THE CHEMISTRY OF THIOL OXIDATION AND DETECTION

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This chapter is dedicated to the memory of Professor William S. Allison.

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1. Introduction

The thiol functional group of the amino acid cysteine can undergo a wide array of oxidative modifications and perform a countless number of physiological functions. In addition to forming covalent cross-links that stabilize protein structure and functioning as a powerful nucleophile in many enzyme active sites, cysteine appears to be the principal actor in redox signaling, functioning as a regulatory reversible molecular switch. It is increasingly appreciated that the thiol group of cysteine in subset of proteins undergoes oxidative modification in response to changes in the intracellular redox environment. To understand these complex, but pivotal biological phenomena, the chemistry of the thiol functionality and related oxidation products must be taken into consideration. Along these lines, selective methods to monitor and quantify discrete cysteine modifications are key to understanding their regulatory and pathophysiological function. This chapter focuses on the chemistry of thiol oxidation and detection.

2. The biological chemistry of thiols

The cysteine side chain is generally considered the most potent nucleophile of all amino-acid side chains under physiological conditions. This notable level of reactivity is due to the presence of a thiol functional group. Thiol is a sulfur analogue of alcohol, but the smaller difference in electronegativity between the sulfur atom and the hydrogen atom makes the S-H bond less polarized than the O-H bond, leading to a diminished propensity to form hydrogen bonds. In contrast, thiols are much more acidic in comparison to alcohols, and this property can be explained by the weakness of the S-H bond and the greater likelihood that the negative charge will be distributed within sulfur 3d orbitals. Cysteine can be considered a triprotic acid in which the pKₐ of thiol group has been determined to be 8.2 (Tajc et al. 2004). In glutathione, the most abundant low molecular weight thiol in the cytosol, cysteine residue has a pKₐ of 9.1 (Tang & Chang 1996). In general, therefore, thiols are mild acids, but the protein microenvironment can dramatically influence the pKa value. The presence of a positively charged residue, such
as lysine or arginine (Copley et al. 2004), as well as the formation of a hydrogen bond (Wang et al. 2001), may increase thiol acidity by 3-4 orders of magnitude. The reactivity of thiols is correlated with its pKa value (Szajewski & Whitesides 1980). In cysteines, a thiolate side chain becomes a stronger nucleophile and readily reacts with oxidants and electrophilic species, although interactions with specific residues or metals can also stabilize the thiolate form. With its remarkable reactivity, the cysteine thiol group can play a key biological role in catalysis and serve as an important site for many post-translational modifications. Considering the propensity of thiols to undergo oxidative reactions, the need for methods and chemical tools to monitor both reduced and oxidized cysteine residues has become clear. The key challenge for effective detection of bio-functional groups is summarized by the concept of chemoselectivity (Trost 1983) and, more specifically, in bioorthogonality (Bertozzi 2011). Indeed, chemists and biologists have sought to identify highly selective and facile reactions to detect biomolecules in living systems without interfering with native biochemical processes. Moreover, the relatively low abundance of cysteine, in comparison to other amino acids, combined with its remarkable nucleophilicity has made cysteine the most common target for selective protein bio-conjugation (Chalker et al. 2009), creating fertile ground for the development of site-specific strategies for protein modification. In this context, the thiol reacts as a soft nucleophile with alkyl and aryl halides, carbonyl, phosphoryl, and sulfonyl groups as well as with unsaturated compounds (Fig. 1a-f). There are a large number of reagents that selective modify thiols, even in the presence of other strong nucleophiles such as lysine or histidine (Fig. 1, 1-12). Such thiol conjugations can be further subdivided into two categories: reversible and irreversible.

