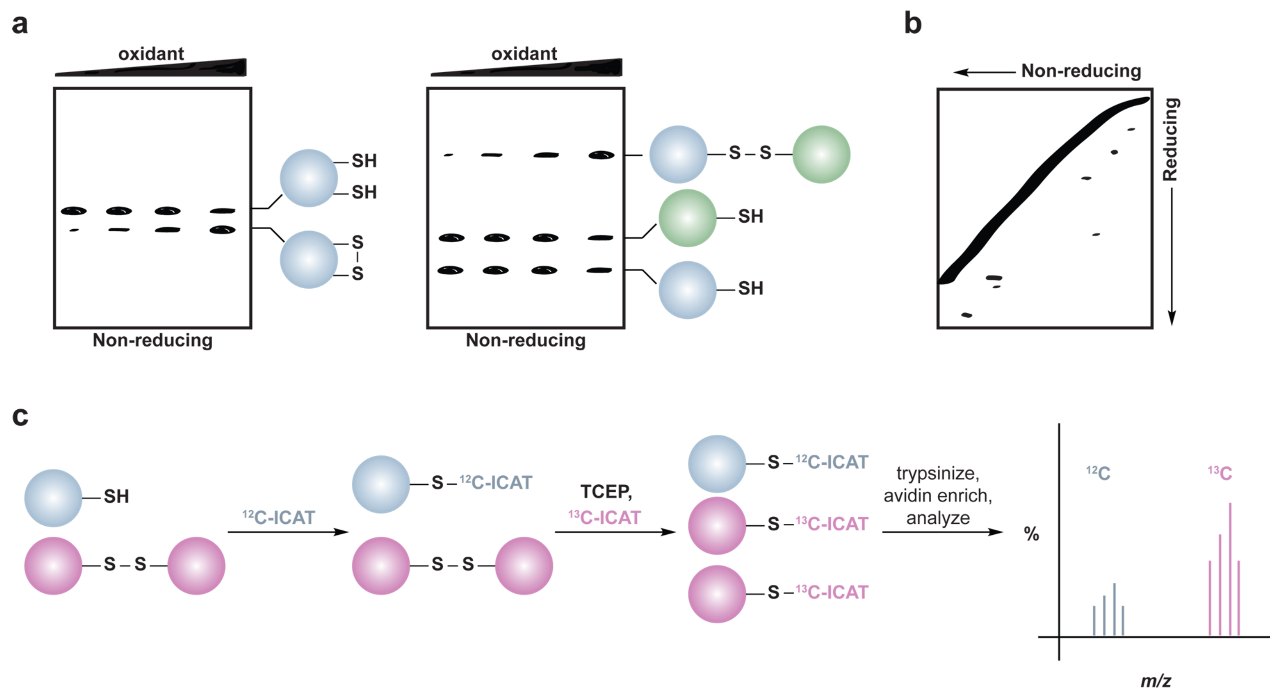


Figure 7. Methods for detection and identification of protein disulfides. (a) Differential migration of proteins containing intra- and intermolecular disulfide bonds. Samples are resolved under nonreducing SDS-PAGE conditions. Intramolecular disulfides can facilitate enhanced protein migration in some proteins as compared to the reduced species (left). Intermolecular disulfide complexes migrate at the combined molecular weight of the individual proteins (right). (b) Redox 2D-PAGE. Protein samples are first separated by nonreducing gel electrophoresis to separate disulfide-bonded complexes by size (top). The proteins are subsequently reduced in-gel with DTT, alkylated with NEM or IAM, and separated in the second dimension under reducing conditions (down). Proteins that are not involved in intermolecular disulfide complexes run at the diagonal. Proteins involved in disulfide complexes migrate off the diagonal and can be identified by in-gel digestion and LC-MS/MS (not shown). (c) OxICAT method combines the ICAT technology with differential alkylation of reduced and oxidized thiols to permit quantification of oxidized residues. Cell lysates are generated in the presence of trichloroacetic acid and detergents to facilitate exposure of all protein cysteines while inhibiting thiol/disulfide exchange. Reduced thiols (blue) are subsequently blocked with the light (^{12}C) ICAT reagent (blue), oxidized proteins (purple) are reduced with TCEP, and nascent thiols are alkylated with the heavy (^{13}C) ICAT reagent (purple). Samples are trypsinized and labeled peptides are avidin enriched. Eluted peptides are analyzed by LC-MS and heavy and light ICAT-labeled peptides are chemically identical, but differ in mass by 9 Da. The percentage of a particular thiol that is oxidized in a sample is determined by the ratio of heavy (^{13}C) to light (^{12}C) signal intensity from the corresponding peptide. While TCEP can reduce all reversible oxoforms (e.g., disulfides, sulfenic acid, *S*-nitrosothiols), sulfenic acids and *S*-nitrosothiols are often acid-labile and likely lost during sample preparation. As such, OxICAT is likely most suitable to detect cysteines involved in disulfide bonds.

The differential migration of disulfide-containing proteins by nonreducing and reducing gel electrophoresis have also been exploited to develop the only direct and high-throughput method to identify oxidant induced, disulfide-bonded protein complexes. This approach, termed diagonal SDS-PAGE[136] or redox 2D-PAGE[137] involves sequential nonreducing/reducing two-dimensional SDS-PAGE (Figure 7b). The protein mixture is first resolved by nonreducing gel electrophoresis to separate complexes by size, followed by excision of a narrow gel strip in the sample lane over the entire molecular weight range. The proteins are then reduced and alkylated in-gel to prevent disulfide bond reformation, the gel strip laid at a 90° angle across a second gel, and the proteins are subsequently resolved under reducing conditions. Proteins that are not involved in disulfide bond formation will lie in a diagonal line on the 2D gel, whereas proteins that form disulfide bonds will appear as distinct spots above or below the diagonal line. Protein identity is subsequently determined by LC-MS/MS analysis. A major limitation of this method, as with all 2D SDS-PAGE based methods, is that it cannot reliably visualize or produce analytical quantities of low abundance proteins that are present in less than 1000 copies per cell.[138] Nonetheless, this procedure has been used to detect disulfide-linked proteins in whole cell lysates derived from oxidant-treated rodent nerve cell cultures[139] and cardiac myocytes.[140] As outlined above, redox 2D-PAGE identifies proteins that form disulfides but does not provide information as to which proteins form which complexes. An alternative approach is to first isolate the protein of interest using a protein-specific antibody or affinity tag. This procedure permits identification of proteins that form disulfides with a protein of interest, and was recently used to identify of a novel reducing system in the bacterial periplasm.[105]

One limitation of the redox SDS-PAGE approach is that it does not provide quantitative information about the extent or fraction of cysteine oxidized under a given condition. To enable identification and quantification of reversibly oxidized protein cysteine residues, including disulfides, the Jakob group has reported an extension of the ICAT technology, known as OxICAT (Figure 7c).[111b] Lysates are first generated in the presence of TCA to precipitate proteins and prevent thiol/disulfide exchange. Free thiols are then alkylated with a light (^{12}C) ICAT reagent, followed by reduction of with TCEP, which serves to reduce reversible modifications (Chart 1). Nascent thiols are subsequently labeled with a heavy (^{13}C) ICAT reagent, protein samples are digested and ICAT-modified peptides are isolated by avidin affinity chromatog-

raphy. The eluted peptides are then analyzed by LC-MS/MS and the extent of oxidation for a particular cysteine is determined by the ratio of the heavy to light MS signals. While this procedure is not specific for disulfide-bonded cysteines per se, sulfenic acids and *S*-nitrosothiols are exquisitely sensitive to changes in pH and may be lost during sample preparation.[104a,141] Consequently, the OxICAT method seems best suited for disulfide detection, including both protein and low molecular weight (e.g., S-glutathionylation) disulfides.

3.3.2.2. S-Glutathionylation. The thiol-containing tripeptide, GSH is maintained at millimolar concentrations inside cells. Under normal conditions, 98% or more of GSH is maintained in its reduced state, however, in oxidative stress-associated disorders like cancer and neurodegenerative diseases, an appreciable amount of the GSH pool exists in the oxidized state, GSSG.[142] The GSH/Grx/GR system maintains protein thiols in their reduced state through thiol-disulfide exchange and redox reactions. Additionally, GSH undergoes nucleophilic addition and displacement reactions to purge the cell of toxic electrophilic and oxidizing reagents as catalyzed by glutathione *S*-transferase (GST), glyoxalase, GR, and Grx.[143]

Protein S-glutathionylation can occur during reduction of disulfides by the GSH/Grx/GR system and is readily reversible. When the GSH/GSSG redox balance shifts toward a more oxidizing state, protein *S*-glutathionylation can function as a regulatory mechanism or protect against irreversible oxidation.[120] If the GSH/Grx/GR system is compromised during oxidative stress, the accumulation of S-glutathionylated proteins can occur and has been associated with aging.[144] Within the context of redox signaling, protein S-glutathionylation can take place through two possible mechanisms: (i) thiol−disulfide exchange of GSSG with a thiolate or (ii) condensation of GSH with a sulfenic acid (Figure 8) or an *S*-nitrosothiol. In a study of

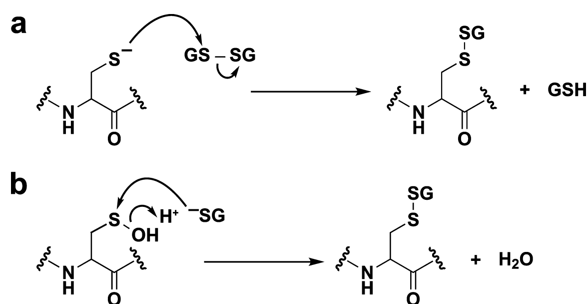


Figure 8. Mechanisms for glutathionylation. Protein glutathionylation products can be formed by (a) thiol/disulfide exchange of a protein thiolate with oxidized glutathione (GSSG) or (b) condensation of GSH with a protein sulfenic acid.

sulfenic acid-modified HSA, S-glutathionylation was estimated to occur with a rate constant of 2−100 M^{-1} s^{-1}.[106c] Thiol−disulfide exchange between GSSG and a protein thiolate is very slow,[145] but may be catalyzed by Grx, which appears to promote S-glutathionylation of the ETC complex I.[146] In this case, Grx-mediated S-glutathionylation may occur through free radical formation.[147] Specificity in S-glutathionylation may depend upon the steric properties, surrounding environment, and oxidation sensitivity of the cysteine. Like disulfides, S-glutathione protein adducts are stable to nonthiol nucleophiles. Deglutathionylation is catalyzed by members of the Grx family,[148] but Srx,[149] Trx,[150] and PDI[150a] may also perform this function, albeit with decreased efficiency.[151]

Enzymes such as trypsin,[152] collagenase,[153] and fructose-1,6-bisphosphatase[154] are activated by S-glutathionylation, whereas glyceraldehyde 3-phosphate dehydrogenase (GAPDH),[155] 26S proteasome,[156] cysteine protease caspase-1,[157] and ETC complex I[158] are inactivated by this modification. As previously mentioned, many PTPs are regulated by intramolecular disulfide bond formation at their catalytic cysteine.[159] However, some PTPs do not contain a second cysteine proximal to their active site. In some of these cases, for example in PTP1B, the phosphatase undergoes *S*-glutathionylation to guard against hyperoxidation (defined as oxidation to irreversible sulfinic and sulfonic acid states).[160] In addition to regulating enzyme activity, S-glutathionylation can also influence protein−DNA and protein−protein interactions. For instance, S-glutathionylation of cysteines in the DNA binding domain of transcriptional regulator, p53 weakens its association with DNA.[161] Similarly, S-glutathionylation of the transcriptional regulator, interferon regulatory factor 3 (IRF3) inhibits its interaction with CBP/p300 coactivators and prevents activation of target genes involved in induction of an antiviral response.[162]

To date, several methods have been developed to detect protein S-glutathionylation based on immunological, metabolic labeling, and differential alkylation approaches.[138] A common method to detect S-glutathionylation in proteins employs an antibody specific for the protein-*S*-GSH adduct.[162,163] This antibody is amenable to immunoprecipitation, Western blot on nonreducing gels, and immunofluorescence analysis. The anti-GSH antibody has also been used in conjunction with 2D SDS-PAGE, where samples are separated by isoelectric focusing in the first dimension and by molecular weight in the second dimension, with ensuing MALDI-TOF MS to identify S-glutathionylated proteins in HeLa cells.[163b] Given the differences in the surrounding environment of the modified cysteine, a limitation of the antibody is that not all protein-*S*-GSH adducts are detected with the same affinity.[164] An alternative immunological approach, called GST overlay, exploits the specificity and affinity of GST for GSH. In this method, Western blots from nonreducing SDS-PAGE gels are exposed to biotinylated-GST, which recognizes and binds selectively to protein-*S*-GSH disulfides; biotin-GST is subsequently detected by avidin blot (Figure 9a).[165] Protein S-glutathionylation can also be monitored indirectly by differential alkylation. In this workflow, free thiols are alkylated, protein-*S*-GSH adducts are selectively reduced by Grx, and nascent thiols are tagged by a biotinylated or fluorescent alkylating reagent (Figure 9b).[166] In theory, this approach could also be coupled to the OxICAT method to measure the extent of protein-*S*-GSH disulfides.

Approaches have been developed to facilitate detection of S-glutathionylated proteins in cells. One such method involves inhibiting protein synthesis with cycloheximide, which does not affect GSH synthesis, with subsequent metabolic labeling of the GSH pool through ^{35}S-cysteine incorporation.[167] Cells are subsequently lysed in the presence of a thiol alkylating agent to minimize thiol-disulfide exchange, samples are separated under nonreducing conditions, and analyzed by radiography. This technique has been used to identify proteins, such as enolase and 6-phosphogluconolactonase, that undergo *S*-glutathionylation in human T lymphocytes exposed to exogenous oxidants (e.g., H_2O_2 and diamide).[5b] Alternatively, Finkel, Eaton and colleagues have used biotinylated-GSH ethyl ester (BioGEE, **3**)[155,167] and *N,N*-biotinyl glutathione disulfide (**4**)[164] (Figure 9c) to monitor protein *S*-glutathionylation in lysates, isolated cells, and tissues. While the biotin tag facilitates

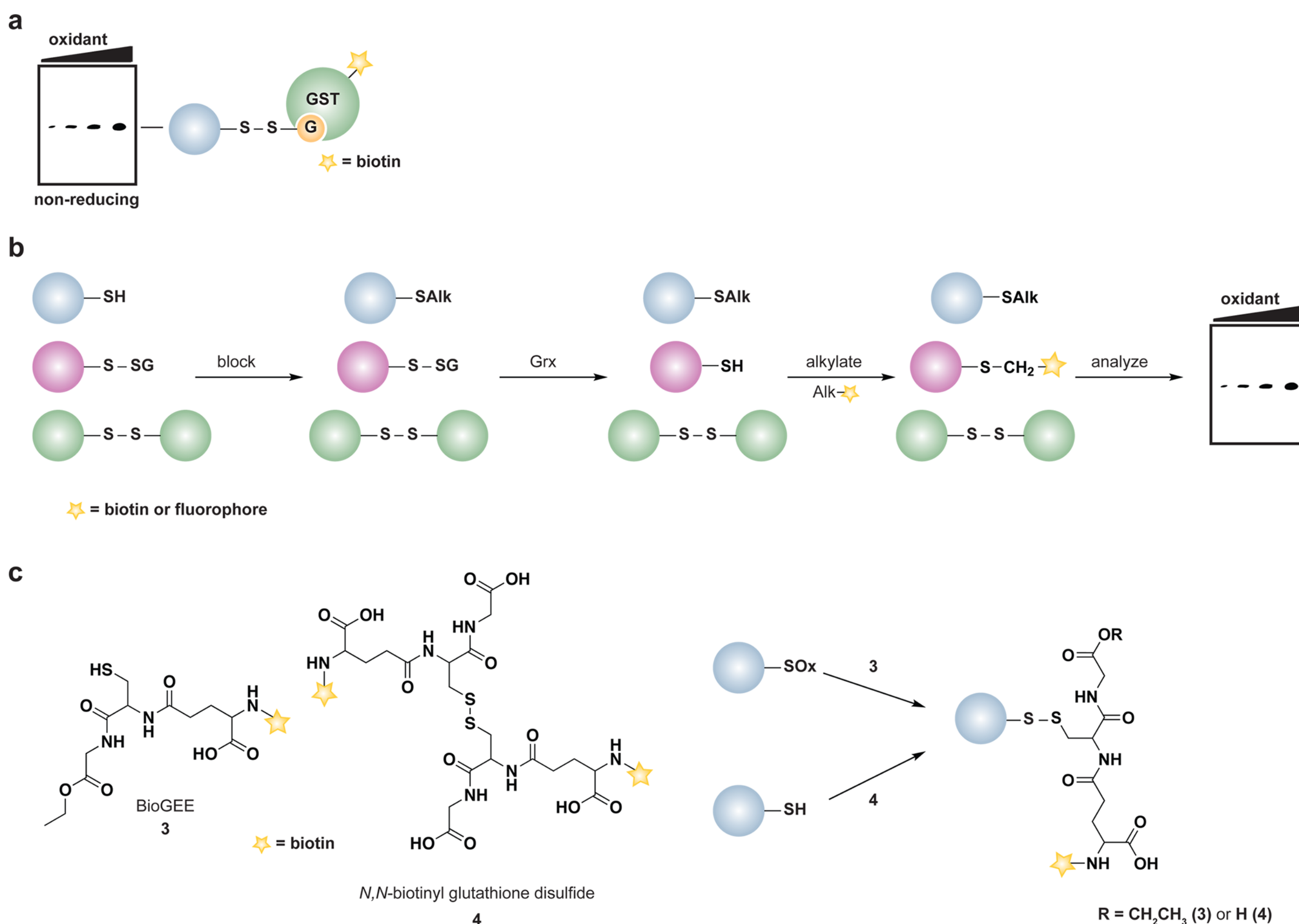


Figure 9. Methods to detect protein S-glutathionylation. (a) GST overlay. Samples are separated by nonreducing SDS-PAGE to preserve protein-GSH disulfides. Blots are subsequently treated with biotinylated glutathione S-transferase (GST), which binds selectively to GSH and permits detection of S-glutathionylated proteins by avidin blot. (b) Indirect differential alkylation of S-glutathionylated proteins. Free thiols are blocked with NEM or IAM, protein-GSH disulfides are selectively reduced with glutaredoxin (Grx), and nascent thiols are labeled with an alkylating agent conjugated to biotin or a fluorophore. S-Glutathionylated proteins are detected by avidin blot or in-gel fluorescence. (c) Biotinylated glutathione ethyl ester (BioGEE, **3**) enables in situ detection of glutathionylated proteins. *N*,*N*-Biotinyl glutathione disulfide (**4**) permits detection of proteins that become S-glutathionylated by thiol/disulfide exchange with GSSG.

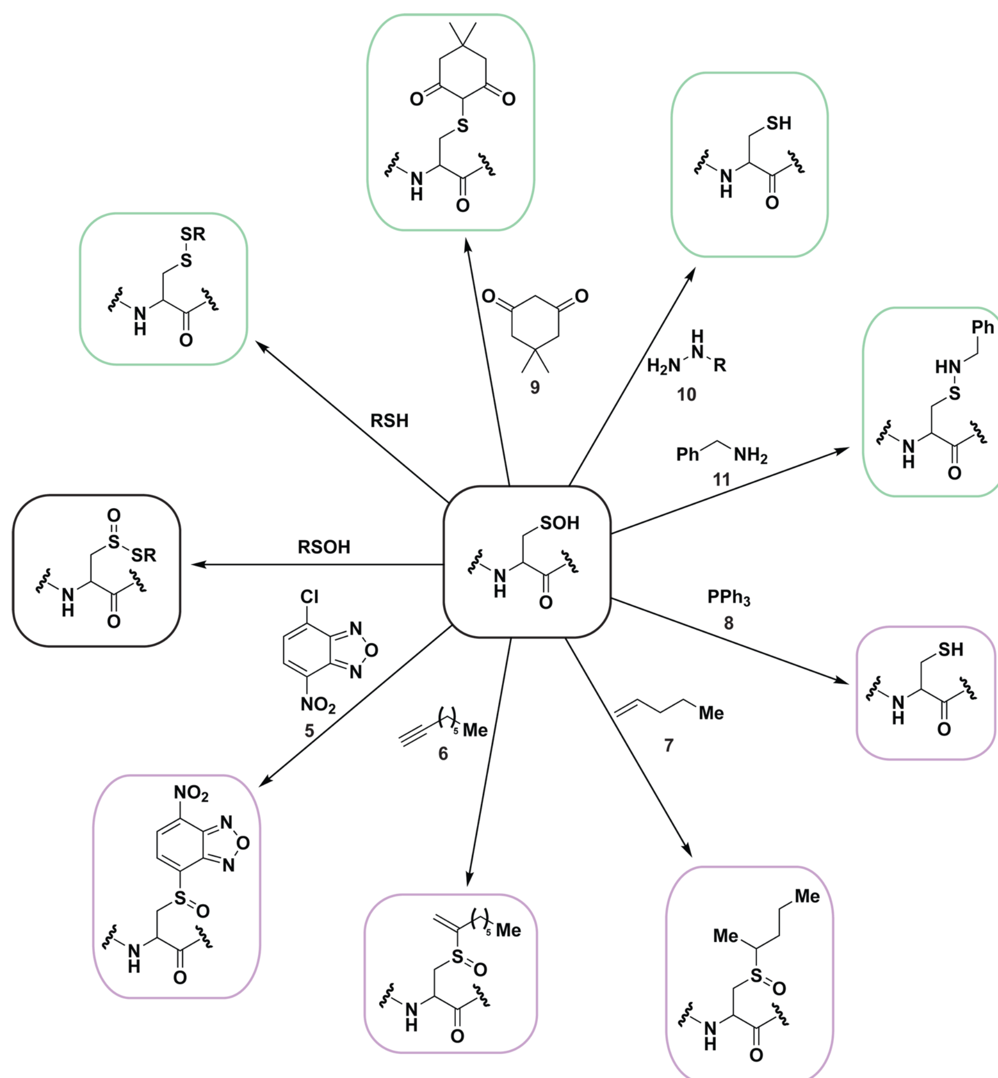
enrichment and identification of proteins that undergo *S*-glutathionylation, limitations of these methods include steric occlusion of biotinylated GSH analogues and poor cellular trafficking of biotinylated probes.[168]

3.3.2.3. Sulfenic Acids. Because of their reactive nature, sulfenic acids are often deemed unstable intermediates en route to additional cysteine modifications (Figure 2 and Chart 4). The formal oxidation state of the sulfur atom in a sulfenic acid is 0, enabling it to function as both a weak nucleophile and a soft electrophile (Chart 4 and 5, eq 1).[3b] The dual nature of its reactivity is clearly illustrated by the condensation of two sulfenic acids to generate a thiosulfinate (Chart 4). Thiosulfinate formation via sulfenic acid condensation may be most facile when sulfenate and sulfenic acid states are equally present.[119b] As previously discussed, the prevalence of thiosulfinates in cells is currently unknown; however, given the abundance of cellular thiols, interfacing of two sulfenic acids is likely to be a rare event.[3a]

Analogous to the reactivity of sulfur in a cysteine thiol, the nucleophilic character of a sulfenic acid is likely to be influenced, in part, by pK_a. Studies of sulfenic acids in small molecules have shown that electron-withdrawing substituents reduce the pK_a to favor sulfenate formation and enhance the stability of this species.[169] The pK_a of sulfenic acids in proteins could be similarly modulated to regulate their stability and reduce its reactivity toward a thiol. Stabilization of the sulfenate anion through decreased pK_a could also enhance the nucleophilic character of the sulfur atom, marking potential sites of cysteine hyperoxidation.