To date, the most well studied example of cysteine modification is disulfide formation between two thiol groups and, thus it is no coincidence that one of the first strategies to detect thiols was inspired by the process of thiol-disulfide exchange (Fig. 1a). Bearing in mind that disulfide exchange is an equilibrium reaction (see Section 4.3), a series of specific disulfides were designed in order to favor one direction over the other. The well-known 5,5’-dithiobis-(2-nitrobenzoate) (DTNB) or Ellman’s reagent is perhaps most common disulfide-based reagent used to monitor and quantify protein free thiols (Ellman 1959). The notably low pKa of the 5-mercapto-2-nitrobenzoate leaving
group (4.4, Little & Broicklehurst 1972) ensures that this reaction occurs rapidly and, at the same time, does not perturb naturally occurring disulfides in the protein of interest.

Thiopyridyl disulfides are another class of common disulfide-exchange based detection reagents (Olsen et al. 2003). Thiopyridyl disulfides such as 1 (Fig. 1) react rapidly with thiols to afford an electro-stabilized compound, pyridine 2-thione, which can be followed by colorimetric analysis (i.e., absorption at 420 nm). Based on the same concept, a series of selenyl sulfides (Gamblin 2004) and thiosulfonates (Kenyon & Bruice 1977) have also been developed to selectively functionalize cysteine side chains. For example, methyl methane thiosulfonate (2) is the classic thiol-targeted reagent used to block free proteins thiols in the biotin switch assay (Jaffrey et al. 2001). All of these reagents (e.g., 1-3) react with the thiol side chain to form a mixed disulfide that can subsequently be reduced to regenerate the free cysteine residue. The reversible nature of the disulfide linkage is acceptable in some circumstances, but in other situations this feature can be problematic. As a result, specific alkylating agents have been designed to irreversibly modify the cysteine side chain (4-11). Alkyl halides react with thiols to yield stable thioethers (Fig. 1c). Such reagents are usually not exclusive in their reaction with thiols; however, with careful control of pH, α-halo carbonyl compounds (4-6) can provide a satisfactory degree of bioorthogonality. Iodoacetamide (4) is employed in standard protein digestion protocols to block cysteine residues (Shevchenko et al. 1996). Despite the popularity of iodoacetamide, side-reactions with lysine, methionine, tyrosine, and histidine residues have been observed (Nielsen et al. 2008). In such cases, this issue can be resolved by using chloroacetamide (Weerapana et al. 2008).

Another unusual class of organo-halides is halo-nitrophenyl derivatives (Fig. 1d and 7). The strong electro-withdrawing nitro group makes haloarene extremely reactive toward thiols, although other sulfur species may also be modified in the presence of similar compounds (see Section 4). Thiolates are good Michael donors and the addition reaction to an α,β-unsaturated system is an alternative way to alkylate cysteine residues (Fig. 1e). Historically, maleimides have been the most widely used tools in biochemistry for the modification of thiols. The popularity of N-ethyl maleimide (8) is due to its good cell permeability and the ease with which the ethyl group can be elaborated by other substituents (e.g., 9). However, what must be considered is that the reaction is only
selective toward the thiol group at pH 7 (Crankshaw & Grant 1996). Vinyl sulfones are an alternative to the use of maleimide derivatives. Although they offer the advantage of yielding a single stereoisomer as a product (i.e., in theoretical models, maleimides can yield a mixture of two diastereoisomers), vinyl sulfones may cross react with the ε-NH₂ of lysine (Masri & Friedman 1988). A selective new reagent for thiol modification reported by Carroll’s research group is based on the α-halo 1,3-diketone scaffold (Fig. 1f). In this reaction, 2-iodo-5,5-dimethyl-1,3-cyclohexandione (10, distinguished from the aforesaid α-halo carbonyl compounds by virtue of its secondary halogen) and related compounds (e.g., 11,12) react with a thiol to give the sulfenyl iodide species; this intermediate is rapidly attacked by the 1,3-diketone carbon nucleophile to afford a stable thioether (Seo & Carroll 2011).

3. Two-electron oxidants

Reactive oxygen species (ROS) are a family of molecules that are continuously generated in cells as consequence of aerobic life. Traditionally, their production has been strictly associated with oxidative stress, aging, and disease. However, growing evidence indicates that controlled generation of ROS contributes to physiological intracellular signaling events. ROS mediate redox modification of various biomolecules via two-electron oxidation or radical-based reactions, and they react in particular with cysteine residues. The term ROS encompasses a wide class of activated oxygen molecules which, can undergo further reaction with nitrogen or sulfur compounds, to produce reactive nitrogen species (RNS) and reactive sulfur species (RSS). Here, we focus on two-electron oxidants without mentioning the radical species, which will be described in subsequent chapters of this book.

3.1 Hydrogen peroxide

A handful of enzymes generate H₂O₂ directly. For example, significant quantities of H₂O₂ are produced in the peroxisome by the action of enzymes, such as D-amino acid oxidase or uric acid oxidase. More generally, however, H₂O₂ is formed in cells via the
disproportionation of superoxide (O$_2^-$). The major sources of O$_2^-$ production are the electron transport chain (ETC) complex in the mitochondria (Murphy 2009) and the family of NADPH oxidases (NOXs) (Bernard et al. 2007). Superoxide disproportionation may occur spontaneously ($7.6 \times 10^5$ M$^{-1}$ s$^{-1}$, Kutula et al. 2008) or may be catalyzed by superoxide dismutase (SOD) (Forma & Fridovich 1973; Fukai & Ushio-Fukai 2011). Finally, several studies have showed the importance of monoamino oxidase (MAO) as an important source of H$_2$O$_2$ in neurons (Marker et al. 1981) and in relationship to neurodegenerative diseases (Ardensen 2004). Hydrogen peroxide has the ability to move between different cellular compartments. Although H$_2$O$_2$ has long been thought to diffuse freely across membranes, its dipole moment of 2.2 $\times$ 10$^{-18}$ C m (Cohen & Pickett 1981) is only slightly higher than that of water (1.9 $\times$ 10$^{-18}$ C m), rendering its passive diffusion similarly limited. Recent studies have demonstrated the implication of aquaporin water channels as specific mediators of H$_2$O$_2$ passage across membranes (Bienert et al 2007) and the involvement of these channels in intracellular signaling (Miller et al. 2010).

The unusual reactivity of H$_2$O$_2$ is generally attributed to relatively low O-O bond energy (47 kcal/mol) – hence, the ease with which it is homolytically or heterolytically cleaved (Bach et al. 1996). H$_2$O$_2$ is a strong oxidant and may behave as an electrophile as well a nucleophile depending on the nature of the reactant. In cells, the principal targets of H$_2$O$_2$-mediated oxidation are thiols by means of two electron nucleophilic substitution. A thiol with a low pK$_a$ value reacts rapidly to yield sulfenic acid and water (Eq. 1).

\[
R\text{-SH} + \text{HO-OH} \rightarrow R\text{-S-OH} + \text{H}_2\text{O} \tag{1}
\]

Although glutathione (GSH) is the most abundant low molecular weight thiol, GSH contributes little to the reduction of H$_2$O$_2$ in cells. In fact, the total protein thiol concentration in human cells is at least two times higher than GSH (Hansen et al. 2009). At steady state, cellular H$_2$O$_2$ is kept in check by enzymes of the peroxiredoxin (Prx) family (Winterbourn & Hampton 2008). The rate of H$_2$O$_2$ reduction catalyzed by Prx is exceptionally fast (1 $\times$ 10$^7$ M$^{-1}$ s$^{-1}$ at pH 7), but this reactivity cannot be explained solely by the low pK$_a$ of the peroxidative cysteine (~5.7, Ree & Woo 2011). More generally, the reactivity of peroxides (RO-OH) shows an inverse relationship with the pKa of the
leaving group RO (for this reason CH$_3$CO$_2$H is much more reactive than H$_2$O$_2$). The presence of specific proton-donating moieties within the protein microenvironment can serve to stabilize the poor hydroxide-leaving group and thus, accelerate the reaction rate. Along these lines, a recent series of experimental and computational studies by Nagy et al. suggests that two highly conserved arginine residues contribute to reactivity of Prx toward H$_2$O$_2$ via hydrogen bonding (Nagy et al. 2011).