The pK_a of sulfenic acids in small molecules has been estimated to be in the range of 4.5−12.5.[104a,170] The pK_a of protein sulfenic acids has not been as extensively studied, but two measurements have been made, both with bacterial Prxs. There are three classes of Prxs: typical 2-Cys, atypical 2-Cys, and 1-Cys Prxs. Both typical and atypical 2-Cys Prxs form sulfenic acid at their active site cysteine after reaction with H_2O_2, which then condenses with a second cysteine in the same (atypical) or neighboring (typical) Prx to generate a disulfide that is reduced by Trx/TrxR to complete the catalytic cycle.[171] 1-Cys Prxs do not contain a resolving cysteine and the sulfenic acid intermediate may be reduced by GSH or ascorbate.[172] The first pK_a measurement reported for the sulfenyl group of a protein sulfenic acid was obtained using a mutant form of 2-Cys Prx from *Salmonella typhimurium*, AhpC in which the resolving cysteine was changed to serine. Key to the success of these experiments, the sulfenic acid and sulfenate

Chart 4. Sulfenic Acids Exhibit Both Nucleophilic and Electrophilic Character, As Illustrated by Condensation of Two Sulfenic Acids to Afford a Thiosulfinate (Black Box)[a]



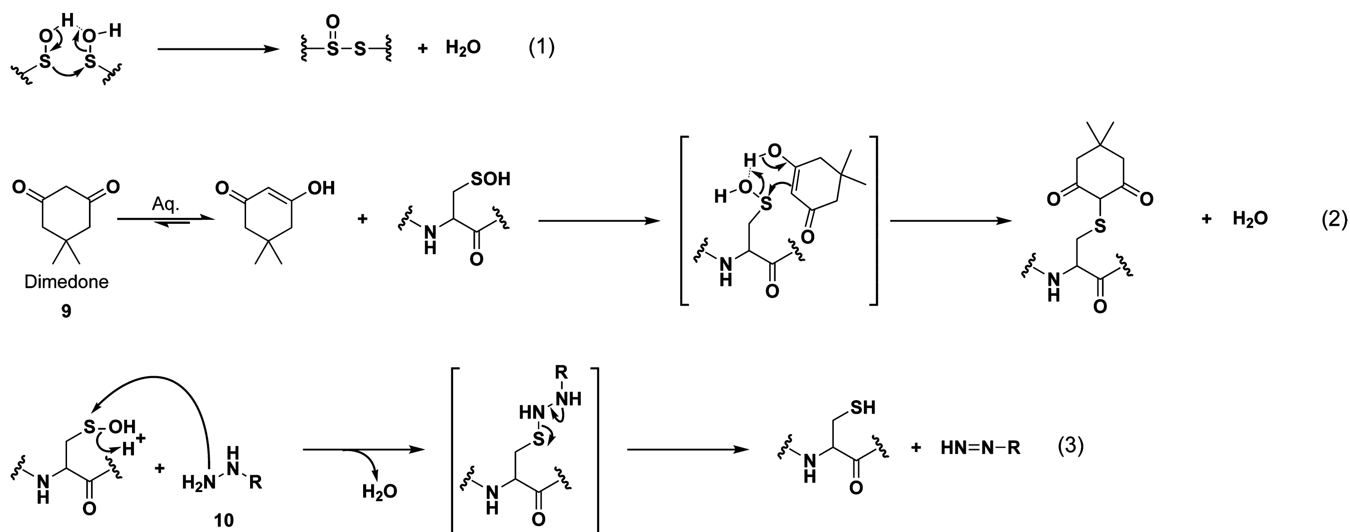
[a]As a nucleophile (purple boxes), sulfenic acids can undergo S_N2 displacement with halogenated compounds, such as 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, **5**), reaction with alkynes (**6**) and alkenes (**7**) to form the corresponding sulfoxides, and reaction with two equivalents of triphenylphosphines (**8**) to afford the free thiol and oxidized phosphine (not shown). Sulfenic acids can also function as an electrophile (green boxes) to react with thiols to yield a disulfide, and with 1,3-cyclohexadiones including dimedone (**9**), to yield a thioether adduct. As an electrophile, sulfenic acids can also react with hydrazines (**10**) to yield the thiol and azo compound (not shown), or with amines (**11**) to yield sulfenyl amides.

forms exhibit distinct spectral shifts in AhpC, allowing a pK_a determination of 6.1.[173] Consistent with this measurement, a tryptophan fluorescence study revealed a pK_a of 6.6 for the sulfenic acid in a 1-Cys Prx from *Mycobacterium tuberculosis*.[106a]

Analogous to cysteine thiolate reactivity with H_2O_2, the propensity for sulfenic acid to undergo further oxidation to sulfinic acid can be strongly influenced by the local protein environment. Relative to their prokaryotic counterparts, 2-Cys Prxs from eukaryotic organisms appear uniquely sensitive to hyperoxidation and may be related, at least in part, to sulfenic acid pK_a.[91,174] For example, oxidation of bacterial peroxiredoxin AhpE sulfenic acid by H_2O_2 occurs at 40 M^{-1} s^{-1}, whereas HSA sulfenic acid reacts at 0.4 M^{-1} s^{-1}.[106a,c] While the pK_a of the protein sulfenic acids were not reported in these studies, it is interesting to note that initial formation of sulfenic acid was also significantly slower in HSA (2.7 M^{-1} s^{-1})[106c] compared to AhpE (8.2×10^4 M^{-1} s^{-1}).[106a] To better understand how some protein environments facilitate sulfenic acid oxidation, additional physical organic and computational studies of both small-molecule and protein model systems will be required.

Sulfenic acids have been identified in the catalytic cycle of multiple enzymes, including Prx, NADH peroxidase, and methionine sulfoxide- and formylglycine-generating enzymes.[66c,71,106a] Formation of sulfenic acid has also been linked to oxidative stress-induced transcriptional changes in bacteria due to altered DNA binding of OxyR and OhrR and changes in the activity of the yeast Prx and Yap1 protein.[66c,175] Less is known about the mechanisms that underlie sulfenic acid-mediated regulation of mammalian protein function and signaling pathways; however, cysteines from several transcription factors (i.e., NF-κB, Fos, and Jun), or proteins involved in cell signaling or metabolism (e.g., GAPDH, GR, PTPs, kinases, and proteases) can be converted to sulfenic acid in vitro. Sulfenic acid formation has also been implicated in the

Chart 5. Reaction Schemes of Condensation of Two Sulfenic Acids to Yield a Thiosulfinate (Equation 1) and Electrophilic Reaction of Sulfenic Acid with Dimedone (9, Equation 2) and Hydrazines (10, Equation 3)



regulation of apoptosis, immune cell activation and proliferation, and growth factor (GF) signaling pathways.[12,123c,176]

Although sulfenic acids are often transient, an advantage to studying this modification is that it represents the initial product of two-electron oxidants with the thiolate anion and can therefore serve as a marker for oxidant-sensitive cysteine residues. A variety of indirect and direct chemical methods have been developed to detect protein sulfenic acid modifications (also termed sulfenylation[5a,12]). An early indirect chemical method that was reported involves thiol alkylation, reduction of sulfenic acids by arsenite, and labeling of nascent thiols with biotinylated NEM (Figure 10a).[177] This methodology was subsequently used to profile sulfenic acid formation in rat kidney cell extracts;[178] however, as with other indirect differential alkylation methods, a significant limitation is the debatable selectivity of the arsenite-mediated reduction step.[179]

Direct methods for sulfenic acid detection have been developed that take advantage of the chemical reactivity of this oxyacid. Nucleophilic substitution of halonitroarenes, such as 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, **5**), and nucleophilic addition to electron-deficient alkynes (**6**), alkenes (**7**), and triphenyl phosphines (**8**) are reported to trap sulfenic acids (Chart 4).[169] Of these, the most commonly used in the detection of protein sulfenic acids is NBD-Cl. This reagent reacts with thiols, sulfenic acids, and at higher pHs, amine-containing residues, but the resulting products are distinguished on the basis of their spectral properties and molecular weight.[180] As NBD-Cl can react with a variety of protein functional groups, this reagent appears best suited for use with recombinant proteins, especially those with a single cysteine residue.[16] Consequently, NBD-Cl does not have utility in global detection of protein sulfenic acids in complex protein mixtures, necessitating the development of methods for selective detection that exploit the electrophilic properties of the sulfur atom in sulfenic acid.

As first reported by Benitez and Allison in 1974, protein sulfenic acids react with cyclic 1,3-diketone carbon nucleophiles, like 5,5-dimethyl-1,3-cyclohexadione (dimedone, **9**) and with hydrazines (**10**) or amines (**11**) (Chart 4 and 5)[181] Dimedone has proven useful in revealing the requirement for protein sulfenic acid modifications in the *S. cerevisiae* Yap1-Gpx3 H_2O_2-sensing pathway,[66c] T cell activation,[123c] and EGFR signaling.[12] Unlike sulfur, nitrogen, or phosphorus-based nucleophiles, under aqueous conditions cyclic 1,3-diketones do not cross react with cysteine thiols, sulfinic acid, or other functional groups commonly found in biomolecules, making this reaction an extremely attractive avenue for developing chemically selective detection methods. All chemoselective methods for detecting protein sulfenic acids reported to date depend upon this chemistry.[138] Two recent reports expand the scope of reactive templates to 1,3-cyclopentadione[182] and linear β-ketoester[183] analogues (though caution should be exercised with linear derivatives since they have been reported to cross react with amines, such as lysine[184]). The lack of an enrichment or visualization "handle" for protein-*S*-dimedone adducts subsequently motivated the development of biotinylated (**12**,**13**)[185] and fluorophore-conjugated (**14**) analogues[185b,186] (Chart 6). These probes have been used in a proteomic study with isolated rat hearts[185a] and to identify AKT2 as a target of PDGF-induced H_2O_2.[187] Depending upon the application, one potential drawback for direct conjugation of any probe to biotin or a fluorophore is that the bulky chemical tags can reduce cell-permeability.[168b] Naturally, not all conjugated probes are entirely impermeant (e.g., DCFH diacetate, DCP-Bio1) however, comparative studies show time and again that tagged derivatives often suffer from diminished cell uptake and trafficking properties.[168,188] Alternative mechanisms of uptake are possible (e.g., active transport of BioGEE), but may limit probe distribution to specific cellular compartments. A further consideration when functionalizing probes with large chemical tags is that increased steric bulk can lead to a significant bias in protein target labeling.[188a,189] The poor permeability of many biotin- and fluorophore-tagged probes typically necessitates labeling of proteins in lysates and is, therefore, subject to the aforementioned limitations. In this context, it is also important to bear in mind that labile or transient sulfenic acid modifications may be further oxidized or insufficiently trapped during the lysis procedure.

A subsequent alternative approach that has emerged is the development of azido- and alkyne-functionalized dimedone analogous (Chart 6), termed DAz-1 (**15**),[168b,180c] DAz-2 (**16**),[190] DYn-1 (**17**), and DYn-2 (**18**),[12] which enable the

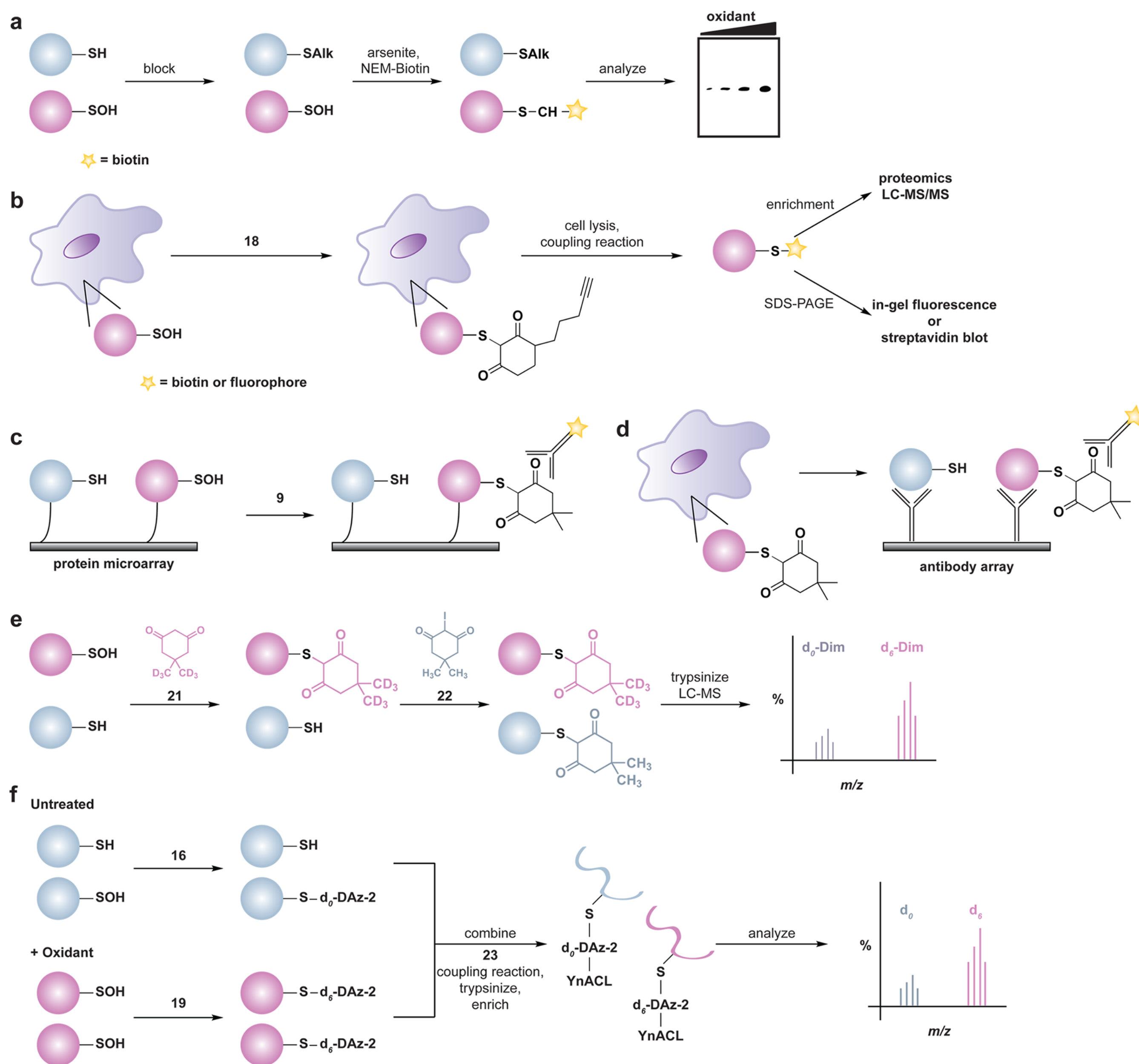
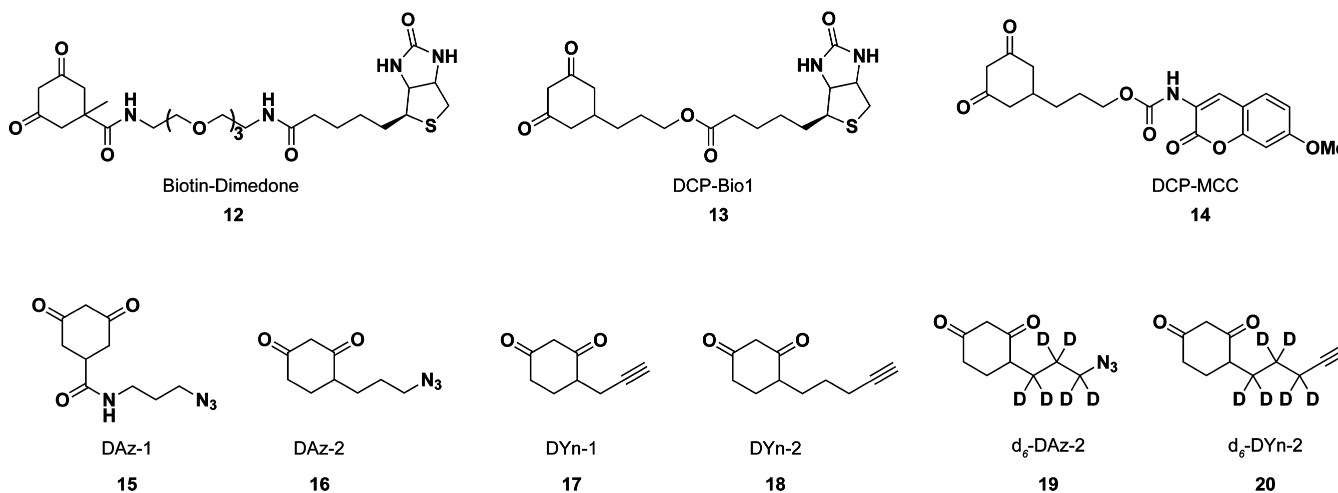


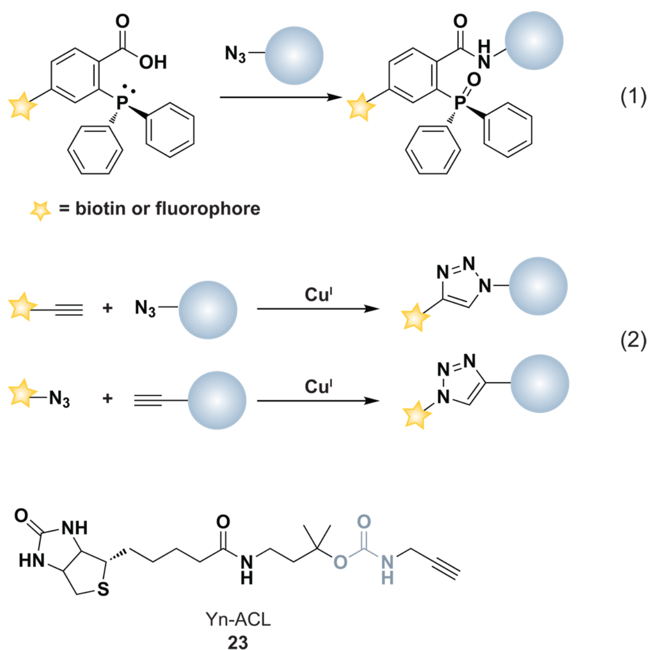
Figure 10. Methods to detect protein sulfenic acids. (a) Indirect differential alkylation of protein sulfenic acids. Free thiols (blue) are blocked with NEM or IAM, protein sulfenic acids (purple) are reduced with arsenite, and nascent thiols are labeled with biotinylated NEM (NEM-Biotin). Sulfenylated proteins are detected by avidin blot where increased protein oxidation is observed as an increase in signal intensity. (b) Direct in situ labeling of protein sulfenylation. Cells are treated with or without stimulant (e.g., oxidant, growth factor) and subsequently incubated with azido or alkyne dimedone analogues, such as **18** to chemically modify sulfenylated proteins. Afterward, excess probe is removed, cell lysates are generated, and probe-modified proteins are conjugated to biotin or a fluorophore by a coupling reaction (e.g., Staudinger ligation or Huisgen [3 + 2] cycloaddition). The samples can then be avidin enriched and subjected to proteomics analysis or analyzed by avidin blot or in-gel fluorescence where increased protein sulfenylation correlates to enhanced signal intensity. (c and d) High-throughput immunological detection of dimedone (**9**)-modified proteins using arrays. (c) Proteins immobilized on a microarray that are susceptible to sulfenylation are irreversibly modified by **9**. The protein−dimedone adduct forms an epitope for selective detection by the antibody. (d) Cells are treated with or without stimulant (e.g., oxidant, growth factor) and are subsequently incubated with **9** to irreversibly modify sulfenylated proteins. Subsequent to cell lysis, proteins within a given signaling pathway are immobilized on an antibody array and dimedone-modified proteins are detected by addition of the antibody. (e) Isotope-coded dimedone 2-iododimedone (ICDID) permits quantification of protein sulfenylation. Sulfenic acids are labeled by d_6-dimedone (**21**, purple), then excess reagent is removed and free thiols are labeled by d_0-2-iododimedone (**22**, blue) generating chemically identical adducts that differ by 6 Da. The samples are trypsinized and analyzed by LC-MS where the extent of sulfenic acid occupancy is determined by the ratio of d_6-dimedone to d_0-dimedone peak intensities. (f) Quantification and site-identification of protein sulfenic acids with d_6-DAz-2 or d_6-DYn-2 and an acid-cleavable linker (ACL) coupling reagent. Sulfenic acids are labeled with d_0-DAz-2 (**16**) in the untreated sample and with d_6-DAz-2 (**19**) in the oxidant-treated sample. Excess probe is removed and the samples are combined and biotinylated by coupling with the alkyne-ACL (**23**) to generate chemically identical adducts that differ by 6 Da. The sample is then trypsinized, avidin enriched, and trifluoroacetic acid-eluted peptides are analyzed by LC-MS/MS where the increase in sulfenic acid modification in response to oxidant is determined by the ratio of d_6 to d_0 peak intensities. Biotin can complicate spectra and decreases peptide recovery, and the removal of biotin with the ACL permits increased sample elution and direct identification of modified peptides in the MS.