Although the recombinant form of proteins may undergo H$_2$O$_2$-mediated thiol oxidation in the test tube, in many cases, it has been difficult to reconcile relatively low oxidation rates observed in vitro with intracellular regulation of enzymatic activity. For example, the rate constant measured for inactivation of recombinant protein tyrosine phosphatase 1B (PTB1B) is $\sim$10 M$^{-1}$ s$^{-1}$ (Denu et al. 1998), which would appear to be incompatible with intracellular signaling events. With respect to this long-standing issue, recent work by Gates and coworkers has proposed that more reactive species, such as peroxymonocarbonate and peroxymonophosphate, oxidize PTPs in cells (Zhou et al. 2011). Peroxymonocarbonate, which may be spontaneously generated from bicarbonate anion in the presence of localized high concentrations of H$_2$O$_2$ (Eq. 3), can increase the rate of PTB1B oxidation by a factor of 20; the elevated reactivity is easily explained by the higher $pK_a$ value of the hydrogen carbonate leaving group.

$$H_2O + CO_2 \rightleftharpoons H_3O^+ HCO_3^- \quad (2)$$

$$HCO_3^- + H_2O_2 \rightarrow H_2O + HO_2COOH^- \quad (3)$$

Peroxymonophosphate is even more powerful and reacts with PTB1B more than 7,000 times faster than H$_2$O$_2$, although the generation and existence of such a species in vivo remains to be evaluated. One very speculative possibility is that peroxymonophosphate could be biologically accessible via direct enzymatic phosphorylation of H$_2$O$_2$ (LaButti et al. 2007). On the other hand, the proximity of proteins to the source of ROS/RNS can also have an obvious influence on target selectivity and rates of thiol oxidation within the cell (Chen et al. 2008; Paulsen et al. 2011).

Besides thiol oxidation, H$_2$O$_2$ can also easily react via one electron reduction with transition metals such as iron and copper. This reaction, known as the Fenton reaction,
generates hydroxyl radical (Eq. 4; where L = ligand), a highly reactive species that can indiscriminately damage different biological target molecules such as DNA, proteins, or lipids via radical-mediated reactions and contributes significantly to the development of oxidative stress (Prousek 2007).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{LFe}^{3+} + \text{HO}^\cdot + \text{HO}^- (4)
\]

Although \( \text{H}_2\text{O}_2 \) has a relatively short half-life in cells \( (t_{1/2} = 10^{-5} \text{ s}) \), high variations in concentration can form locally and temporarily in cells (Giorgio et al. 2007). Molecular imaging is a powerful method for real-time monitoring of \( \text{H}_2\text{O}_2 \) in biological systems. Traditional tools for ROS detection, such as 2’-7’-dichlorodihydroflurescein, are not specific for \( \text{H}_2\text{O}_2 \), but recent years has seen the development of many chemoselective probes, in particular by Chang’s research group (Lippert et al. 2011). Because of its ambiphilic reactivity, \( \text{H}_2\text{O}_2 \) can be chemically differentiated from other ROS. With a pKa value of about 11, \( \text{H}_2\text{O}_2 \) is also a good nucleophile owing to the \( \alpha \)-effect of adjacent nonbonding orbitals on its oxygen atoms (Jencks et al. 1960), particularly in the deprotonated form. \( \text{H}_2\text{O}_2 \)-mediated deprotection of aryl boronates to phenols (Kuivila & Armour 1957, Fig. 2a) has served as the starting point for the development of selective probes for \( \text{H}_2\text{O}_2 \) imaging. The installation of boronic esters on the 3’ and 6’ positions of the fluorescein core, for example, produces masked fluorescein (e.g., 13a) in which the two boronates block the molecule in the closed lactone form, eliminating its absorptive and emissive properties. The reaction with \( \text{H}_2\text{O}_2 \) transforms the boronates into phenols (e.g., 13b) with a strong increase in fluorescence intensity (Chang et al. 2004). Although boronates should also react with alkyl peroxides (like lipid peroxides), the reaction with \( \text{H}_2\text{O}_2 \) should be faster because hydroxide anion is a better leaving group than alkoxides \( (\text{RO}^-) \). Obviously, the reaction is accelerated at higher pH levels, and local pH changes can alter the probe response. Based on the same approach, several other probes have been developed with enhanced sensitivity (Dickinson et al. 2011) or which are targeted to specific subcellular compartments (Dickinson et al. 2010). Other bioorthogonal reactions have inspired alternative probes for selective \( \text{H}_2\text{O}_2 \) detection. Chang’s group, for example, has developed a hyperpolarized \( ^{13}\text{C} \) probe based on oxidative decarboxylation
of α-ketoacids (Fig. 2b) for $^{13}$C MRI resonance imaging of H$_2$O$_2$ (14a, Lippert et al. 2011). Finally, Nagano’s group has taken advantage of the less known Baeyer-Villiger benzil oxidations (Fig. 2c) to design a highly sensitive probe for H$_2$O$_2$ (15a, Abo et al. 2011). Together, these novel chemical tools have revealed fundamental new insights into the role of H$_2$O$_2$ in cells (Dickinson et al. 2011; Miller et al. 2010).

3.2 Hypo(pseudo)halous acid

The heme-containing enzymes myeloperoxidase (MPO) and lactoperoxidase (LPO) use H$_2$O$_2$ to oxidize halides (Cl$^-$, Br$^-$ and I$^-$) and thiocyanate (SCN$^-$) into their respective hypohalous acids (HOXs). These species are more reactive than H$_2$O$_2$ and are used as antimicrobials by the immune system (Albrich et al. 1981). Although chloride is the most abundant negative electrolyte in living systems (1-60 mM in cell, 100 mM in extracellular fluids), the major substrate of MPO is SCN$^-$, whose affinity for the enzyme is a thousand-fold greater than is that of Cl$^-$ (Hawkins 2009). Generally SCN$^-$ has a concentration of 20-250 µM (Van Dalen et al. 1997) but can reach a concentration of 1 mM in saliva, where LPO produces almost exclusively HSCN (Ashby 2008). Br$^-$ is nearly sixty-fold more specific for MPO but, with a concentration of 20-100 µM, the HOBr generated from neutrophils is estimated to be ~10% of HOCl production (Chapman et al. 2009). Finally, since the iodide concentration in cells is usually low (~1 µM), the production of hypoiodous acid in vivo can be considered negligible. Hypohalous acids are weak acids; their pKa values are 7.6 for HOCl, 8.7 for HOBr, 10.4 for HOI, and 5.3 for HOSCN (Davies et al. 2008). Except for HOSCN, which is completely deprotonated, these species exist as a mixture of their acid and anion forms at physiological pH. HOCl and, to a lesser extent HOBr, are extremely strong oxidants and indiscriminately react with a large variety of functional groups in proteins, nucleotides, and membrane lipids. HSCN is several times less reactive and selectively oxidizes thiols (Lloyd et al. 2008).

HOX species may also interconvert, and this reaction reflects the relative oxidizing strengths of these acids. As a result, if HOCl can oxidize Br$^-$, I$^-$, and SCN$^-$,
HOBr reacts solely with I\(^-\) and SCN\(^-\) (Spalteholz \textit{et al.} 2005), though only the interconversion with SCN\(^-\) appears to be relevant \textit{in vivo} (Nagy \textit{et al.} 2006). HOXs are electrophilic oxidants and undergo two-electron nucleophilic substitution. Thiols react with HOCl and HOBr \textit{via} formation of a very unstable sulphenyl halide (Eq. 5-6), which readily rearranges to sulfenic acid (Nagy & Ashby 2007). Otherwise, HOSCN generates a sulfonyl-thiocyanate (Eq. 8), which can react with another thiol to yield a disulfide and SCN\(^-\) (Eq. 9, Ashby & Aneetha 2004). Kinetic studies indicate that reaction proceeds \textit{via} thiolate and hypohalocyanous acid reactants, indicating that this mild oxidant is very selective for proteins with low pK\(_{a}\) thiol residues (Nagy \textit{et al.} 2009).