Chart 6. Biotin, Fluorophore, and Chemical Handle Derivatives of Dimedone



trapping and tagging of protein sulfenic acid modifications directly in living cells. In later steps, proteins covalently modified by DAz or DYn probes can be coupled to biotin or fluorophores by Staudinger ligation[191] or Huisgen [3 + 2] cycloaddition reactions (Chart 7, eqs 1 and 2, and Figure 10b).[192] Application of DAz-2 to identify proteins that undergo sulfenic acid modifications in HeLa cells identified upward of 200 candidates, including the majority of known sulfenic acid-modified proteins.[190] Cross-comparison of these data with those from disulfide and S-glutathionylation proteomes revealed modest overlap between these "redoxomes", suggesting that a significant portion of sulfenic acid modifications may not be intermediates en route to S-thiolated forms and, instead, can be stabilized by the protein local environment.[138] Alternatively, or in addition, it is also possible that (i) lysate-based approaches employed in the S-thiolation proteomic studies resulted in fewer identifications and, therefore, lower overlap with the "sulfenome", and (ii) the modest rate constant for the reaction of many dimedone analogues with sulfenic acid (10^3 M^{-1} min^{-1})[111a] may not be sufficient to trap especially transient modifications. Azido dimedone analogues have also been used to show that sulfenic acid modification of the thiol peroxidase, Gpx3 is essential for yeast to sense oxidative stress[66c] and to identify a unique reducing system in the bacterial periplasm that protects single cysteine residues from oxidation.[105]

Chart 7. Bioorthogonal Coupling Reactions Staudinger Ligation and Huisgen [3 + 2] Cycloaddition[a]



[a]Staudinger ligation (equation 1) functionalizes an azide-modified molecule, while Huisgen [3 + 2] cycloaddition (equation 2) couples azide-modified or alkyne-modified proteins to detection tags. Alkyne acid cleavable linker (Yn-ACL, **23**) reagent for Huisgen [3 + 2] cycloaddition with azide-modified proteins. Blue, acid cleavable moiety.

More recently, DYn-2 was used in global profiling studies to reveal dynamic protein sulfenylation during EGF signaling in human epidermoid A431 cells and to identify the EGFR kinase as a prominent target of endogenous signaling H_2O_2 (Figure 11).[12] Three PTPs involved in the regulation of EGFR signaling, PTP1B, PTEN, and SHP2 were also shown to undergo sulfenic acid modification in response to EGF stimulation of cells. Interestingly, PTPs and EGFR displayed differential sensitivity to oxidation by EGF-induced endogenous H_2O_2 that correlated with the relative proximity of each enzyme to the oxidant source itself, NOX2 (Figure 11). This study was the first of its kind to provide evidence for sulfenic acid modification of PTP in cells during growth factor signaling. Prior studies performed in lysates had led to speculation as to the likelihood of PTP oxidation due to their modest reactivity with H_2O_2 and their low abundance in comparison to the abundant and reactive Prxs.[2d,123c,193] Interestingly, while this study found ER-localized PTP1B to be only moderately sensitive to H_2O_2 derived from plasma membrane-bound NOX2, a study by Keaney and colleagues indicates that this oxidation reaction becomes relevant during ER-localized NOX4 activation.[194] As it is unlikely that the intrinsic reactivity of the active site cysteine in PTP1B differs in these two systems, these data suggest that proximity of PTP1B (and other proteins) to the NOX oxidant source may be an important determinant of target selectivity. Hence, the apparent sensitivity and physiological relevance of PTP1B oxidation, and protein thiols in general, is likely to be a signaling pathway and cell type-specific phenomena.

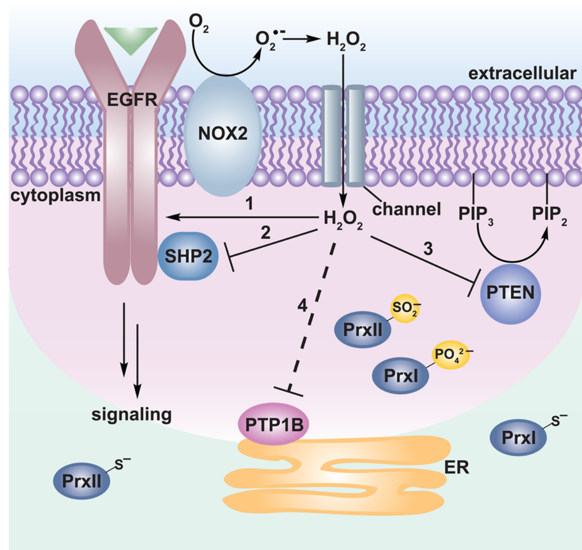


Figure 11. Redox regulation of epidermal growth factor (EGF) signaling by protein sulfenylation. Binding of EGF to the EGF receptor (EGFR) facilitates receptor dimerization, activation (not shown), and promotion of NOX2 complex assembly. NOX2-derived H_2O_2 translocates into the cytoplasm likely through channels such as aquaporins where it has been shown to regulate the activity of proteins involved in the EGFR signaling cascade. EGFR and the phosphatases SHP2, PTEN, and PTP1B were all found to be sulfenylated in response to EGF stimulation, albeit with differential sensitivities (ranked 1−4 in order of decreasing susceptibility). The sensitivity of each protein to oxidation correlates to their relative proximity to the oxidant source. In this way, EGFR, which forms a complex with NOX2, exhibited the highest sensitivity. Moreover, the EGFR-associated phosphatase SHP2 exhibited increased susceptibility to sulfenylation as compared to the cytoplasmic phosphatase PTEN, which regulates the levels of PIP_3, and PTP1B, which is localized to the cytoplasmic face of the endoplasmic reticulum (ER). Co-localization of antioxidant enzymes such as peroxiredoxins (Prx) to the signaling regions is thought to limit the range of H_2O_2 diffusion (green area). Interestingly, NOX-derived reactive oxygen species (ROS) have also been shown to inactivate Prxs by hyperoxidation (PrxII) or phosphorylation (PrxI).[11b] These regulatory mechanisms have been proposed to permit localized accumulation of ROS for redox regulation of proteins located near the oxidant source (pink area).

In 2009, our group reported the first immunological method for detecting protein sulfenic acid modifications. Antibodies were elicited by a synthetic hapten mimicking dimedone-modified cysteine conjugated to KLH (Figure 10c) and are highly specific and sensitive for detecting protein-S-dimedone adducts by Western blot and immunofluorescence.[195] Application of this immunochemical approach to protein arrays and breast cancer cell lines revealed considerable differences in the level of protein sulfenic acid modifications among tumor subtypes (Figure 10c). This method has also been used to demonstrate the cysteine sulfenylation and colocalization of oxidized proteins with NOX2 during EGF signaling.[12] Subsequently, in 2011, Eaton and colleagues reported a similar antibody and used this reagent to study sulfenic acid modification of GAPDH in cardiac myocytes exposed to exogenous H_2O_2.[196] A future application of these antibodies will be to combine their use with antibody arrays to facilitate unbiased investigation of protein sulfenic acid modifications in signaling pathways (Figure 10d).

Beyond detection, one approach to determine which protein sulfenic acid modifications are relevant to signaling in normal cells as well as in pathological processes is to quantify the extent of oxidation. To this end, our laboratory has recently developed two methods to facilitate relative quantification of sulfenic acid modifcations: (1) isotope-coded dimedone and 2-iododimedone (ICDID) (Figure 10e),[197] and (2) isotopically light and heavy derivatives of DAz-2 (**19**) and DYn-2 (**20**) (Chart 6).[198] The ICDID workflow uses deuterium-labeled dimedone (d_6-dimedone, **21**) to trap sulfenic acids, followed by alkylation of free thiols with 2-iododimedone (**22**). Importantly, the covalent adducts afforded by these two reagents are structurally/chemically identical, and have identical efficiencies of ionization. Nevertheless, the thiol and sulfenic acid-tagged species are differentiated from each other by 6 Da and the extent of sulfenic acid modification at a cysteine is determined from the ratio of heavy to light isotope-labeled peak intensities (Figure 10e). Alternatively, isotopically light and heavy forms of DAz-2 can be used to monitor relative changes in sulfenic acid modification. This strategy has been combined with an acid cleavable linker (ACL) that is suitable for Huisgen [3 + 2] cycloaddition coupling (Chart 7).[198] With this method, samples are labeled by heavy or light DAz-2, combined and conjugated to the alkyne-biotin ACL reagent (Yn-ACL, **23**) digested with trypsin, enriched on avidin cartridges, and tagged peptides are eluted by trifluoroacetic acid (TFA)-mediated cleavage. Peptides are then mapped by LC-MS/MS analysis to identify the sulfenic acid-modified protein and map the site of modification. The relative change in protein sulfenic acid modfication between two samples is determined by the ratio of heavy to light isotope-labeled peak intensities (Figure 10f).

Analogous to irreversible electrophilc inhibitors that modify semiconserved cysteines residues in protein tyrosine kinases (PTKs) currently in phase II and III cancer clinical trials,[199] we envision the development of nucleophile-functionalized small molecules that target a sulfenic acid-modified cysteine in a specific protein. Our design strategy is to conjugate the nucleophile "warhead" to a high affinity ligand that binds proximal to the target cysteine sulfenic acid. As proof of principle, we have developed small molecules that target PTPs, termed redox-based probes (RBPs, Figure 12, **24**−**26**), comprised of three parts: (i) a cyclohexanedione nucleophile, (ii) a chemical scaffold that binds to the conserved PTP active site, and (iii) an azide (or alkyne) chemical reporter to facilitate downstream detection and isolation of labeled PTPs (Figure 12a).[189] The RBPs exhibited enhanced binding and sensitivity for detecting sulfenic acid modification of the catalytic cysteine in the YopH and PTP1B phosphatases, compared to the parent compound, DAz-1 (**15**), which lacks the additional binding element. The RBP approach should facilitate cellular investigations of PTP redox regulation. Methods to study PTP redox modulation are often thwarted by issues of low abundance and studies of this nature would greatly benefit from a targeted approach, as exemplified by RBPs. Neel and colleagues have also reported an indirect immunochemical method for global proteomic assessment of the PTP "redoxome" that relies on performic acid hyperoxidation of cysteine oxyacids (Figure 12b).[200]

In addition to pan-PTP recognition, RBPs can be refined to target a single member of the PTP family. Such a reagent would not only be useful to study redox-regulation of a specific PTP, but might possibly serve as lead compounds for the development of a new class of therapeutics to ameliorate

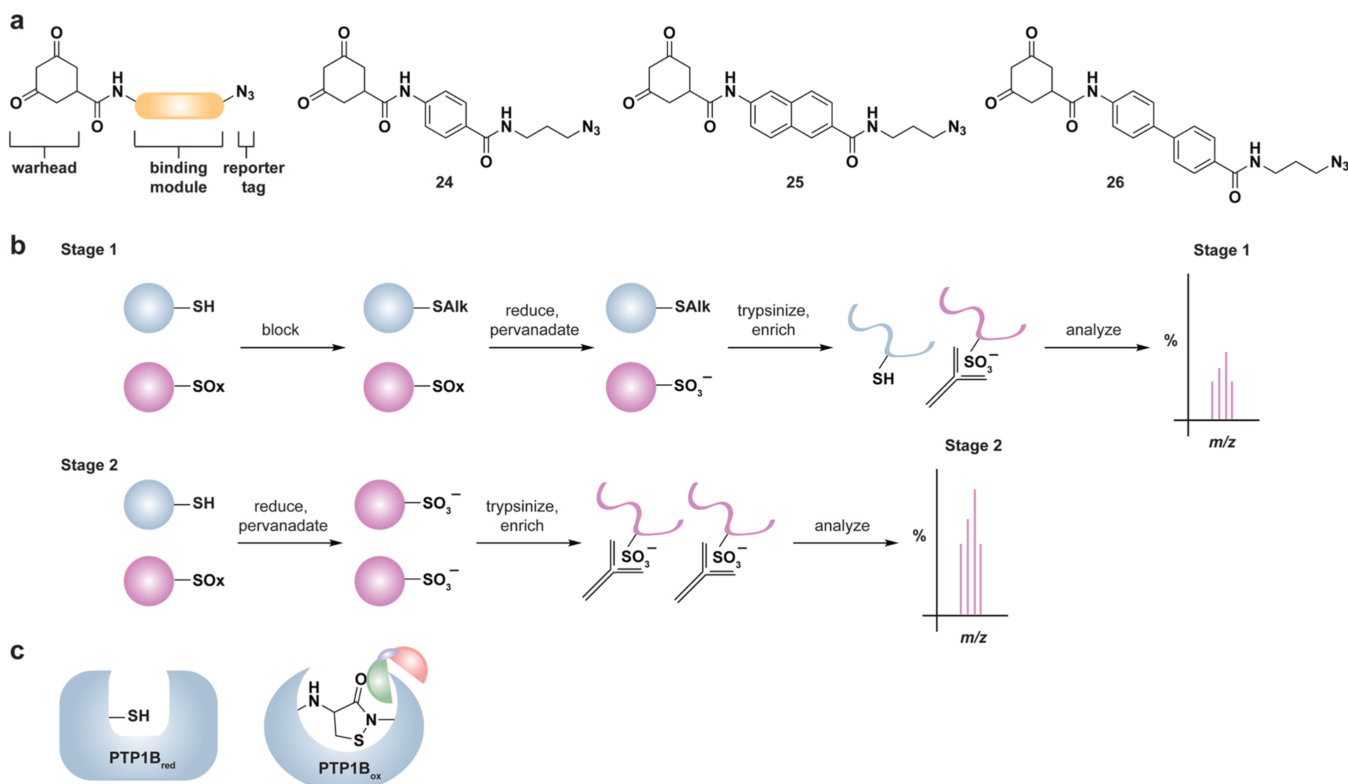
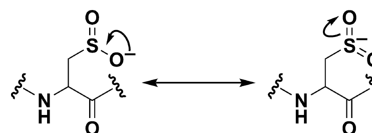


Figure 12. Targeted approaches to monitor cysteine oxPTM with in specific or family of proteins. (a) Redox-based probes (RBPs, **24**–**26**) for protein tyrosine phosphatases (PTPs) are comprised of three parts: a warhead that permits chemoselective reaction with sulfenic acid, a PTP-directed scaffold that exhibits enhanced affinity for target binding, and an azide chemical reporter to facilitate downstream detection and isolation of labeled protein. (b) Indirect two-stage immunochemical approach to monitor PTP oxidation. In stage 1, free thiols in one aliquot of sample are alkylated with NEM, oxidized cysteines are reduced with DTT, and nascent thiols are hyperoxidized to sulfonic acid with pervanadate. The proteins are subsequently trypsinized, and sulfonic acid-modified peptides are isolated with a monoclonal antibody that recognizes hyperoxidized PTPs. In stage 2, a second aliquot of sample is reduced with DTT and all thiols are oxidized to sulfonic acid with pervanadate and processed as in stage 1. The enriched peptides are analyzed by LC-MS/MS and the extent of PTP oxidation is determined by the ratio of stage 1 to stage 2 signal intensities. (c) Conformation-sensing single-chain variable antibodies that selectively recognize the unique conformation of the sulfenyl amide oxoform of PTP1B.

diseases associated with aberrant PTP activity, as in diabetes.[201] In support of this approach, Tonks and colleagues recently reported the development of antibodies as single-chain variable fragments that selectively recognize the unique conformation that PTP1B adopts when its activate site cysteine exists in the sulfenamide form (Figure 12c).[202] These conformation-sensing antibodies were able to trap PTP1B in the inactive conformation permitting sustained insulin signaling in human embryonic kidney (HEK) cells. Lastly, the RBP or "nucleophilic inhibitor" approach can be extended to other classes of proteins that contain a redox-sensitive cysteine, such as EGFR.[12]

3.3.2.4. Sulfinic Acids. In the presence of excess oxidant, sulfenic acid can be oxidized to sulfinic acid (Figure 2). The formal oxidation number of the sulfur atom in sulfinic acid is +2. On this basis, this oxyacid might be expected to have enhanced electrophilicity compared to sulfenic acid. However, sulfinic acid does not undergo self-condensation or nucleophilic attack by thiols. This can be explained by the increase in partial positive charge on the sulfur in sulfinic acid, which converts the sulfur atom into a harder electrophile making it less prone to reaction with soft nucleophiles, such as thiols. With a pK_a of 2, sulfinic acid is deprotonated at physiologic pH and can exist in two resonance forms (Chart 8).[203] Sulfinic acids function as nucleophiles (Chart 9), reacting largely from sulfur to undergo alkylation (**27**), as well as nucleophilic addition to activated

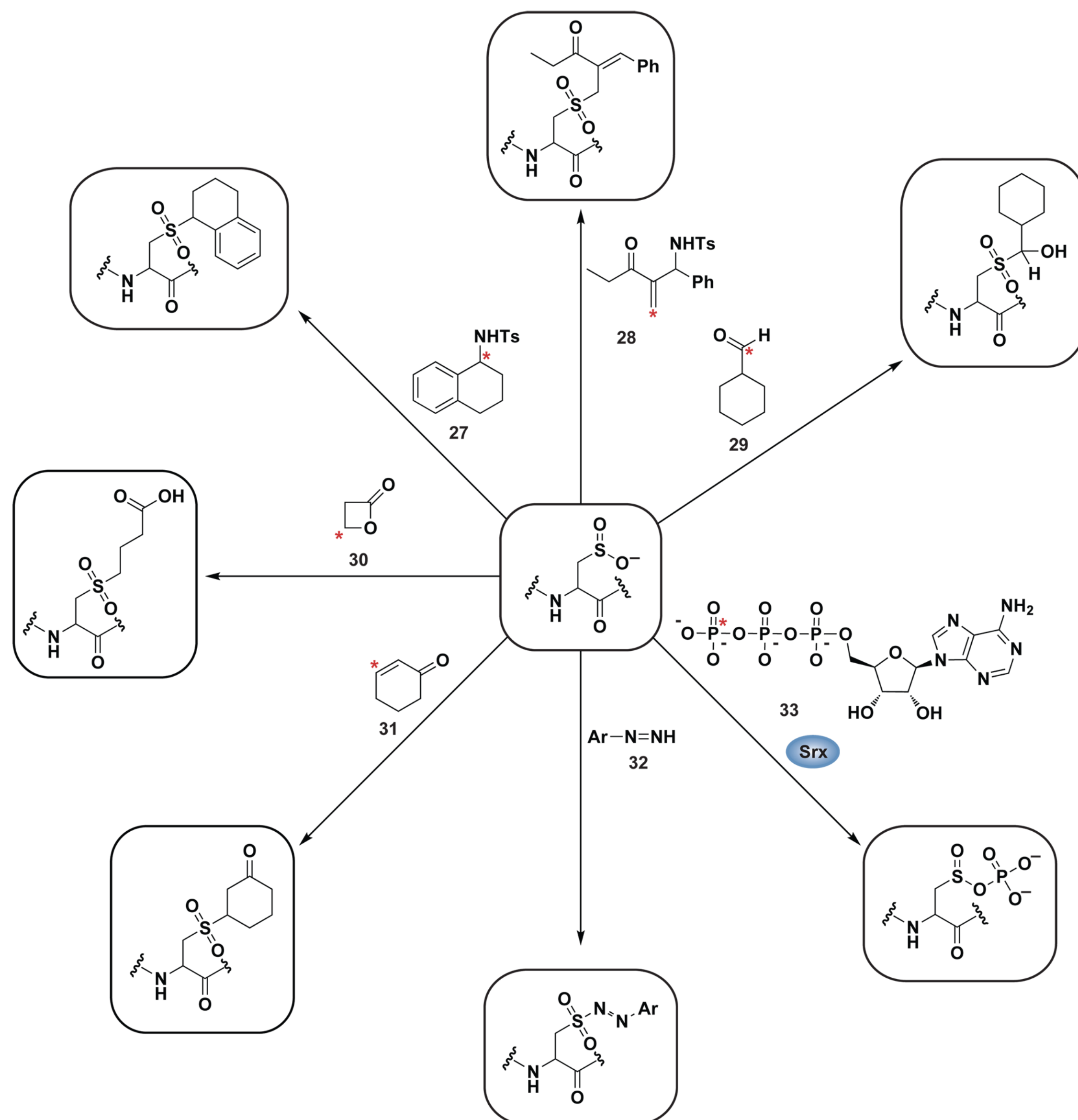
Chart 8. Resonance Structures of Sulfinic Acids



alkenes (**28**), aldehydes (**29**), lactones (**30**), *α*,*β*-unsaturated compounds (**31**), and diazonium salts (**32**) to give the corresponding sulfones.[3b,204] The preceding reactions are established for sulfinic acids under synthetic organic conditions, but it is not established whether all of these reactions would take place with protein sulfinic acids. The reactions in Chart 9 exhibit a wide range of rates and some go to completion on the hour time scale (**27**,[205] **28**,[206] **30**,[207] **31**[208]), while others, such as **29** and **32**,[209] undergo rapid equilibrium-based transformations. Of note, the reaction of sulfinic acid with aldehydes serves as the basis for the Schiff's test. As an ambidentate nucleophile, sulfinic acid can also react at oxygen as illustrated by nucleophilic attack of the sulfinate oxygen on the *γ*-phosphate of ATP (**33**) to form the sulfinic acid phosphoryl ester intermediate in the Srx catalytic cycle (Chart 9).[122a,210]

Cysteine sulfinylation can also modify protein metal binding properties. Oxidized sulfur ligands are weaker donors and can increase the Lewis acidity of the liganded metal center, which influences affinity and coordination. In matrix metalloproteases

Chart 9. Sulfinic Acids Function as Soft Nucleophiles Reacting Primarily from the Sulfur to Undergo Alkylation (27) or Nucleophilic Addition to Activated Alkenes (28), Aldehydes (29), Lactones (30), α,β-Unsaturated Compounds (31), and Diazonium Salts (32)[a]

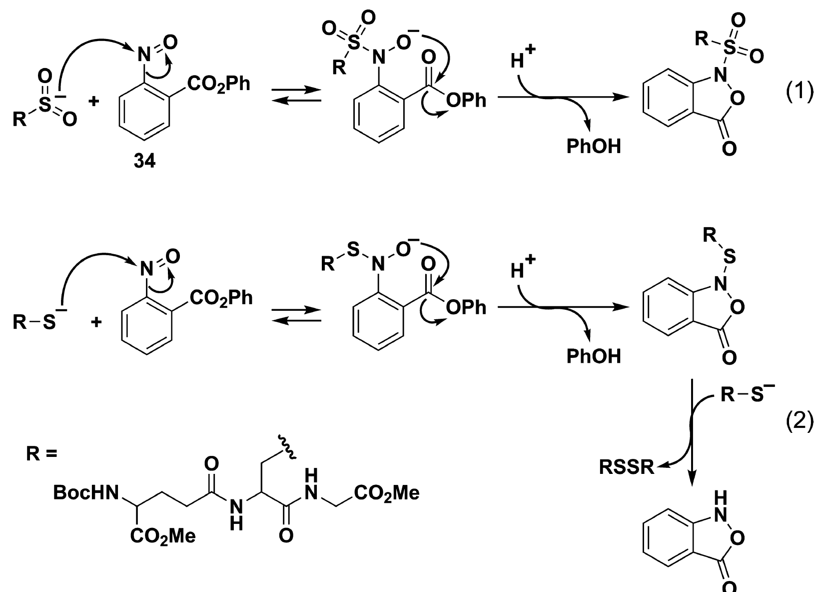


[a]Sulfinic acids can also function as nucleophiles involving reactivity from the oxygen as exemplified by the sulfiredoxin (Srx)-catalyzed reaction with ATP (**33**) to yield a transient sulfinic acid phosphoryl ester. Though not shown, reactions **27** and **28** undergo acid-catalyzed SN1 reactions and thus require the protonated sulfinic acid species.

(MMPs), sulfinic acid oxidation of a zinc-coordinated active site cysteine thiolate activates protease function, in part by reducing the ability to coordinate the zinc cation.[211] In contrast, nonheme Fe^{III} coordination in nitrile hydratases (NHases) is accomplished by a unique CXXCXC binding sequence in which two cysteines are present as the sulfinic and sulfenic acid states.[154,212] Cysteine oxidation is necessary for hydratase activity, and the increased Lewis acidity of the Fe^{III} afforded by cysteine oxidation is believed to regulate the affinity of a catalytic water molecule for the metal center.[3b,212a,213] A similar coordination motif has also been identified in the unique noncorrin cobalt center in a NHase from *Pseudonacardia thermophila*[214] and in thiocyanate hydrolase.[215] It has also been suggested that cysteine oxidation alters the metal coordination from zinc (thiol) to iron or cobalt (sulfenic/sulfinic acid). This preference may not be as strictly defined as once thought, however, as a peptide mimetic inhibitor of neurotoxin F from *Clostridium botulinum* was recently shown to coordinate to an essential zinc by a cysteine sulfinate ligand.[216]

To date, the cysteine sulfinic acid modification has been most extensively characterized in Prxs and the Parkinson's disease protein, DJ-1.[217] Eukaryotic Prxs appear most susceptible to sulfinic acid modification,[91,174] a feature that evolutionarily coincides with Srx expression, the only known sulfinic acid reductase.[109a,218] Srx was recently identified in cyanobacteria,[219] which also appear to have 2-Cys peroxiredoxins that are susceptible to hyperoxidation.[220] Recent work shows that Srx-mediated reduction of Prx proceeds by a sulfinic acid phosphoryl ester that undergoes nucleophilic attack by Srx Cys84 to form a thiosulfinate intermediate that is subsequently

Chart 10. Chemoselective Approach to Detect Sulfinic Acids[a]



[a]Reaction of a sulfinic acid with ester-protected aryl-nitroso compound **34** and subsequent intramolecular nucleophilic attack of the nitroso anion intermediate on the ester yields a stable *N*-sulfonylbenzisoxazolone adduct (equation 1). In contrast, analogous reaction with a thiol yields a sulfenyl amide adduct that is susceptible to subsequent reaction with a second thiol or reducing agent (equation 2).

resolved by Srx Cys48 to release Prx sulfenic acid and oxidized Srx, which are both recycled by the Trx/TrxR system.[122a,210,221] The reaction of Srx with sulfinic acid is slow ($k_{cat} \approx 0.2$ min^{-1}), and it is currently unknown whether any accessory proteins enhance this reaction in vivo.[109b]

The biological reversibility of sulfinic acid (at least in some proteins) hints at a regulatory function, analogous to a disulfide or sulfenic acid. In this vein, it has been proposed that reversible inactivation of Prx by sulfinic acid modification facilitates the accumulation of endogenous H_2O_2 to regulate signaling events in the so-called "floodgate hypothesis".[91] While Prx II appears to be particularly sensitive to hyperoxidation, it has recently been shown that phosphorylation inactivates Prx I,[11b] with both mechanisms of Prx inactivation serving to facilitate localized accumulation of H_2O_2 for signaling purposes. Reversible Prx oxidation has also been proposed to regulate eukaryotic circadian rhythms, though the molecular details remain largely unknown.[222]

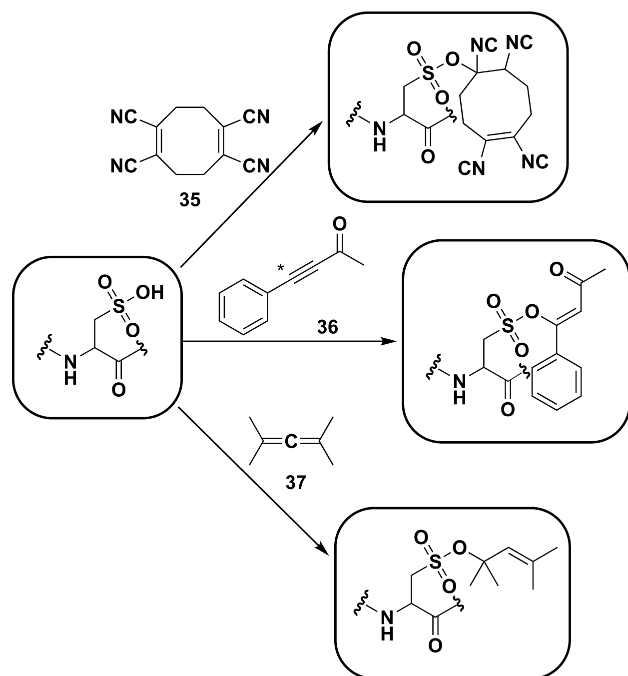
Evidence of a regulatory role for reversible sulfinic acid Prx inactivation also stems from the observation that many signaling pathways, including neuronal *N*-methyl-D-aspartate (NDMA) receptor activity[223] and macrophage activation by lipopolysaccharides[224] induce Srx expression. In both cases, induction of Srx was dependent upon redox-regulated transcription factors, AP-1 and Nrf2.[225] Srx can also translocate to the mitochondria, to reduce hyperoxidized Prx III and protect against oxidative damage and apoptosis.[226] The molecular details are not entirely clear, however, it is possible that Srx-mediated reactivation of Prx III maintains low mitochondrial ROS levels to prevent opening of the mPTP. Srx overexpression also stabilizes PTEN and PTP1B,[109c] which is reminiscent of Prx I-mediated protection of PTEN tumor suppressor activity.[227] The aforementioned studies suggest an important biological role for the reversibility of Prx hyperoxidation. Nonetheless, further studies are required, including the development of an Srx knockout mouse model to assess the physiological relevance of Prx reactivation. It should also be noted that Srx may also carry out other biological functions independent of sulfinic acid reduction.[109c,224]

Although sulfinic acid has gained recognition as a regulatory modification, the full scope of its biological formation remains poorly understood, due in part, to the lack of methods that are suited to general detection. Methods to detect protein sulfinic acids include the a molecular mass increase of 32 Da,[228] acidic electrophoretic gel shifts,[335,336,228] and antibodies that recognize a sulfinic/sulfonic acid peptide from a specific protein.[200,229] Such approaches facilitate study of sulfinic acids in individual proteins, but have limited utility in global analysis. As thiols are good nucleophiles, a challenge to developing chemical methods for sulfinic acid detection lies in is its behavior as a weak nucleophile. An alternative approach is to design a reaction in which the product of the reaction with sulfinic acid is uniquely stabile. Along these lines, our lab has recently investigated the reaction sulfinic acids with aryl-nitroso compounds (Chart 10, eq 1). The initial sulfinic acid-derived *N*-sulfonyl hydroxylamine product is reversible, but can be trapped by ester-functionalized aryl-nitroso **34** to give an irreversible *N*-sulfonylbenzisoxazolone adduct (Chart 10, eq 1).[203a] The reaction of **34** with a thiol yields a sulfenamide species that can be cleaved with nucleophiles (Chart 10, eq 2) and, importantly, **34** does not cross react with other sulfur and nonsulfur containing biological functional groups.

3.3.2.5. Sulfonic Acids. While the sulfinic acid modification is relatively stable, it can undergo further oxidization to give sulfonic acid, the most highly oxidized thiol species (Figure 2). Like sulfinic acid, the sulfur atom in sulfonic acid (formal oxidation number of +4) functions as a hard electrophile and does not undergo self-condensation or nucleophilic attack by thiols. With a pK_a <2, the sulfonic acid is a both strong acid and a weak base, which makes it a good leaving group in S_N1, S_N2, E1, and E2 reactions.[3b] Moreover, organic sulfonic acids can undergo nucleophilic attack on alkenes (**35**), alkynes (**36**), and allenes (**37**) to generate the corresponding sulfonic acid esters (Chart 11), where the reaction initiates exclusively at the

oxygen.[230] However, it is currently unknown whether any of the reactions presented in Chart 11 are amenable to protein studies.

Chart 11. Reactions of Sulfonic Acids[a]



[a]Sulfonic acids function as soft nucleophiles and react exclusively from the oxygen atom to undergo acid-catalyzed reaction with alkenes (**35**), alkynes (**36**), and allenes (**37**) to generate the corresponding sulfonic acid esters. These reactions occur only in organic solvent as the resulting products are largely unstable in water.

The sulfonic acid modification has been characterized in a small group of proteins, including mammalian Cu,Zn-SOD, where it has been speculated that damage resulting from hyperoxidation plays an important role in diseases like familial amyotrophic lateral sclerosis.[229b] Sulfonic acid is also present in mammalian cells as the naturally occurring low molecular weight compound, taurine. This biomolecule plays functions as a general osmolyte and modulator of neuronal activity.[231] As with sulfinic acid, elucidation of the biological and pathological role of sulfonic acid modification has been hindered by a lack of means to selectively detect this oxyacid. Recently, a method has been developed that permits selective enrichment of sulfonic acid-modified peptides using poly arginine (PA)-coated nanodiamonds as high affinity probes.[232] BSA, used as a model system in this study, was oxidized with performic acid, digested, and sulfonic acid-containing peptides were enriched and eluted from PA-coated nanodiamonds with phosphoric acid, with subsequent identification of oxidized peptides by MALDI-MS analysis (Figure 13a). This methodology might have an application in the characterization of protein sulfonic acids in cell lysates by first alkylating reduced and reversibly oxidized thiols with IAM or NEM. Sulfinic acid and sulfonic acid-modified peptides might also identified through a scheme involving performic acid oxidation (Figure 13b). A limitation of this method, however, is that sulfonic acid-modified peptides are in competition with phosphorylated peptides for binding to the PA-coated nanodiamonds,[233] though this could potentially be reduced by phosphatase treatment of lysates.

4. REACTIVE NITROGEN SPECIES (RNS) IN BIOLOGICAL SYSTEMS

The prototypical RNS produced in biological systems is nitric oxide (•NO). In cells, the estimated steady state concentration and half-life of this species is 100 pM−5 nM and ∼0.1−2 s, respectively.[234] Although •NO is more stable than H_2O_2 in cells, protein and small molecule NO-donors are believed to be a relevant source of •NO in biological systems. In general, •NO is a modestly reactive radical and does not inflict indiscriminate damage on biomolecules. Due to its gaseous and neutral nature, •NO is four times more soluble in membranes than in aqueous solution,[235] which permits its diffusion across membranes and, in this context, •NO can function as an autocrine and a paracrine signal within a 100−200 μm radius of the production

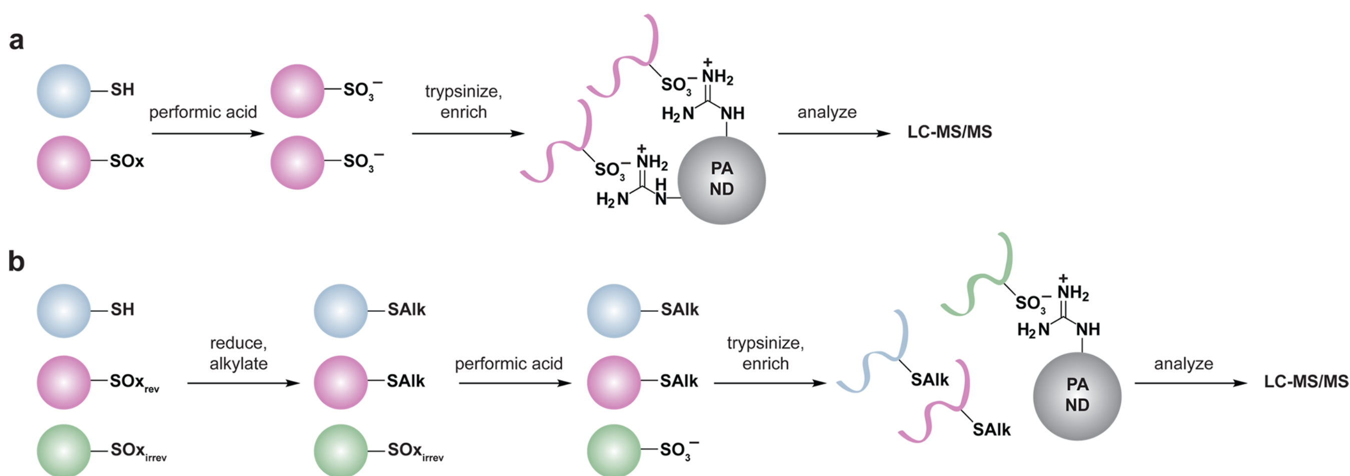
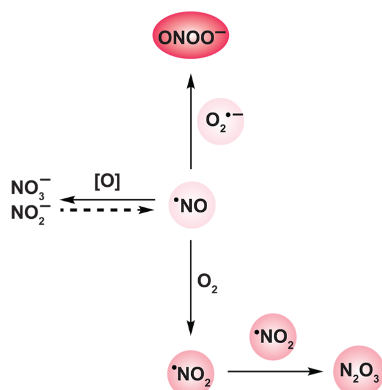


Figure 13. Method for selective enrichment of sulfonic acid-modified peptides. (a) All cysteine residues are oxidized to sulfonic acid with performic acid. Proteins are then trypsinized and sulfonic acid-modified peptides are enriched using polyarginine (PA)-coated nanodiamonds (ND). Eluted peptides are analyzed by LC-MS/MS. (b) A plausible extension of the PA-ND enrichment technology to identify sulfinylated and sulfonylated cysteines. Samples are first treated with a reducing agent to reduce all reversibly oxidized cysteines (purple), and alkylated with NEM or IAM. Irreversibly oxidized cysteines (green) are subsequently oxidized to sulfonic acid with performic acid. The sample is then trypsinized, sulfonic acid-modified peptides are enriched with PA-ND, and eluted peptides are analyzed by LC-MS/MS to identify sites of hyperoxidation.

site.[236] For example, •NO was recently shown to function as a paracrine signal to regulate active T cell expansion in lymph nodes.[237] Initially deemed toxic, •NO was later identified as the first gas known to act as a biological second messenger in mammals where it regulates vasodilation/relaxation of underlying smooth muscle cells.[238] Since these seminal discoveries, roles for •NO have been established in a range of biological processes including proliferation, apoptosis, angiogenesis, and host defense.[239] •NO appears to be metabolized by autoxidation to nitrite (NO_2^-) and nitrate (NO_3^-), which occurs about 30-fold faster within the interior of lipid bilayers than in aqueous solution (Chart 12).[240] •NO can also react

Chart 12. Formation and Transformation of Biologically Relevant Reactive Nitrogen Species (RNS)[a]



[a]Metabolism of nitric oxide (•NO) ultimately involves oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) and there is evidence for this process being reversible (dashed arrow). Though a generally unreactive radical, •NO can react with molecular oxygen to generate nitrogen dioxide radical (•NO_2). Radical-radical coupling of •NO_2 with a second molecule of •NO affords dinitrogen trioxide (N_2O_3) via an ultimately trimolecular reaction. The trimolecular nature of this reaction makes N_2O_3 generation poorly favored at low •NO concentrations. In the presence of $O_2^{•-}$, •NO can react to yield peroxynitrite ($ONOO^-$). Color intensity correlates to relative RNS reactivity.

rapidly (1.1×10^9 M^{-1} s^{-1}) with nitrogen dioxide (•NO_2) to generate additional nitrosating compounds such as dinitrogen trioxide (N_2O_3) (Chart 12).[241] The production of N_2O_3 is a trimolecular reaction with oxygen and two molecules of •NO and is, therefore, not favorable at low •NO concentrations (Chart 12). In turn, these •NO oxidation products play important roles in physiological and pathological processes.[241,242] In addition to autoxidation, •NO reacts rapidly (10^{10} M^{-1} s^{-1}) with $O_2^{•-}$ to generate peroxynitrite ($ONOO^-$), which is reactive and damaging to biomolecules, analogous to •OH.[242c,243] Though it will not be discussed further here, $ONOO^-$ is an important RNS in many biological settings; the interested reader is referred to the following source for additional information.[242c]

4.1. •NO Production and Metabolism

4.1.1. •NO Synthases (NOS). Enzymatic •NO production is predominantly mediated by the heme- and flavin-containing •NO synthases (NOSs), which catalyze the formation of •NO from NADPH, molecular oxygen, and L-arginine (Figure 14a).[244] The linear arrangement of NOSs reveal three domains: the N-terminal oxygenase domain, C-terminal reductase domain, and the connecting calmodulin (CaM)-binding site.

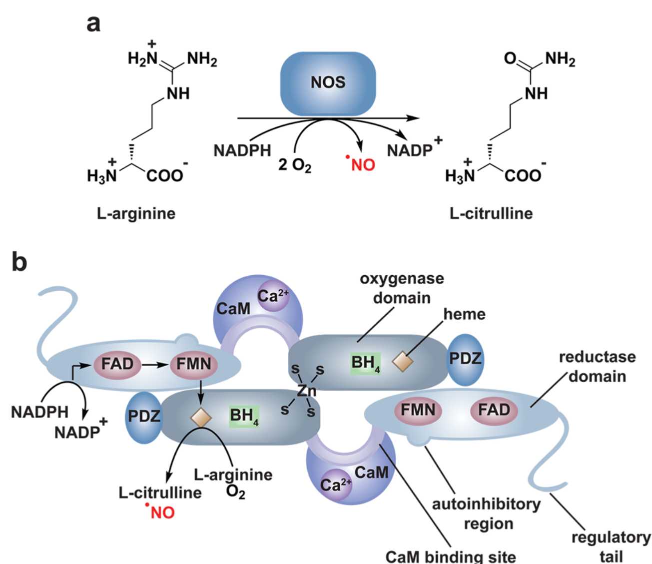


Figure 14. Nitric oxide (•NO) production by nitric oxide synthases (NOS). (a) Reaction catalyzed by NOS. (b) Linear arrangement of NOS. NOS contain three distinct domains: the N-terminal oxygenase domain (gray), the C-terminal reductase domain (light blue), and the connecting calmodulin (CaM) binding site (purple). All three NOS isoforms encode a C-terminal regulatory tail and endothelial NOS (eNOS) and neuronal NOS (nNOS) additionally contain an autoinhibitory region in the reductase domain. Activation of eNOS and nNOS requires binding of Ca^{2+}/CaM whereas inducible NOS (iNOS) is expressed with Ca^{2+}/CaM tightly bound. The production of •NO by NOS involves translocation of electrons from NADPH bound in the reductase domain through the FAD and FMN cofactors. The electrons are then transferred to the heme prosthetic group in the oxygenase domain where L-arginine and molecular oxygen bind. The tetrahydrobiopterin (BH_4) cofactor in the oxygenase domain appears to regulate the nature of reactive intermediates generated by NOS (e.g., •NO versus $O_2^{•-}$). The CaM binding site, autoinhibitory region, and regulatory tail are believed to regulate enzyme activity by influencing the efficiency of electron transfer between the reductase and oxygenase domains. All NOS isoforms function as homodimers that are stabilized by a zinc ion coordinated by two cysteine residues from each monomer and there is evidence suggesting that electrons are transferred between monomers (as depicted). NOS enzymes additionally contain sequences such as PDZ domains (deep blue) that facilitate protein−protein interactions, which are involved in subcellular targeting of NOS and for mediating direct interactions with protein targets of •NO.

The oxygenase domain contains the heme and (6*R*)-5,6,7,8-tetrahydrobiopterin (BH_4) cofactors, and the L-arginine binding site, while the reductase domain has a binding site for NADPH and houses the FAD and FMN flavin cofactors (Figure 14b).[245] •NO is produced by the flow of electrons derived from NADPH through the flavins in the reductase domain to the heme in the oxygenase domain, where oxygen and L-arginine are bound. NOS functions as a dimer in which the large (3000 Å) dimer interface in the oxygenase domain includes the BH_4 binding site and is stabilized by a zinc ion that is coordinated by two cysteine residues in a conserved CXXXXC motif per monomer.[246] Dimerization helps to structure the active-site pocket containing the heme cofactor and the L-arginine binding site, and there is evidence for electron flow occurring between monomers (Figure 14b).[247]

There are three known NOS isoforms that exhibit 51−57% sequence homology among the human enzymes: inducible

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NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). iNOS is expressed in a wide range of cell types and tissues including phagocytic cells where it produces •NO for cytotoxic purposes. eNOS is expressed primarily in vascular endothelial cells where •NO functions in a paracrine manner to regulate vasodilation. Lastly, nNOS is expressed primarily in the brain where •NO is involved in neurotransmission.[245d,e,248] The NOS isoforms can be classified as those that exhibit constitutive (eNOS, nNOS) and inducible (iNOS) expression as well as those that are activated in a Ca^{2+}-dependent (eNOS, nNOS) and independent (iNOS) manner. All NOS isoforms have a C-terminal tail that appears to regulate enzyme activity.[249] Moreover, eNOS and nNOS additionally contain an autoinhibitory loop in the flavin-binding domain that is believed to hinder efficient electron transfer between the FAD and FMN cofactors.[250] In response to receptor activation, a number of growth factors, cytokines, and G-protein coupled receptor (GPCR) agonists have been shown to induce an increase in intracellular calcium, which binds tightly to CaM.[251] The Ca^{2+}/CaM complex then binds to the CaM binding site in NOS and is believed to induce a conformational change involving the C-terminal tail and the autoinhibitory loop to optimally orient the reductase and oxygenase domains for efficient electron transfer, which is the rate limiting step in •NO production.[249,250,252] In contrast to eNOS and nNOS, iNOS, whose expression is controlled by cytokines and interleukins, is expressed with tightly bound Ca^{2+}/CaM and thus functions independent of the intracellular calcium concentration. Rate constants for •NO production range from 200 min^{-1} for iNOS, which produces high concentrations of •NO over the course of hours in immune cells, to 100 min^{-1} for nNOS and 20 min^{-1} for eNOS.[249,253] The diverse •NO production rates suggest structural and regulatory differences between isoforms that influence inherent electron flux rates. Moreover, NOS isoforms appear to be regulated, in part, by the rate of product (•NO) release.[254]

In addition to intrinsic factors, extrinsic factors such as phosphorylation and protein−protein interactions also regulate NOS activity.[245e,248] The serine/threonine kinase AKT has been shown to phosphorylate eNOS in the reductase domain and the C-terminal regulatory tail.[255] Accessibility of these phosphorylation sites appears to be regulated by Ca^{2+}/CaM binding, and both AKT-mediated eNOS phosphorylation and sustained eNOS activation were recently found to be necessary for the tumorigenic properties of oncogenic Ras.[256]

Additional extrinsic factors that regulate NOS activity are protein−protein interactions, as illustrated by Ca^{2+}/CaM-mediated eNOS and nNOS activation. Interestingly, eNOS and nNOS have also been shown to interact with the ATP-dependent molecular chaperone, Hsp90, which may facilitate Ca^{2+}/CaM-induced conformational changes.[257] Protein−protein interactions have also been shown to mediate membrane localization of the cytoplasmic NOS enzymes for cell signaling. In contrast to certain NOX complexes, which have been shown to assemble at activated membrane receptors,[12,70] NOS appears to preassociate with receptors and distinct membrane microdomains prior to ligand stimulation. For example, nNOS has a unique N-terminal PDZ domain, which mediates protein−protein interactions and directs intracellular proteins into multiprotein complexes.[258] In neuronal cells, nNOS is targeted to postsynaptic sites through binding of its PDZ domain to corresponding domains of proteins including PSD-95 and PSD-93.[259] PSD-95 binds to the NMDA receptor (NMDAR) thereby mediating a link between the receptor and nNOS, and this complex forms in the absence of NMDA.[260] By an independent mechanism, eNOS is localized to the membrane through direct interaction with the bradykinin 2 receptor (B2R).[261] All three NOS isoforms contain the conserved sequence FXXFXXXXW, which is a putative caveolin binding site, in their oxygenase and reductase domains.[246b,262] In endothelial cells and cardiac myocytes, eNOS is localized to caveolae, a specialized form of lipid raft, by direct interaction with caveolin-1 and caveolin-3.[263] Interestingly, eNOS is held in an inactive conformation by its interaction with caveolin and B2R, which is released upon Ca^{2+}/CaM-binding or receptor activation, respectively.[261,264]

Membrane localization of eNOS regulates •NO production in endothelial cells by mediating L-arginine availability. In endothelial cells, eNOS forms a complex with the cationic amino acid transporter CAT-1 and arginosuccinate lyase (ASL).[265] CAT-1 is responsible for arginine transport[266] and ASL works in concert with arginosuccinate synthase (ASS1) to recycle L-citrulline, the amino acid product formed by NOS, back to L-arginine. Additionally, ASL funnels L-arginine imported by CAT-1 to eNOS.[265] In this way, eNOS complex formation with CAT-1 and ASL regulates •NO production by modulating local substrate availability, somewhat like the regulation of flavin availability through NOX complex formation with riboflavin kinase.[70]

Under certain circumstances, NOS can form $O_2^{\bullet-}$ instead of •NO.[267] Such conditions include the absence of the BH_4 cofactor[268] and uncoupling of electron transfer within NOS via conformational changes that permit direct oxygen interaction with the flavins in the reductase domain[264b] or *S*-glutathionylation of Cys689 and Cys908 in the reductase domain of eNOS.[163a] The more recent finding that *S*-glutathionylation influences reactive intermediate production by NOS is interesting given that coproduction of ROS and RNS can result in generation of the aggressive oxidant $ONOO^-$ and might be a mechanism to deter $ONOO^-$ production.[163a] Although further studies are required to determine whether ROS mediate NOS glutathionylation, the cross-regulation proposal is also supported by the observation that •NO can inhibit NOX-mediated oxidant production in plant immune cells.[269]

•NO has been shown to regulate a range of processes including proliferation, apoptosis, angiogenesis, host defense, and regulation of vasodilation.[239] To elucidate the role of unique NOS isoforms in regulating these diverse biological processes, mouse models of NOS deficiency have been generated.[270] Additionally, much effort has been aimed at developing selective small molecule inhibitors for individual NOS isoforms.[245d] Selective inhibitors have been developed for iNOS that act in competition with L-arginine in which selectivity is achieved through interactions with the novel substrate-binding site in this isoform, as compared to nNOS and eNOS.[271] Two iNOS inhibitors have been used to probe the roles of this isoform in several animal models of diseases in which iNOS has been implicated. More recently, a therapeutic role for iNOS selective inhibitors has been shown for lung regeneration in a mouse model of full-established emphysema.[272] Selective iNOS inhibitors have also been used in clinical studies for medical conditions involving lung damage including chronic obstructive pulmonary disease (COPD) and asthma.[273] The continued development of NOS inhibitors will further our understanding of distinct roles for each of these isoforms in

diverse biological processes and will certainly continue to uncover additional avenues for therapeutic intervention for diseases where NOS are implicated.

In plants, •NO has been shown to be involved in seed germination, root growth, respiration, stromal closure, and adaptive responses to biotic and abiotic stresses.[274] Although the existence of bona fide NOS isoforms in plants remains controversial, the cytoplasmic enzyme nitrate reductase is a recognized source of •NO in these organisms, as reviewed elsewhere.[275] Alternative pathways for NOS-independent •NO production have also been identified in different plant cell compartments, such as peroxisomes, mitochondria, and the apoplasm.[275]

The discovery that RNS are produced as second messengers to regulate a number of biological processes has spurred the development of methods to specifically detect these species in cells. Historically, •NO production has been detected indirectly by monitoring its oxidation products, namely N_2O_3, NO_2^-, and NO_3^- by colorimetric, spectroscopic and fluorescent means.[276] The field has more recently seen the development of direct methods to specifically detect not only •NO, but also $ONOO^-$ and nitroxyl (HNO) by exploiting the unique reactivity of each of these species. These methods include nanotube-,[277] cell-,[278] protein-,[279] small molecule-,[280] and electrochemical-based[281] assays. To date, no RNS probes are available that permit species detection in specific subcellular compartments or organelles. Improvements to the current technology including reversibility are required for regio- and spatiotemporal resolution of RNS production and the interested reader is referred to the following review for additional information.[282]

4.1.2. •NO-Metabolizing Enzymes.

Unlike $O_2^{\bullet-}$ and H_2O_2, for which ROS metabolizing enzymes exist to regulate their levels, far less is known about enzymatic regulation of •NO availability. As previously mentioned, •NO autooxidizes to NO_2^- and NO_3^-, however, it was recently shown that •NO oxidation to NO_2^- can also be catalyzed by the abundant plasma multicopper oxidase, ceruloplasmin.[283] NO_2^- and NO_3^- have traditionally been thought of as inert byproducts of •NO; however, there is increasing evidence for enzymatic reduction of NO_2^- to regenerate •NO by xanthine oxidase,[284] by nitrate reductase in plants,[275] or through reaction with deoxyhemoglobin in the vasculature.[285] NO_2^- reduction could also facilitate •NO release at sites distant from NOS. Along these lines, fatty acids and proteins modified by •NO can similarly be reduced to release •NO or act to transfer •NO to sites distal from NOS. Through protein−protein interactions, NOS has been found to localize to the plasma membrane, endoplasmic reticulum, sarcoplasmic reticulum, and sarcolemmal caveolae where NOS regulates a distinct set of proteins in each location.[286] This has spurred the hypothesis that NOS is placed where it is needed for local action of •NO, akin to NOX.[287] However, it is possible that the aforementioned alternative mechanisms of •NO release and transport may extend •NO signaling to subcellular regions that are inaccessible by NOS, such as the nucleus,[288] or may enhance the paracrine activity of •NO.[289]

4.2. Modification of Protein Cysteine Thiols by RNS

Similar to $O_2^{\bullet-}$, •NO is a relatively unreactive radical and its primary targets in cells include other radical species such as $O_2^{\bullet-}$ and metals. Indeed, the propensity for •NO to coordinate to metals has been exploited in the development of •NO-specific small molecule fluorescent detectors.[280a−i] The first identified cellular target of •NO was soluble guanylyl cyclase (sGC) in which •NO activates sGC through binding reversibly to the prosthetic heme.[290] In endothelial cells, the •NO produced migrates through the vasculature to activate sGC in the underlying vascular smooth muscle cells to promote vasodilation.[291] •NO-mediated sGC activation also stimulates mitochondrial biogenesis in brown adipose tissue.[292] In addition to sGC, •NO can regulate other heme-containing proteins including ETC Complex IV, where •NO binding inhibits cellular respiration and ROS production under hypoxic conditions.[293] •NO can also control protein function through iron−sulfur clusters, as documented for bacterial transcriptional regulators, such as NsrR, SoxR, and FNR.[294] This form of regulation is thought to occur via •NO-mediated iron−sulfur cluster nitrosylation and degradation.[295]

It was recognized early on in the field that, in addition to regulating protein function by coordination to metal-based prosthetic groups, •NO could covalently modify protein cysteines, a modification subsequently termed S-nitrosylation.[5c] Analogous to other oxPTMs, specificity in modification appears to be imparted by cysteine reactivity, local protein environment, and proximity to the oxidant source.[3b,12,194] In contrast to NOX signaling, (or, more likely, as is less well established for NOX signaling) the proximity of protein targets of •NO to the RNS source is frequently imparted by direct interaction with NOS. As discussed above, NOS enzymes contain structural features that facilitate protein−protein interactions, and a number of NOS-interacting proteins including caspase-3,[296] cyclooxygenase-2,[297] and the postsynaptic scaffolding protein PSD-95,[260] have been shown to be S-nitrosylated after NOS activation.

Though still an active area of research, three prominent mechanisms have been proposed to account for de novo *S*-nitrosothiol formation, none of which involve direct reaction of •NO with thiols (Figure 15a−c). As mentioned above, •NO can be converted to the nitrosating compound N_2O_3 (Chart 12). The initial reaction of •NO with molecular oxygen to generate $^{\bullet}NO_2$ and subsequent radical−radical combination of •NO with $^{\bullet}NO_2$ permits N_2O_3 production with a rate constant of 10^9 M^{-1} s^{-1}.[241] N_2O_3 can subsequently react with a protein or low molecular weight thiolate to yield an *S*-nitrosothiol (Figure 15a). Given the requirement for two molecules of •NO in this reaction, the route is not favorable at low concentrations of this species. Alternatively, $^{\bullet}NO_2$ or other radical species such as $O2^{\bullet-}$[298] can promote the one-electron oxidation of a protein or low molecular weight thiolate to generate a thiyl radical that can undergo radical−radical combination with •NO to yield the *S*-nitrosothiol (Figure 15b). While evidence exists to support both of these mechanisms,[299] a third route has been postulated to account for *S*-nitrosylation of some proteins. This mechanism, which has been demonstrated for both hemoglobin[300] and nitrophorin,[301] relies on the propensity of •NO to bind to heme prosthetic groups in which the heme-bound NO undergoes reductive nitrosylation of the heme prosthetic group and autotransfer to a thiol within the same protein to generate an *S*-nitrosothiol (Figure 15c). Though it will not be further discussed here, •NO and •NO-derived species can also modify other amino acids, including tyrosine.

Like sulfenic acid, the formal oxidation number of the sulfur atom in *S*-nitrosothiol is 0; despite this apparent similarity, there are many important differences between these modifications. The *S*-nitrosothiol group is not ionizable,[302] can undergo hydrolysis to give sulfenic acid,[303] or react with a thiol

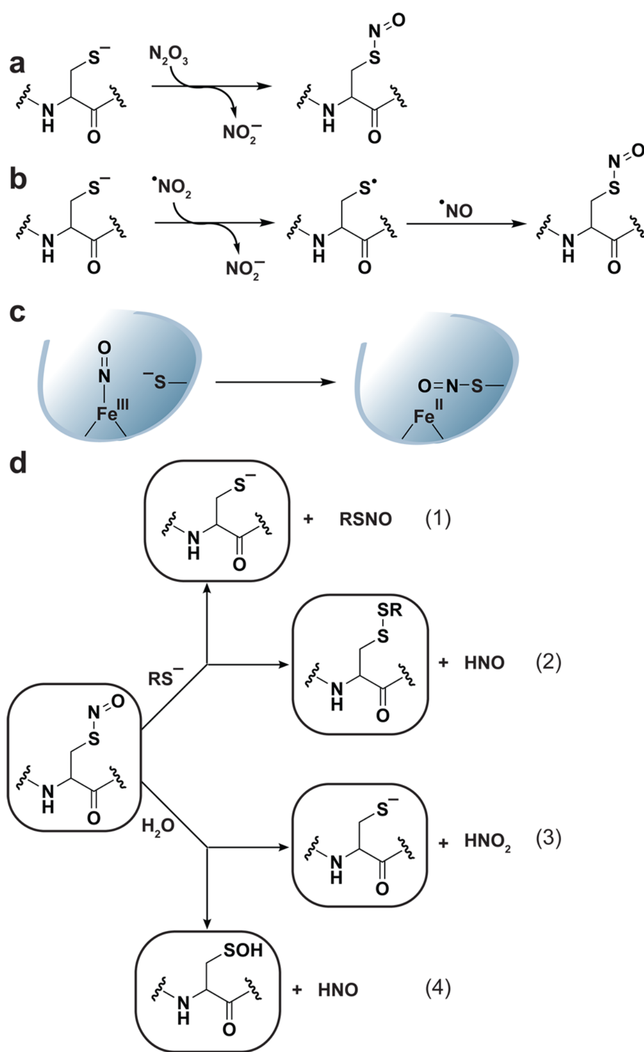

Figure 15. Formation and subsequent reactions of *S*-nitrosothiols. Three prominent mechanisms for *S*-nitrosothiol formation include (a) reaction of a protein or low molecular weight thiolate with N_2O_3, (b) formation of a thiyl radical upon initial reaction of a thiolate with $^{\bullet}NO_2$ and other radical species and subsequent radical−radical combination with $^{\bullet}NO$, and (c) autotransfer of heme-bound ^{+}NO to a nearby cysteine thiolate as has been demonstrated for hemoglobin and nitrophorin. (d) Once formed, an *S*-nitrosothiol can react with a neighboring cysteine residue either within the same or an adjacent protein, or with GSH (not shown) to undergo transnitrosylation (eq 1) or disulfide bond formation (eq 2). Alternatively, an *S*-nitrosothiol can be hydrolyzed to release the free thiolate and nitrite (HNO_2) (eq 3) or a sulfenic acid and HNO (eq 4). In each case, the pK_a of the sulfur in the *S*-nitrosothiol, in part, influences which product is formed. In most cases, transnitrosylation and release of a free thiolate are favored upon reaction with a second cysteine or water, respectively due to the high pK_a of the HNO leaving group.

(Figure 15d). Interestingly, reaction of an *S*-nitrosothiol with a protein thiol or GSH does not always yield the mixed disulfide, but can instead (and perhaps more frequently) facilitate a process known as transnitrosylation (Figure 15d). The ability to undergo transnitrosylation is due to the different chemical properties of ^{+}NO compared to the hydroxyl in sulfenic acid, and will be discussed further in the following subsection. Transnitrosylation is increasingly viewed as another physiologically relevant mechanism for *S*-nitrosothiol formation[5c,287,304] and studies of protein *S*-nitrosylation often use ^{+}NO donors such as GSNO, *S*-nitrosocysteine (SNOC), and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP).[260,297,305] In vitro rate constants for de novo thiol S-nitrosylation in human and bovine serum albumin are on the order of 10^3 to 10^4 M^{-1} s^{-1}.[306] In contrast, in vitro S-nitrosylation rate constants for glutathione and other low molecular weight thiols are on the order of 10^5 to 10^7 M^{-1} s^{-1}.[306,307] Since de novo *S*-nitrosothiol formation depends on the combined reactivity of two $^{\bullet}NO$, molecular oxygen, and a thiol (Chart 12, Figure 15a and b), GSH and abundant proteins such as Trx, albumin, and hemoglobin could be primary targets of S-nitrosylation. Indeed, as previously mentioned, the aforementioned protein and low molecular weight thiols can function as ^{+}NO donors, and are proposed to extend $^{\bullet}NO$ signaling to proteins distal to its site of production both within cells and as a paracrine signal.[288,289]

The tendency for a particular cysteine residue to undergo transnitrosylation appears to be regulated, in part, through steric (*e.g.*, accessibility to ^{+}NO donors) and electrostatic factors.[287,305h,308] Computational studies to identify a consensus sequence for S-nitrosylation have uncovered an acid−base motif, located distal to the modified cysteine in the protein tertiary structure among some S-nitrosothiols.[304,309] This charged nature of the acid−base motif has been proposed to engage in protein−protein and protein-GSH interactions to facilitate transnitrosylation. A recent structural study, has revealed to two additional sequence motifs proximal to the *S*-nitrosothiol that facilitate reduction by Trx, though whether these particular cysteines also participate in transnitrosylation from nitrosylated Trx has not been established.[304] Beyond these putative protein−protein interaction motifs, manual inspection of *S*-nitrosothiol sites incidate that S-nitrosylated cysteines may be directly flanked by an acid−base motif that enhance reactivity or decrease pK_a.[310] An additional feature of the environment surrounding S-nitrosylation sites is hydrophobicity.[311] Hydrophobic protein surfaces could potentially concentrate nonpolar $^{\bullet}NO$ and molecular oxygen, permitting the formation of N_2O_3 directly at the site of S-nitrosylation. Neither the local acid−base motif nor the hydrophobic environment are uniformly conserved, which is consistent with a similar lack of sequence bias for sulfenylation[9b] and may be reflective of the numerous mechanisms for de novo and transnitrosylation.[304,312]

Given the propensity for *S*-nitrosothiols to participate in transnitrosylation reactions, reducing systems are in place to reverse *S*-nitrosylated thiols and the interested reader is referred to the following reviews for a more thorough coverage of the topic.[287,313] GSH can reduce a *S*-nitrosothiol to give the free thiol and GSNO. In turn, GSNO is reduced to regenerate GSH and release HNO by GSNO reductases (GSNOR).[314] GSNOR acts exclusively on GSNO and deficiency of this enzyme increases the steady-state level of protein *S*-nitrosylation (which can be further enhanced by iNOS activation and may support a physiological role for GSNO as an ^{+}NO donor).[314a,315] Protein *S*-nitrosothiols can also be reduced by the Trx/TrxR system.[305e] Enzymes with primary functions unrelated to protein S-nitrosylation may also act as denitrosylases, including PDI, xanthine oxidase, and SOD,[313] though the physiological relevance of these activities remains unclear.

Since its discovery, S-nitrosylation has been implicated in the regulation of proteins involved in cellular trafficking,[316] muscle contractility,[317] apoptosis,[305a,e] circulation,[318] neural transmission,[260,319] and host defense.[320] However, it is important to keep in mind that most S-nitrosylated proteins that have

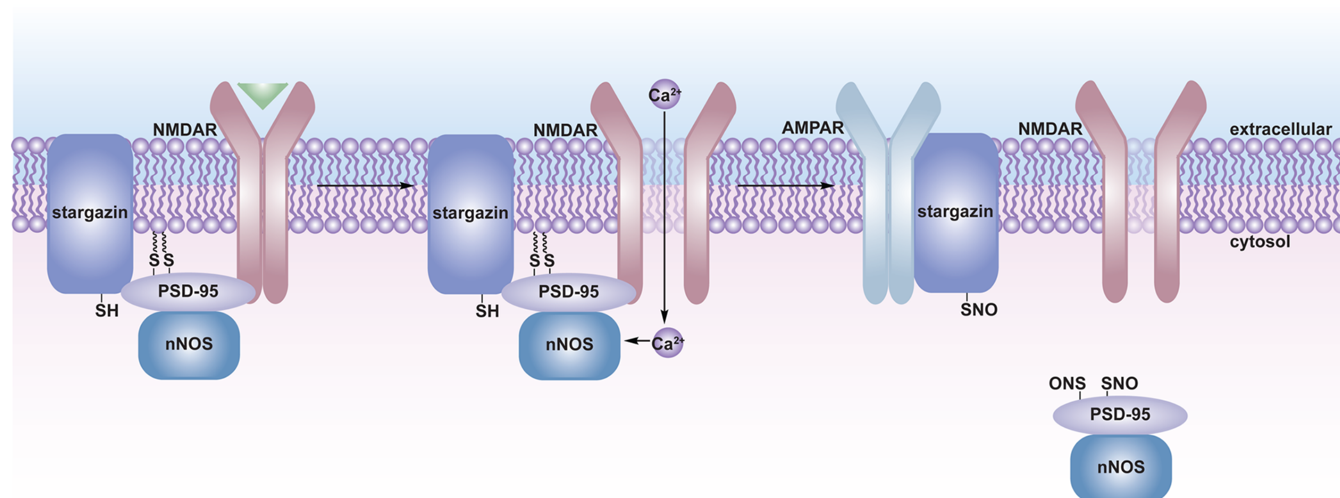

Figure 16. Regulation of neuronal signaling by S-nitrosylation. PSD-95 is a scaffolding protein that localizes to postsynaptic densities by reversible S-palmitoylation of two cysteine residues in the N-terminal region. In the absence of ligand, nNOS is physically linked to the NMDA receptor (NMDAR) at the neuronal plasma membrane *via* a mutual interaction with PSD-95. PSD-95 similarly localizes stargazin near NMDAR. NMDA binding to NMDAR facilitates calcium entry, which activates nNOS mediating •NO production and subsequent S-nitrosylation of PSD-95 on the same cysteine residues that undergo S-palmitoylation. S-nitrosylation thereby prevents PSD-95 lipidation and decreases membrane association of PSD-95 and, hence, nNOS. Stargazin is similarly S-nitrosylated, which enhances its interaction with the AMPA receptor facilitating its recruitment to the postsynaptic densities.

been identified to date are derived from studies with exogenous $^+$NO donors employed at unphysiological concentrations (though protein S-nitrosylation from endogenous •NO production has been observed in neurons and immune cells, the latter of which produce high concentrations of •NO for bactericidal purposes).[245d,248]

In plants, attempted microbial invasion triggers the hypersensitive response, a programmed execution of plant cells at the sites of infection. This process involves the generation of NOS-derived •NO with subsequent production of NOX-derived $O_2^{\bullet -}$ and the chemical messenger, salicylic acid.[320] Interestingly, S-nitrosylation of NPR1, a master regulator of salicylic acid-mediated defense genes, promotes its oligomerization and cytoplasmic retention. S-nitrosylation is reversed by Trx in a salicylic acid-stimulated manner to facilitate NPR1 monomerization and nuclear translocation.[305f] Whether microbial invasion in plants induces cell death appears to be regulated, in part, by the extent of •NO and $O_2^{\bullet -}$ production, which together produce the more reactive $ONOO^-$. Interestingly, both NOS[321] and NOX[269] have been found to be inhibited by *S*-nitrosylation, shedding light on a potential regulatory mechanism to control ROS and RNS coproduction in immune responses, which could be conserved across species. More recently, it was shown in a mouse model of *Clostridium difficile* infection that host-derived •NO S-nitrosylates and inhibits clostridial small molecule-activated glucosylating toxins, thereby preventing toxin cleavage and cell entry.[322] This represents a unique mechanism for •NO-mediated pathogen detoxification.

In addition to being involved in the immune response, NOS may also play a role in synaptic plasticity (the strength of connection between two neurons), which is relevant to processes, such as spatial learning.[323] nNOS is recruited to the membrane prior to synaptic signaling through its interaction with PSD-95, which physically links nNOS to NMDAR (Figure 16).[259a,324] Stimulation of NMDAR triggers calcium entry and activates •NO production via the proximal nNOS. PSD-95 is localized to the membrane through a dynamic reversible cycling of S-palmitoylation, a posttranslational lipid modification, of two N-terminal cysteine residues.[325] It was recently shown that nNOS activation mediates S-nitrosylation of these same cysteine residues in PSD-95 thereby preventing S-palmitoylation and reducing PSD-95 and hence nNOS membrane localization subsequent to neuron activation (Figure 16).[260] This study highlights the intriguing possibility that differential modification of cysteines may represent a general paradigm in cell signaling and, in this context, S-nitrosylation of PSD-95 may function to regulate the duration of NMDA signaling. NMDAR activation also regulates the recruitment of AMPA receptors (AMPAR) to the synapse to propagate signaling. PSD-95 regulates AMPAR through its interaction with stargazin[326] and was recently shown to be S-nitrosylated in response to NMDA signaling, thereby enhancing its binding to AMPAR (Figure 16).[319a] Lastly, nNOS-derived •NO can also regulate neural cells at the level of gene transcription. For example, S-nitrosylation of histone deacetylase 2 was found to induce its release from chromatin, permitting increased acetylation of histones surrounding genes involved in neural development and promoting transcription.[305d]

Expression of NOS isoforms is regulated by Ca^{2+}/CaM binding. S-Nitrosylation of calcium transporters has been increasingly demonstrated, revealing a potential positive feedback loop. In one instance, S-nitrosylation of ryanodine receptors in skeletal muscle[327] and neurons[328] releases intracellular calcium stores to potentiate signaling that, in the latter case, are required for neural synaptic plasticity and can also contribute to neuronal cell death. S-Nitrosylation can also regulate entry of extracellular calcium as S-nitrosylation of transient receptor potential (TRP) cation channels mediates a conformational change in endothelial cells that opens the pore to permit calcium entry,[329] which may similarly function as a positive feedback loop to potentiate NOS activity.

In addition to promoting cell signaling responses, dysregulation of *S*-nitrosylation has been implicated in disease,

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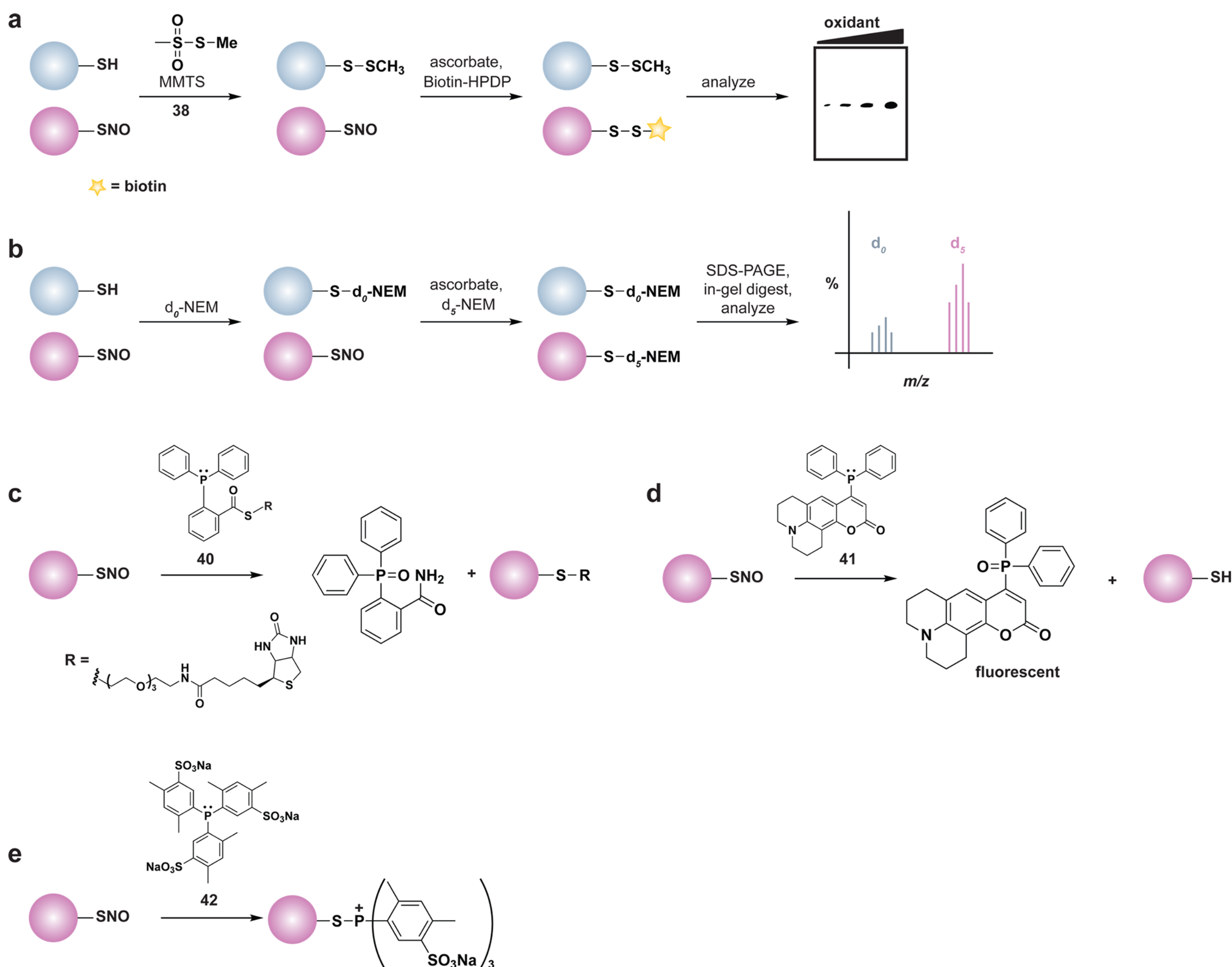


Figure 17. Indirect and direct chemical methods for *S*-nitrosothiol detection. (a) The biotin switch technique (BST) is an indirect differential alkylation method that involves blocking free thiols (blue) with methylmethane thiosulfonate (MMTS, **38**), reducing *S*-nitrosothiols (purple) with ascorbate, and labeling nascent thiols with Biotin-HPDP. The samples are then analyzed by nonreducing avidin blot in which *S*-nitrosylation correlates to increased signal intensity. (b) Quantification of protein S-nitrosylation with d-Switch. The d-Switch technique combines the BST with isotopically labeled NEM in which free thiols (blue) are blocked with d_0-NEM, *S*-nitrosothiols (purple) are reduced with ascorbate and labeled with d_5-NEM. The samples are subsequently separated by SDS-PAGE, digested in-gel with trypsin, and the resulting peptides are analyzed by LC-MS/MS. The extent of protein S-nitrosylation is determined by the ratio of d_5-NEM to d_0-NEM signal intensity. (c−e) Triarylphosphine-based methods to directly modify *S*-nitrosothiols. (c) Triarylphosphine reagent **40** reacts with *S*-nitrosothiols to yield a disulfide-bonded biotin adduct. (d) Compound **41** is oxidized upon reaction with *S*-nitrosothiols to yield a fluorescent compound that comments on the presence of *S*-nitrosothiols, but does not covalently modify oxidized proteins. (e) Water-soluble triarylphosphine **42** appears to form a stable *S*-alkylphosphonium adduct as monitored by ^{31}P NMR and mass spectrometry.

including neurodegenerative disorders.[256,287,305a,b,330] The E3 ubiquitin ligase parkin, which regulates the degradation of proteins important to survival of dopamine neurons, is S-nitrosylated in a mouse model of Parkinson's disease (PD) and in brains of patients with PD.[305b] Parkin S-nitrosylation inhibits its ubiquitin ligase activity, which impairs ubiquitination of its substrate proteins and may contribute to the degenerative process. It has also been shown that PDI, an ER-resident enzyme that facilitates proper protein folding and protects neuronal cells against ER dysfunction, is S-nitrosylated in brain samples manifesting sporadic PD or Alzheimer's disease.[305c] PDI S-nitrosylation inhibits its activity, resulting in activation of ER stress pathways (including the unfolded protein response) and abrogates PDI-mediated attenuation of neuronal cell death triggered by ER stress, which could contribute to neurodegenerative disorders. More recently, amyloid-β, a key mediator in Alzheimer's disease was found to induce $^{\bullet}$NO production, which triggered mitochondrial fission, synaptic loss, and neuronal damage.[305a] This effect was attributed, in part, to S-nitrosylation of dynamin-related protein 1 (Drp1), a protein involved in regulation of mitochondrial fission. S-nitrosylated Drp1 is increased in brains of human Alzheimer's disease patients where it is postulated to contribute to disease pathogenesis.

4.3. Methods for Detecting RNS-Modified Cysteines

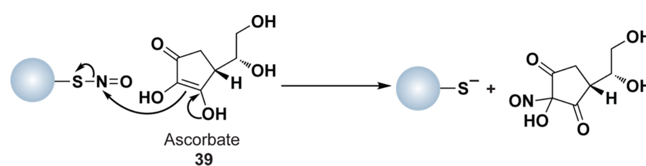
The discovery of protein S-nitrosylation has spurred the development of methods for its detection.[138,331] Initial indirect chemiluminescent, colorimetry and electrochemical approaches relied upon detection of NO liberated from *S*-nitrosothiols by mercury.[332] However, these methods are artifact prone because

of interference from other metabolites in the sample, such as NO_2^-. Moreover, indirect spectroscopic methods report only on the total amount of *S*-nitrosothiols and do not permit identification of the target proteins. While NO is released with metal treatment, the protein thiol remains coordinated to mercury and strategies to identify metal-coordinated proteins have been reported.[304,333] A limitation to this method is that other metal-interacting modifications, including protein-*S*-GSH disulfides and, perhaps cysteine sulfenic acid and persulfide, can similarly be detected and complicate selective analysis. While alternative methods for *S*-nitrosothiol detection have since been developed, these indirect spectroscopic methods are still used to quantify the total amount of S-nitrosylated protein in purified samples.[305g] S-Nitrosylated proteins can also be identified using an anti-*S*-nitrosocysteine antibody or by MS, however, these methods are not well suited to identify *S*-nitrosothiols in complex protein mixtures and do not facilitate enrichment of oxidized proteins.

Chemical methods for direct and selective detection of protein S-nitrosylation have also been reported. In contrast to sulfenic acids, which have one electrophilic center, *S*-nitrosothiols contain two, allowing nucleophiles to attack the sulfur or nitrogen (the reaction site is influenced by the relative stability (i.e., pK_a) of the leaving group). As previously discussed, the pK_a of a free thiol group is ∼8.5 but can be significantly modulated in the protein environment.[334] The pK_a of HNO, the alternative leaving group, is approximated at 11.4.[242a,335] On the basis of these relative pK_a values, in the majority of cases, the thiol is predicted as the preferred leaving group. This leaving group preference provides a chemical rationale for transnitrosylation; however, *S*-nitrosothiols can also form en route to disulfide bonds (Figure 15d). In these cases, it is possible that features of the *S*-nitrosothiol environment favor disulfide bond formation by increasing the electrophilicity of the sulfur through pK_a modulation (e.g., increasing the thiol pK_a) or by promoting protonation of HNO in the *S*-nitrosothiol. The latter would be analogous to protonation of a hydroxyl group prior to nucleophilic attack to facilitate expulsion as water. Importantly, the disparate electrophilic centers in sulfenic acids and *S*-nitrosothiols can be exploited to permit chemical discrimination between these forms.

To date, the most popular procedure for *S*-nitrosothiol detection, is known as the biotin switch technique (BST).[336] As shown in Figure 17a, the BST is an indirect method that involves blocking free thiols by S-methylthiolation with methylmethane thiosulfonate (MMTS, **38**), selective reduction of *S*-nitrosothiols with ascorbate, and labeling nascent thiols with *N*-[6-(biotinamido)-hexyl]-3′-(2′-pyridyldithio)-propionamide (biotin-HPDP). The reaction of thiols with biotin-HPDP yields a mixed disulfide adduct that can be detected by avidin blot. Additionally, the biotin handle permits enrichment of labeled proteins for proteomics analysis. As with the two previously described indirect chemical methods, the success of the BST is dependent upon complete blocking of free thiols and the selectivity and efficiency of the reducing agent. Though the mechanism is not entirely clear, *S*-nitrosothiol reduction may involve nucleophilic attack of ascorbate (**39**) at the electrophilic nitrogen center to release the thiol (Chart 13). In accordance with the leaving group bias of transnitrosylation versus disulfide bond formation, some *S*-nitrosothiols cannot be reduced efficiently by ascorbate,[331a,337] which might be due to competing reaction at the electrophilic sulfur center. Recently,

Chart 13. Predicted Mechanism for the Reaction of Ascorbate with *S*-Nitrosothiol

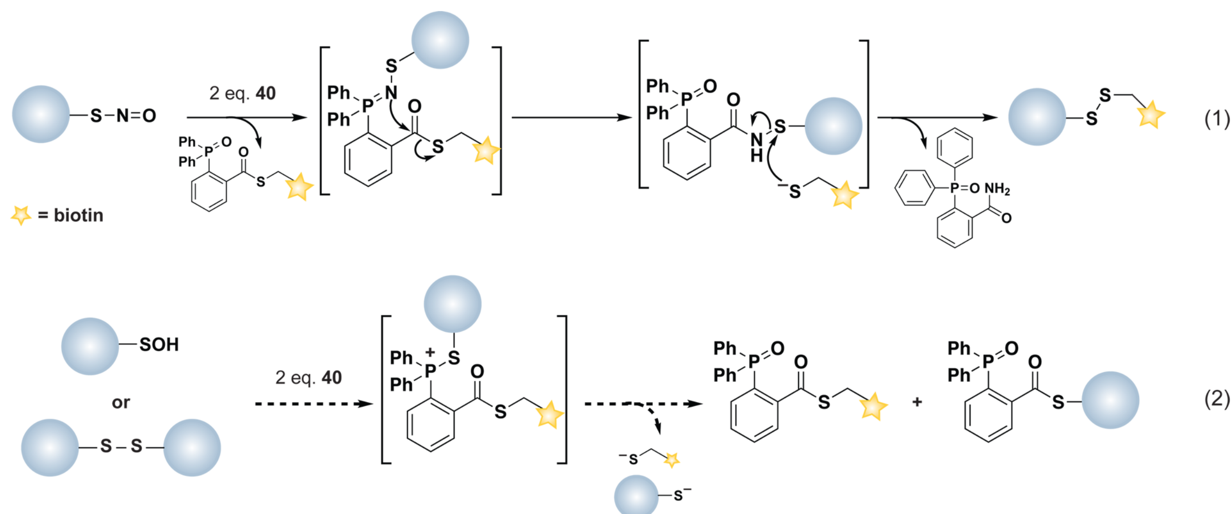


the use of ascorbate as a selective reductant for *S*-nitrosothiols has been questioned, because of the observation that ascorbate can reduce some disulfides[179,338] and sulfenic acid, as recently shown for some 1-Cys Prxs.[172] In one instance, sinapinic acid was used in place of ascorbate as it does not appear to react with disulfides.[339] Despite the limitations of the BST, this technique is routinely used in diverse protein systems and led to important advances in S-nitrosylation research.[226,260,269,288,329]

Improvements to the BST have been made that involve biotin enrichment of trypsin-digested peptides,[310,340] resin-assisted capture,[341] fluorescence labeling,[342] and a microarray-based assay.[305h] The latter case shows a small percentage of false positives and does not cover the entire proteome; nonetheless, it permits rapid identification of candidate *S*-nitrosylated proteins and allows for the direct comparison and assessment of chemically distinct $^+$NO donors. More recently, Thatcher and colleagues developed a quantitative approach termed d-Switch that combines the BST with isotopically labeled NEM (d_5-NEM) (Figure 17b).[343] Future adaptations of the d-Switch technique could incorporate a biotin affinity handle to permit sample enrichment analogous to ICAT.

Methods for direct chemical modification of *S*-nitrosothiols have also been reported. For example, triarylphosphines have shown promise as chemical probes for S-nitrosylation[344] and the interested reader can find additional information about this chemistry from the following review.[331b] In the first demonstration of this approach, a small-molecule *S*-nitrosothiol model underwent reductive ligation with a triarylphosphine ester.[344] A variation on this theme involves reductive ligation of an *S*-nitrosothiol with a biotinylated triarylphosphine thioester (**40**) in a THF-PBS system to generate a disulfide linkage with biotin (Figure 17c).[345] The triarylphosphine reduction reaction has also been adapted to generate a new fluorescent probe (**41**) to monitor *S*-nitrosothiol content in recombinant proteins, but it is not currently amenable to identification of S-nitrosylated proteins in complex mixtures (Figure 17d).[346] King and colleagues have reported the water-soluble triarylphosphine (**42**) that reacts with *S*-nitrosothiols to give a stable *S*-alkylphosphonium adduct detectable by ^{31}P NMR and MS (Figure 17e).[347] A future modification of this reagent could incorporate an affinity handle for protein enrichment (though the anionic nature of **42** likely precludes membrane permeability for cellular studies). Interestingly, while the S−P bond is usually labile, steric hindrance imparted by the substituted aryl ligands and aromatic stabilization of the phospho cation is believed to stabilize the *S*-alkylphosphonium adduct. Future work with triarylphosphine reagents will need to address cross reactivity with disulfides and sulfenic acids. From the perspective of selectivity, only the strategy presented in Figure 17c would be able to rigorously discriminate between *S*-nitrosothiols and disulfides or sulfenic acids, as biotin disulfide formation would be unique to this species (Chart 14).

Chart 14. Reaction Mechanism of Triarylphosphine 40 with a Protein *S*-Nitrosothiol to Yield a Disulfide-Bonded Biotin Adduct (Equation 1) and Potential Reaction of Sulfenic Acids or Disulfides with 40 (Dashed Arrows) Should Not Yield the Same Adduct (Equation 2)



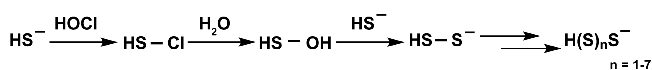
5. REACTIVE SULFUR SPECIES (RSS) IN BIOLOGICAL SYSTEMS

As we have seen, oxidation of protein and low molecular weight thiols generates a wide range of sulfur-containing products including disulfides, thiosulfinates, sulfenic acids, and *S*-nitrosothiols. Each modification is capable of propagating redox transformations that involve oxidation of other thiols analogous to ROS and RNS (Figures 2, 5, and 15d). As follows, these chemically reactive forms of cysteine can be classified as reactive sulfur species (RSS).[213,348] Several "nonspecific" peroxidases, such as horseradish peroxidase, can also oxidize thiol substrates by one-electron oxidation to form thiyl radicals, which also represent an important class of RSS.[349] Once formed, this radical species can participate in a variety of chemical reactions. Of particular note, thiyl radicals can react with a thiolate affording a disulfide radical anion intermediate, which culminates later in disulfide and $O_2^{\bullet-}$ formation.

In addition to reactive cysteine species in proteins, inorganic sulfur-containing species are also classified as RSS. The prototypical inorganic RSS is hydrogen sulfide (H_2S), which is the most stable reactive intermediate considered in this review with a half-life on the minute time-scale.[350] Along with $^{\bullet}$NO and carbon monoxide, H_2S is produced in biological systems where it functions as a gasotransmitter to regulate diverse biological processes as an autocrine, paracrine, and endocrine signal.[351] H_2S is a weak acid with a pK_{a1} and pK_{a2} of 6.9 and >12 and, therefore, exists primarily in the dissociated thiolate form (HS^-) at physiological pH (though H_2S is commonly used to refer to all species: H_2S, HS^-, S^{2-}).[352] Similar to other reactive intermediates, H_2S was first recognized as a toxic species when it was found to emanate from sewers, and is produced as a toxic byproduct of industrial processes. Research over the past two decades, however, has implicated H_2S in a number of physiological and pathological systems. Roles for H_2S in biology were initially suggested in vasodilation/relaxation, subsequently as a synaptic modulator and neuroprotectant, and as a regulator of inflammation.[350,351,352b,353] The latter has motivated the development of H_2S-releasing drugs, which are currently under investigation for their use as anti-inflammatory agents.[354] More recently, H_2S was also implicated in the control of cell proliferation and survival in cardiomyocytes.[355]

Given its role in similar physiological settings, H_2S has been said to engender many of the same effects of $^{\bullet}$NO without the generation of hyperreactive (and possibly toxic) intermediates.[356] H_2S can scavenge reactive intermediates including $^{\bullet}$NO,[357] $ONOO^-$,[358] $O_2^{\bullet-}$,[359] HOCl, or H_2O_2; however a general biological role for this activity remains largely speculative at this stage, owing to the low concentration of H_2S in many systems (vide infra) and modest reactivity compared to GSH.[301,350,352b,360] Nonetheless, Nudler and colleagues recently reported that microbial H_2S production and subsequent oxidant scavenging can serve as defense against oxidant-generating antibiotics.[361] On the other hand, H_2S has also been shown to stimulate the production of ROS in prostate cancer cells[362] in a pathway that depends on p66(Shc).[363] Though a mechanism was not devulged, this study highlights the potential for complex interplay between RSS and ROS signaling, which is likely to be tissue and cell-type specific. Reaction of H_2S with $^{\bullet}$NO and $ONOO^-$ can generate inert nitrosothiols,[358] but in some cases, reaction with reactive intermediates facilitates production of additional RSS including polysulfides (H_2S_n, n = 2−8) as has been recently described with HOCl (Chart 15).[364] Polysulfide production proceeds through a sulfenyl chloride intermediate, which hydrolyzes to sulfenic acid, followed by condensation with a second H_2S (Chart 15). Since H_2S can participate in two nucleophilic reactions, subsequent oxidation of H_2S_2 and condensation with H_2S facilitates higher order polysulfide formation.

Chart 15. Formation of H_2S_2 and Higher Order Polysulfides by Reaction of Hydrogen Sulfide (H_2S) with ROS, Such as Hypochlorous Acid (HOCl) Initially Yields a Sulfenyl Chloride That Is Hydrolyzed by Water to Afford a Sulfenic Acid



In addition to H_2S, production of other inorganic RSS in cells, such as thiocyanate ($^-$SCN), thiocyanogen [$(SCN)_2{}^-$],

trithiocyanate [$(SCN)_3^-$] and hypothiocyanite [^-OSCN] has been shown or postulated.[119b,365] Nevertheless, the physiological significance of these RSS in redox signaling is not well established and, consequently, this section will focus exclusively on the role of H_2S as an RSS in redox biology. For a more extensive overview of RSS, the interested reader is referred to the following sources.[213,348,366]

5.1. H_2S Production and Metabolism

5.1.1. H_2S-Generating Enzymes. H_2S is primarily produced through an alternative metabolic pathway of the cytosolic pyridoxal 5′-phosphate (PLP)-dependent enzymes cystathionine *β*-synthase (CBS) and cystathionine *γ*-lyase (CSE). Together, these enzymes comprise the transsulfuration pathway that regulates cysteine biogenesis and can produce H_2S in a variety of tissues (Figure 18a).[350,351,352b,355,367] Little is known about the regulation of CBS and CSE activities, and it is thus largely unclear how H_2S may be produced for signaling. To some extent, H_2S production by CBS and CSE appears to be controlled by differences in tissue expression. CBS is expressed in many tissues including the liver, kidney, and brain[367b,368] and CSE is expressed in, among others, the liver, kidney, cardiovascular system, and to some extent, the brain.[367b,369] In the brain, H_2S appears to function as an endogenous neuromodulator[370] and CBS knockout mice show altered long-term potentiation.[371] While the molecular mechanism(s) of H_2S action in the context of neuronal signaling are not entirely clear, there is evidence that CBS is regulated at the transcriptional level by the second messenger cAMP.[368a] Moreover, CBS appears to be directly inhibited by binding of $^•NO$ to the heme cofactor,[372] whereas CSE expression and activity have been shown to be enhanced by distinct NO donors,[373] though these latter effects are controversial.[374] Given that H_2S can scavenge reactive intermediates, $^•NO$-mediated regulation of CBS and CSE may influence $^•NO$ availability. Both CSE and CBS are believed to be activated by Ca^{2+}/CaM binding similar to nNOS, suggesting that $^•NO$ and H_2S production may be colocalized in some settings.[371,375] Additionally, CBS has been shown to be abundant in actively proliferating cells where its activity is coupled to cellular metabolic demands through allosteric activation by AdoMET,[370,376] which also appears to regulate CBS-mediated H_2S production in neurons.[377] More recently, H_2S production in the liver of mice treated with the proinflammatory cytokine, tumor necrosis factor *α* (TNF*α*) was shown to be dependent upon CSE, which was regulated at the level of transcription.[353]

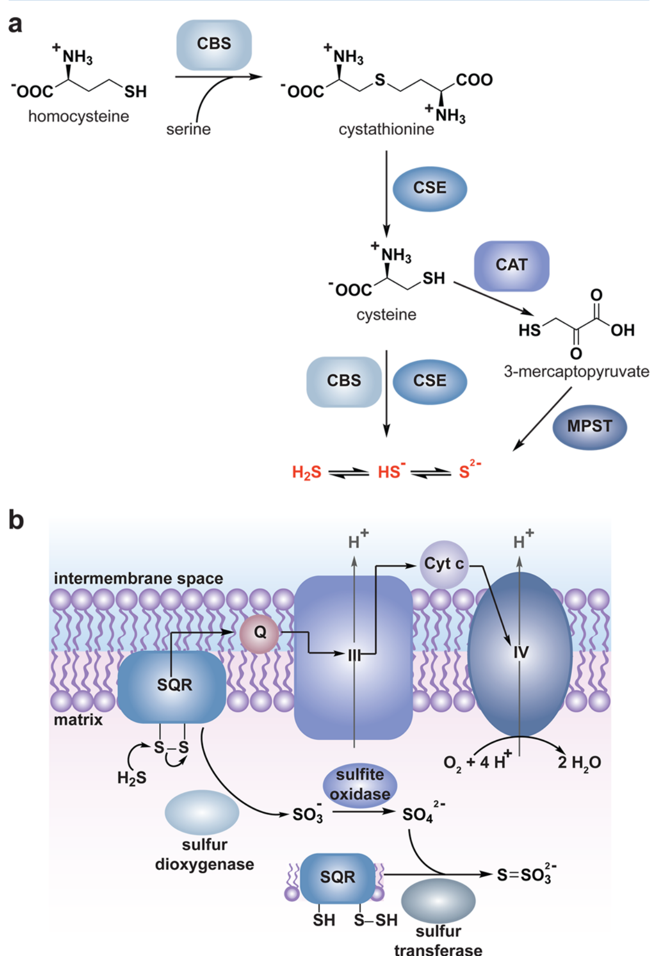
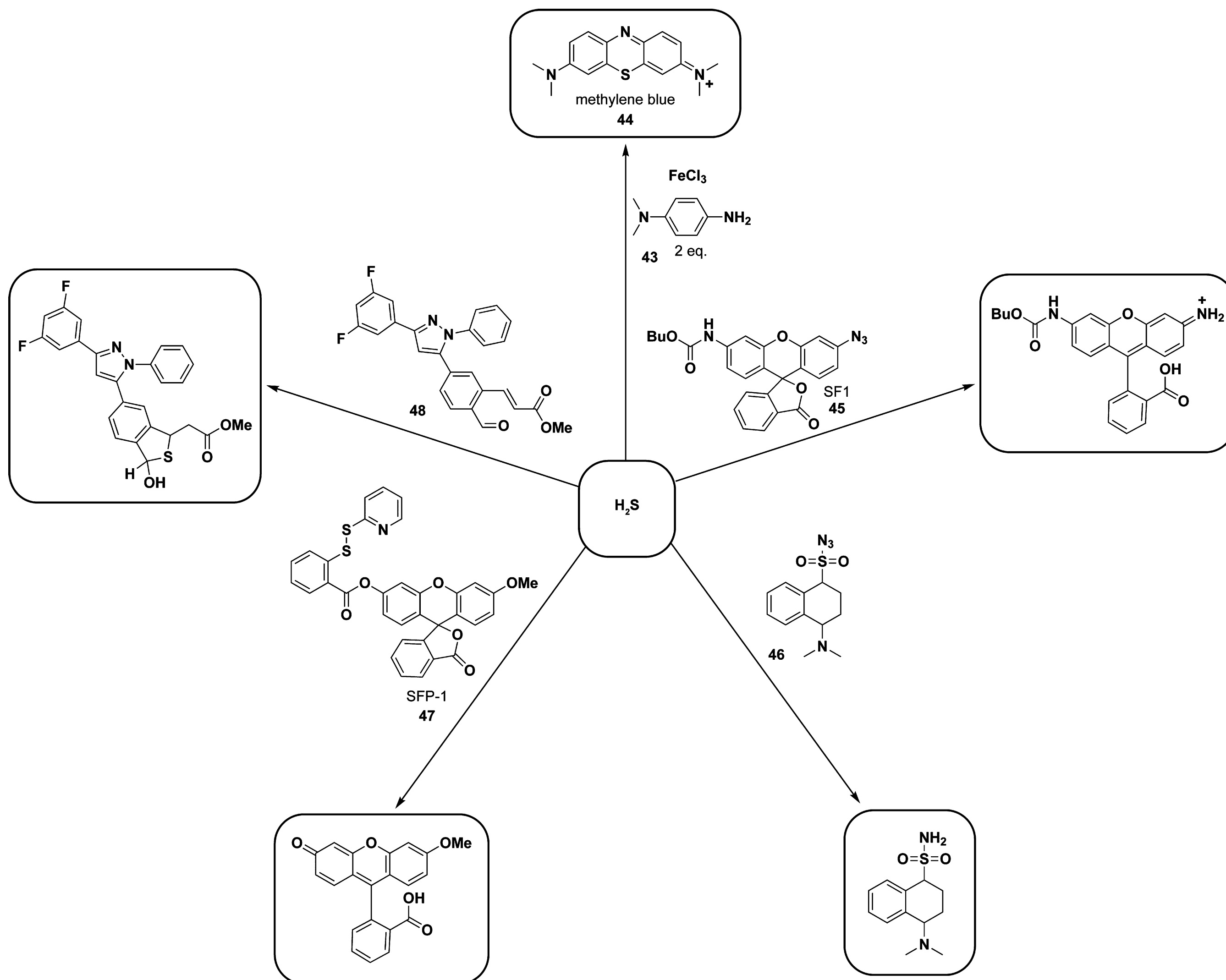


Figure 18. Generation and metabolism of H_2S. (a) Pyridoxal 5′-phosphate (PLP)-dependent enzymes of the transsulfuration pathway, cystathionine *β*-synthase (CBS) and cystathionine *γ*-lyase (CSE) catalyze H_2S production from homocysteine, cystathionine, and cysteine. Additionally, the combined activity of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3MST) in the cytoplasm and mitochondria can generate H_2S. (b) Oxidative metabolism of H_2S in the mitochondrial matrix is catalyzed by a series of enzymes to generate persulfide, sulfite (SO_3^-), thiosulfate ($S_2O_3^{2-}$), and sulfate (SO_4^{2-}). The first step is catalyzed by SQR, which forms a protein-bound persulfide intermediate and funnels electrons from H_2S oxidation directly into the ETC. The subsequent action of sulfur dioxygenase, sulfite oxidase and sulfur transferase are proposed to convert SQR persulfide into SO_3^-, SO_4^{2-}, and $S_2O_3^{2-}$, respectively.

H_2S is also produced by the combined activity of 3-mercaptopyruvate sulfurtransferase (3MST) and cysteine aminotransferase (CAT) in the cytosol and mitochondria (Figure 18a).[352b,378] 3MST is expressed in the liver, kidney, heart, lung, and brain,[378d,379] and has been shown to produce H_2S in brain homogenates from CBS knockout mice.[379b] Additionally, H_2S is produced nonenzymatically from naturally occurring polysulfanes and other therapeutic compounds and the interested reader is referred to the following sources for additional information.[351,380] H_2S release from extracellular polysulfanes naturally occurring in garlic appears to occur via a GSH-coupled mechanism[381] where extracellular H_2S liberation leads to elevated levels of GSSG inside the cell.

To better understand the function of H_2S in physiological and pathological settings, reliable assays are needed to accurately determine H_2S concentrations in biological samples. A number of colorimetric, electrochemical, gas chromatography, and metal-induced sulfide precipitation techniques have been developed.[357,382] One such method for H_2S detection involves the reaction of H_2S with *N,N*-dimethyl-p-phenylenediamide (**43**) and ferric iron under acidic conditions to generate methylene blue (**44**), which is monitored by spectroscopy (Chart 16).[356,383] One limitation to these methods is that they do not allow for rapid, accurate, and real-time determination of H_2S concentrations. Additionally, many of these methods require the generation of cell lysates or tissue homogenates, and therefore afford variable estimates due to rapid H_2S catabolism.[352b] Indeed, a significant challenge in this field is the disagreement as to what constitutes physiological concentrations of free H_2S. Current estimates in biological samples and tissues range over a 10^5-fold concentration range (15 nM[384]–300 μM[355]) with 10–600

Chart 16. Colorimetric and Fluorescent Methods for Detection and Quantification of H_2S^a



^{a}Reaction of H_2S with two equivalents of **43** in the presence of iron(III) chloride ($FeCl_3$) yields methylene blue (**44**). Chemoselective reduction of azides in **45** and **46**, two-step deprotection of **47**, or two-step thioacetal formation from **48** upon reaction with H_2S yields fluorescent compounds.

μM exogenous H_2S frequently used to elicit cellular responses.[370,375,385] At present, the most reliable estimate (∼100 pM in the blood and 15 nM in tissues) has been reported by Banerjee et al.[384] using an innovative gas-chromatographic based chemiluminescent sulfur detection method, which avoids lengthy manipulation steps and the aggressive acidic or basic conditions utilized by early approaches that can leach sulfide from iron−sulfur proteins and lead to cysteine desulfuration through β-elimination.

The need for detection methods with improved sensitivity and that measure H_2S in cells has motivated the development of fluorescent small-molecule sensors. Recently, three approaches have been reported that rely on selective reaction of a caged fluorophore with H_2S. Chang and colleagues have developed an azide-caged fluorophore (**45**) that becomes fluorescent after H_2S-mediated reductive reaction to release the amine (Chart 16).[386] The sensitivity of this probe for H_2S was reported as 5 μM and was capable of detecting H_2S in HEK293 cells treated with exogenous NaHS. Wang and colleagues similarly developed an azide-caged fluorophore (**46**) that underwent rapid and selective reductive reaction by H_2S at concentrations ≥5 μM (Chart 16).[387] Xian and colleagues have developed a two-step deprotection strategy (**47**) to liberate a fluorophore upon reaction with H_2S (Chart 16).[388] This two-step strategy precludes background signal arising from the reaction of their probe with cysteine or GSH. The probe was sensitive to ∼1 μM H_2S in buffer and ∼50 μM in plasma, though lower concentrations were not tested. Invoking alterative chemistry, He and colleagues developed a selective sulfur trapping strategy involving H_2S addition to an aldehyde (**48**) with subsequent Michael addition of the hemithioacetal intermediate with an adjacent unsaturated acrylate ester to give a thioacetal (Chart 16).[389] In this latter case, however, important controls were not included to verify a lack of reactivity of **48** with GSH or cysteine. The resulting products exhibit increased fluorescence upon reaction with NaHS and H_2S and can detect H_2S biogenesis in cells. Implementation of these tools to study H_2S signaling and further development of probes with lower limits of detection should expand our understanding of pathways where H_2S is proposed to function as a second messenger.

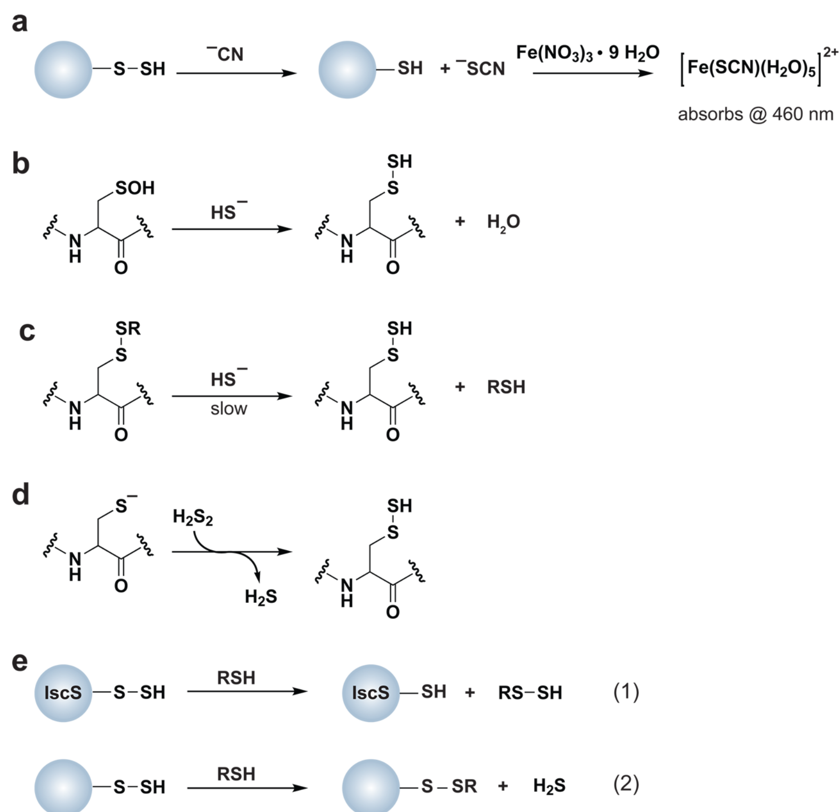


Figure 19. Formation and reactions *S*-sulfhydryls. (a) The cyanolysis assay for persulfides relies upon reaction of persulfides with cyanide (CN^-) to form thiocyanate (SCN^-). SCN^- is subsequently detected with iron nitrate ($Fe(NO_3)_3•9H_2O$) to form $[Fe(SCN)(H_2O)_5]^{2+}$, which absorbs at 460 nm. (b−d) Mechanisms of *S*-sulfhydryl formation. Protein *S*-sulfhydryls can form upon the reaction of H_2S with (b) a protein sulfenic acid or (c) a disulfide, though the latter is very slow owing to the poor reductant activity of H_2S. (d) Alternatively, *S*-sulfhydryls can form from nucleophilic attack of a protein thiolate on H_2S_2. (e) Once formed, protein *S*-sulfhydryls can be shuttled between proteins via transsulfhydration, as is well established for the sulfurtransferase IscS (eq 1). Reaction of a protein *S*-sulfhydryl with a second cysteine can alternatively yield a disulfide (eq 2). Akin to *S*-nitrosothiols, the relative pK_a of the protein and H_2S thiols will influence which reaction occurs.

5.1.2. H_2S-Metabolizing Enzymes. Unchecked H_2S accumulation is toxic and metabolic pathways are in place to regulate its levels. H_2S is metabolized through oxidation, in a process that occurs efficiently in the mitochondria[352b,390] as catalyzed by a series of enzymes to generate persulfide, sulfite, thiosulfate ($S_2O_3^{2-}$), and sulfate (Figure 18b).[352b,391] The electrons from H_2S oxidation funnel directly into ETC complex III by the sulfide:quinone oxidoreductase (which is intriguing given that H_2S is also a potent inhibitor of cellular respiration as will be discussed in a following subsection). H_2S can also be methylated by thiol-*S*-methyltransferase to give methanethiol and dimethylsulfide, and it serves as a substrate for rhodanese, leading to the formation of $^-$SCN.[351]

5.2. H_2S-Mediated Modification of Protein Cysteine Thiols

The first signaling role attributed to H_2S was as a physiologic vasorelaxant.[369b,373] Mice lacking CSE display pronounced hypertension[375] and H_2S was subsequently shown to be produced by CSE in endothelial cells where it suppresses leukocyte-endothelial cell interactions in the circulation, thereby regulating the immune response.[392] Interestingly, both the vasorelaxation and immune response effects of H_2S are due, in part, to activation of the ATP-sensitive potassium channel (K_{ATP}).[373,393] Analogous to protein regulation by •NO, H_2S-mediated K_{ATP} activation is imparted by chelation to the heme prosthetic group.[394] Additional enzymes in which H_2S regulates activity by metal chelation include cytochrome c oxidase (complex IV),[395] carbonic anhydrase,[396] and some NOS isoforms.[397] H_2S-mediated inhibition of cytochrome c oxidase decreases the cellular metabolic rate and $O_2^{•-}$ production, and therefore regulates cell respiration analogous to •NO.[293,398] Higher doses of H_2S induce hypothermia and establishes a state of suspended animation in mice, which has become a topic of significant interest in the medical field.[399] Other membrane channels including the cysteine/glutamate antiporter may also be regulated by H_2S.[400] In this case, activation of this antiporter stimulates cysteine uptake and GSH production to modulate cellular redox balance.

A second mechanism by which H_2S regulates biological processes that has gained increasing attention is by *S*-sulfhydration/persulfide modification of cysteine residues in proteins. Though considered as "new players" in the field of redox signaling, persulfides were first identified as an intermediate that forms to facilitate sulfur delivery in multiple biosynthetic pathways.[401] Within these biosynthetic pathways, the sulfur originates from cysteine, and persulfides form in a number of enzymes, including sulfurtransferases (e.g., rhodanese) and cysteine desulfurases (e.g., IscS) with rhodanese homology domains. Many of these enzymes are involved in delivering sulfide for the production of sulfur-containing vitamins and cofactors, including iron−sulfur clusters through shuttling of persulfide intermediates (e.g., transsulfhydration) between proteins, thus preventing release of free H_2S.[401,402] In addition to shuttling sulfide from cysteine for biosynthetic pathways, rhodanese proteins are also involved in H_2S

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oxidation in the mitochondria and elimination of toxic compounds like cyanide through its thiosulfate:cyanide sulfurtransferase activity, involving sulfur transfer from a persulfide intermediate.[352b,403] A common mechanism for detecting persulfide formation in recombinant protein utilizes a cyanolysis assay based on the reaction catalyzed by rhodanese (Figure 19a).[367a,404]

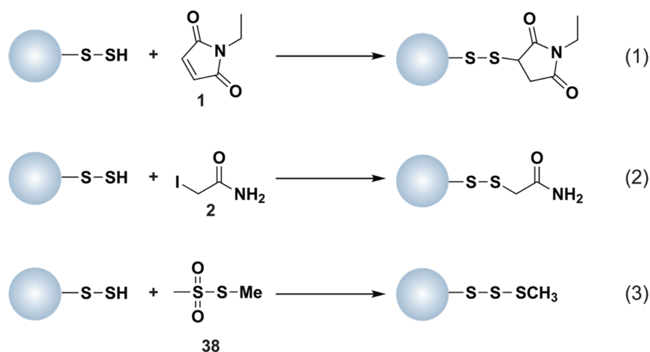
Another mechanism through which H_2S is proposed to regulate biological processes is by neutralization of reactive electrophiles. Though not mentioned previously, reactive electrophiles, such as the lipid oxidation product, 4-hydroxy-2-nonenal (4-HNE), can covalently modify protein cysteine, lysine, and histidine residues.[143] To date, a number of proteins have been shown to be modified by specific reactive electrophiles. These include the transcription factor NF-kB,[405] PTP1B,[406] the MAPK kinases ERK and p38 MAPK,[407] and transient receptor potential (TRP) channels.[408] An additional system that is sensitive to reactive electrophiles is the mammalian Keap1-Nrf2 pathway that regulates expression of genes involved in oxidant and xenobiotic detoxification.[409] A recent study also suggests that H_2S can intercept reactive electrophiles, such as 4-HNE, to prevent protein modification.[410] Though the 4-HNE modified H_2S adduct was not confirmed in this work, the ability to neutralize reactive electrophiles is thought-provoking, as GSH is known to carry out an analogous function.[411]

S-sulfhydration (herein used to differentiate between a mechanism of sulfur transfer for the biogenesis of sulfur-containing cofactors and a redox regulated posttranslational modification) is now recognized as a mechanism by which H_2S can modulate protein activity. Three primary mechanisms for protein *S*-sulfhydration have been postulated (Figure 19b–d). The first two mechanisms involve nucleophilic attack of H_2S on sulfenic acid- or disulfide-modified proteins (Figure 19b and c). In regard to the latter reaction, we note that H_2S is a poor reductant compared to GSH[352b] and reacts very slowly with protein disulfides in vitro.[412] The final mechanism involves oxidation of H_2S to generate H_2S_2 by reaction with ROS such as HOCl (Chart 15) and subsequent nucleophilic attack by a protein thiolate (Figure 19d). Given the low concentration of H_2S that has been estimated in cells relative to the high concentration of GSH, the feasibility of direct reaction between H_2S and a protein thiolate has been questioned.[352b,413] In addition to H_2S, the polysulfide diallyl trisulfide (DATS) and the H_2S oxidation product, $S_2O_3^{2-}$ have also been postulated as sulfur donors by transferring sulfane sulfur (S^0), which could present an additional mechanism for *S*-sulfhydryl formation.[413] Like *S*-nitrosothiols and disulfides, *S*-sulfhydryls contain two electrophilic centers and can undergo reaction with a second thiol. Reaction of *S*-sulfhydryls with a second protein thiol could conceivably yield a disulfide or facilitate transsulfhydration (Figure 19e, eq 2). The latter route is in line with persulfide transfer from IscC to associating proteins as a means for sulfur delivery in the biosynthesis of sulfur-containing cofactors and nucleotides (Figure 19e, eq 1).[401,414] As previously mentioned, the pK_{a1} of H_2S is 6.9,[352a] and therefore a preference for transsulfhydration is less certain than for transnitrosylation (pK_a of 11.4 for HNO).[242a,335] Trans-sulfhydration is thus likely to be highly protein specific, whereby cysteine pK_a influences which thiol is expelled, analogous to thiol–disulfide exchange.

5.3. Methods for Detecting H_2S-Modified Cysteine Thiols

Recently, three chemical procedures have been developed to study proteins susceptible to S-sulfhydration. The first method, developed by Snyder and colleagues, is a modified BST that sought to capitalize on the distinct reactivity of *S*-sulfhydryls in comparison to the other reversible modifications, including disulfides and protein-*S*-GSH adducts.[415] In the first step, free thiols are labeled with MMTS. Next, excess MMTS is removed and *S*-sulfhydrated cysteines are alkylated with biotin-HPDP (the nature of the covalent adduct, disulfide or trisulfide, was not established in this work). The ability of *S*-sulfhydryls to react with biotin-HPDP implies that *S*-nitrosothiols detected by the original BST would be "contaminated" with persulfide-modified proteins. Indeed, the idea to modify the BST method for detection of protein S-sulfhydration originated from the observation that many proteins were still labeled by biotin-HPDP when ascorbate was omitted from the reaction sequence.[336a] Using the modified BST, Snyder and colleagues identified 39 potential targets of S-sulfhydration in liver homogenates treated with NaHS.[415] GAPDH and actin, two targets of S-sulfhydration, exhibited a respective enhancement in activity and polymerization, after treatment. K_{ATP} was also found to be S-sulfhydrated by NaHS, though the effect of this modification on channel activity was not reported. Analogous to other differential alkylation strategies, specificity in this modified BST assay is dependent upon selectivity of the alkylating agents. Recent studies with small molecule and protein *S*-sulfhydryl models have shed light on the previously uncharacterized nucleophilic properties of the terminal sulfur (J. Pan and K. S. Carroll, unpublished results) as this functional group was found to react at the terminal sulfur atom with NEM, IAM, and MMTS (Chart 17). Thus, the underlying chemistry that mediates S-sulhydryl detection in the modified BST is yet unclear.

Chart 17. Protein *S*-Sulfhydryls React with NEM (Equation 1), IAM (Equation 2), and MMTS (Equation 3) to Yield the Corresponding Di- or Trisulfides



Snyder and colleagues subsequently demonstrated that S-sulfhydration of the p65 subunit of transcription factor NF-*κ*B mediates its antiapoptotic activity using a second indirect chemical method to monitor *S*-sulfhydryl formation (Figure 20a).[353] This two-step method involves modifying free thiols and *S*-sulfhydryls (at the terminal sulfur) with a fluorescently labeled maleimide compound. The reaction of the *S*-sulfhydryl with the maleimide reagent yields a disulfide linked adduct that can be reduced by DTT (Figure 20a). In this assay, an increase in *S*-sulfhydryl formation correlates with a decrease in signal, as monitored by in-gel fluorescence. This method was also

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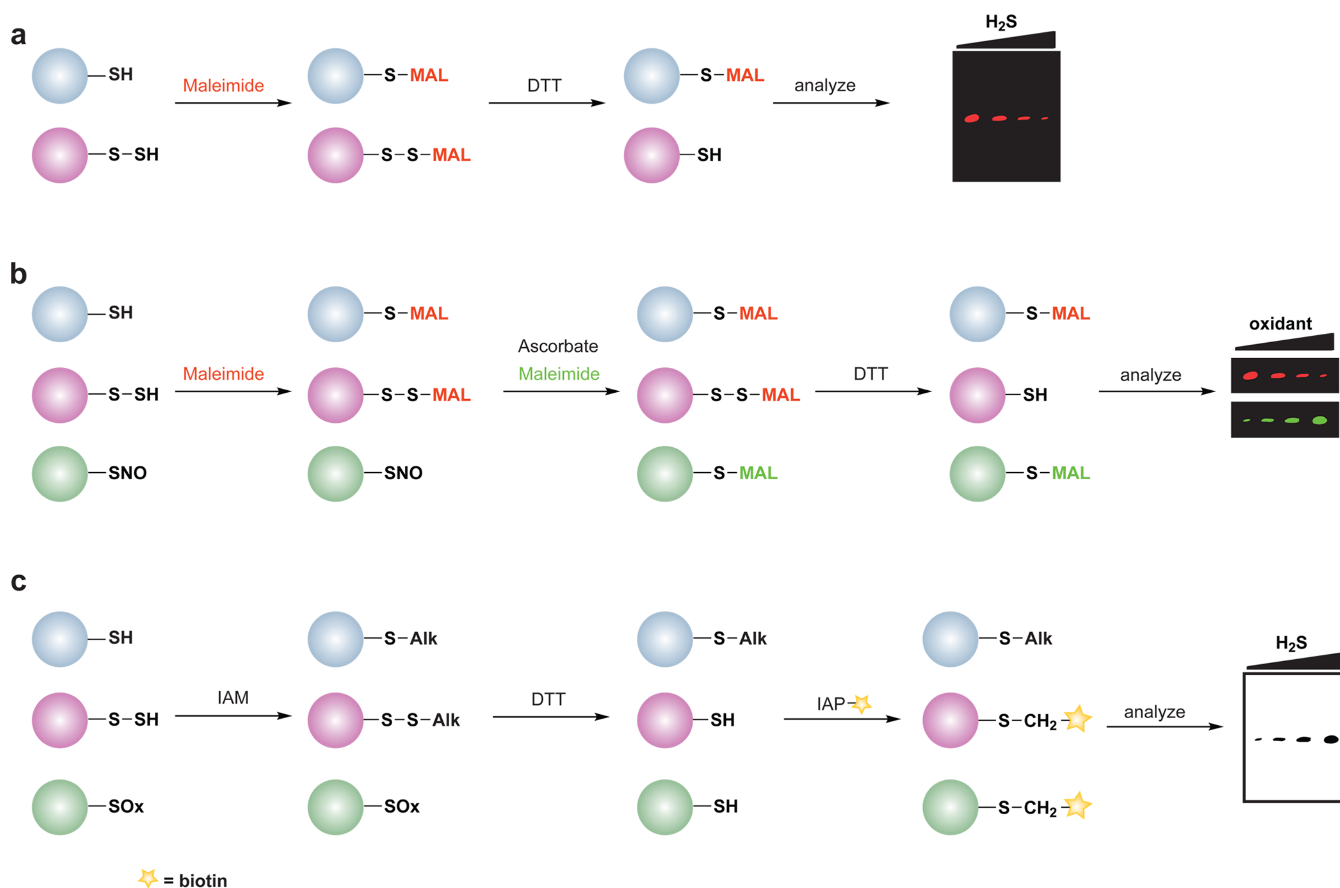

Figure 20. Indirect chemical methods to detect *S*-sulfhydryls. (a) Free thiols (blue) and *S*-sulfhydryls (purple) are modified with a red fluorescent maleimide reagent. The disulfide linked maleimide adducts derived from *S*-sulfhydryls are reduced by DTT. Protein modification by S-sulfhydration is detected as a decrease in signal by in-gel fluorescence. (b) Indirect method to simultaneously monitor *S*-sulfhydryl and *S*-nitrosothiol formation. Free thiols (blue) and *S*-sulfhydryls (purple) are modified with a red fluorescent maleimide reagent. *S*-nitrosothiols (green) are reduced with ascorbate and nascent thiols modified with a green fluorescent maleimide reagent. The disulfide linked maleimide adducts derived from *S*-sulfhydryls are reduced by DTT. Protein modification by S-sulfhydration and S-nitrosylation are detected as a decrease in red signal and increase in green signal by in-gel fluorescence, respectively. (c) Free thiols (blue) and *S*-sulfhydryls (purple) are modified by IAM. The disulfide-linked alkylation adduct and all other reversible cysteine modifications (green) are reduced with DTT and nascent thiols are modified with IAP-biotin. Cysteine oxidation is monitored as an increase in signal by avidin blot.

extended to include the standard BST, such that S-sulfhydration and S-nitrosylation could be simultaneously monitored using two fluorescently labeled maleimide compounds (Figure 20b). This new method underscores the nucleophilic properties of *S*-sulfhydryl groups, however, its subtractive nature precludes its use for global identification of S-sulfhydrated proteins.

An alternative indirect chemical method was recently reported by Tonks and co-workers to demonstrate S-sulfhydration of PTP1B in H_2S-treated human embryonic kidney HEK293T cells.[416] This three-step method involves IAM blocking of free thiols and persulfides, DTT reduction of reversibly oxidized thiols, and labeling of nascent thiols with IAP-biotin (Figure 20c). A limitation to this method is that the DTT reducing step is not selective for alkylated *S*-sulfhydryls, and thus, biotin signal will increase due to the presence of any reversible cysteine modification, including disulfides and sulfenic acids. This study additionally indicated that HeLa cells subjected to ER stress produce H_2S in a CSE-dependent manner, leading to PTP1B modification. In these experiments, *S*-sulfhydration of PTP1B was confirmed in cell lysates by LC-MS/MS analysis. As the authors of this study point out, caution must be taken when assessing *S*-sulfhydration by MS as *S*-sulfhydryl and sulfinic acid modifications give a similar mass increase and necessitates high instrument resolution to differentiate between adducts.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Reactive intermediates, including ROS, RNS, and RSS, are increasingly emerging as major contributors to regulation of numerous physiological and pathological processes. Within this capacity, reactive intermediates function as second messengers to regulate the activity of an ever-expanding number of proteins through covalent modification of cysteine residues. Nonetheless, our understanding of the contribution that each class of reactive intermediate makes is at strikingly different stages. Research over the past five years has provided insight into mechanisms to regulate ROS and RNS production in response to diverse stimuli and the continued development of inhibitors for specific NOX and NOS isoforms will further our understanding of the individualized role of each of these enzymes. In this way, selective inhibitors could prove helpful in further dissecting signaling pathways susceptible to redox modulation and could shed light on novel therapeutic targets. Moreover, the continued improvement of ROS and RNS detection methods will facilitate regiotemporal resolution of

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their production. In stark contrast, our understanding of regulation of H_2S production is still in its infancy. Continued studies to elucidate how and when H_2S production is regulated by diverse signals and the development of probes with enhanced sensitivity for H_2S will further our understanding of how this RSS participates in redox signaling as a physiologically relevant second messenger.

Likewise, work over the past decade has led to the development of methods that permit selective detection of specific cysteine modifications and, more recently, targeted detection within a subclass of signaling proteins, like PTPs. These collective techniques have greatly expanded the known inventory of proteins susceptible to cysteine oxidation and have shed light on diverse ways in which redox regulation can influence signaling. Nonetheless, the repertoire of reactive cysteine residues and related oxPTMs (particularly those identified by chemically selective, biocompatible approaches) is not complete. Future work is also needed to develop more selective methods for detection of sulfonic acid and *S*-sulfhydryl modifications. Techniques for cell-based discovery and identification of *S*-nitrosothiols and *S*-sulfhydryls are vital, as both modifications can be transferred among cysteines, and sample processing is important to minimize these side reactions as a means to accurately identify relevant sites of modification. Knowledge regarding the extent of site-specific cysteine oxidation is also important for understanding the function and regulation of oxPTMs. In addition, the ability to quantify changes in cysteine oxidation should help overcome a major hurdle in the field-namely, the prioritization of proteins within the "redoxome" selected for further characterization and functional analysis. Indeed, the transformative and paradigmatic discoveries in this exciting field lie in elucidating the functional consequences of these oxidative cysteine modifications in physiological and pathological processes.

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Notes

The authors declare no competing financial interest.

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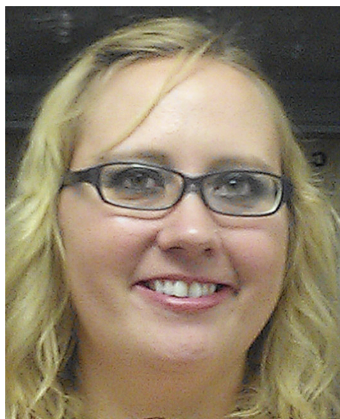

Candice Paulsen, originally from Portland, Oregon, received her B.S. in genetic biology from Purdue University in 2006. Paulsen earned her Ph.D. in chemical biology from the University of Michigan in 2011, where she worked under the advisement of Prof. Kate Carroll to study the role of protein sulfenylation in eukaryotic signal transduction. After continuing as a postdoctoral fellow with Prof. Carroll at Scripps Florida, Paulsen began as a postdoctoral fellow at the University of California, San Francisco in the department of physiology with Prof. David Julius in July of 2012. Her current research interests are focused on elucidating the gating mechanism of transient receptor potential (TRP) ion channels by noxious chemical and thermal stimuli as it pertains to pain signaling.

Kate S. Carroll is an Associate Professor in the Department of Chemistry at The Scripps Research Institute in Jupiter, Florida. She received her B.A. degree in Biochemistry from Mills College in 1996 and Ph.D. in Biochemistry from Stanford University in 2003. Her postdoctoral work was completed at the University of California, Berkeley, where she was a Damon Runyon-Walter Winchell Chancer Fund Fellow with Prof. Carolyn Bertozzi. She was an Assistant Professor at the University of Michigan until 2010, when she joined the Chemistry faculty at Scripps. Her research interests span the disciplines of chemistry and biology with an emphasis on studies of sulfur biochemistry pertinent to disease states. Her lab focuses on the development of novel tools to study redox modifications of cysteine thiols, profiling changes in protein oxidation associated with disease, and exploiting this information for development of diagnostic and therapeutic approaches. In addition, her group investigates sulfur pathways that are essential for infection and long-term survival of human pathogens such as *Mycobacterium tuberculosis*. She has received the Pfizer Award in Enzyme Chemistry (2013), the Camille Dreyfus Teacher-Scholar Award (2010), the Scientist Development Award from the American Heart Association (2008), and the Special Fellow Award from the Leukemia and Lymphoma Society (2006).

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