\[
\begin{align*}
R-S^- + \text{HO-Cl} &\rightarrow R-S-Cl + \text{HO}^- \quad (5) \\
R-S^- + \text{HO-Br} &\rightarrow R-S-Br + \text{HO}^- \quad (6) \\
R-S-X + \text{H}_2\text{O} &\rightarrow R-S-\text{OH} + \text{X}^- \quad (7) \\
R-S^- + \text{HO-SCN} &\rightarrow R-S-\text{SCN} + \text{HO}^- \quad (8) \\
R_1-S^- + R-S-\text{SCN} &\rightarrow R-S-S-R_1 + \text{SCN}^- \quad (9)
\end{align*}
\]

It is generally accepted that the sulfur-containing residues (cysteine and methionine) react most rapidly with HOCl (with rate constants of \(~3-4 \times 10^{-8} \text{ M}^{-1}\text{s}^{-1}\) ), but strong nucleophilic amines (such as lysine and histidine) are also modified at high HOCl concentrations to yield chloramines (Pattison & Davies 2001).

\[
\begin{align*}
R_1R_2\text{NH} + \text{HO-Cl} &\rightarrow R_1R_2\text{N-Cl} + \text{HO}^- \quad (10) \\
R-S^- + R_1R_2\text{N-Cl} + \text{H}^+ &\rightarrow R-S-\text{Cl} + R_1R_2\text{NH} \quad (11)
\end{align*}
\]

Small-molecule amines such as ammonia or glycine may react with HOCl to form membrane-permeable chloramines (Eq. 10), which selectively mediate thiol oxidations (Midwinter \textit{et al} 2006). Taurine-chloramine (TauCl) is another biologically relevant chloramine. This cell-impermeable chloramine is generated especially in neutrophils, in which taurine is the most abundant free “amino acid” (10-30 mM) and also appears to play a scavenger role for chlorinated oxidants (Marcinkiewicz \textit{et al.} 1995).

Although HOCl plays a fundamental role in destroying a wide range of
pathogens, neutrophil-mediated HOCl production has also been implicated in several inflammation-associated diseases. A series of detection methods have been developed in order to shed light on its physiological and pathological roles (Fig. 3). These selective probes are based on the major oxidant strength of HOCl in comparison with other ROS. For example, \( p \)-methoxyphenol is selectively oxidized by HOCl (Fig. 3a) and shows good stability in the presence of other ROS and RNS. Yang’s research group has taken advantage of this reaction to design a BODIPY-based probe (16a) that displays a significant increase of fluorescence after HOCl incubation (Sun et al. 2008). By analogy to chloramine formation, Tae’s research group has instead developed a fluorescent probe based on HOCl-mediated oxidation of hydroxamic acid (Yang et al. 2009). Following generation of an unstable chlorinated intermediate, hydroxamic acid yields an acyl nitroso compound with elimination of HCl (Fig. 3b). Starting from a rhodamine scaffold, Tae’s group designed a hydroxyamido derivative enclosed in a stable spirocyclic non-fluorescent form (17a), which demonstrates a rapid and selective fluorescent response in the presence of exogenous HOCl in cells as well as in mice. Recently, a thioether-rhodamine derivative was designed as a chemoselective probe for HOCl. In fact, thioethers and thiols are the major target of HOCl-mediated oxidation and, although thiols indiscriminately react with any ROS and RNS, thioethers show selective oxidation (Fig. 3c). The thioether group maintains the Si-rhodamine probe in the cyclic non-fluorescent form (18a), and the system recovers its highly conjugated structure through the generation of a sulfone (18b), followed by elimination of sulfinic acid (which then oxidizes to sulfonic acid) and a robust increase in fluorescence (Kolde et al. 2011). Finally, although many efforts have been made, few methods (with the exception of probe 17a) appear to have the appropriate characteristics for biological application in vivo.

3.3 Nitric oxide and peroxynitrite

NADPH-dependent nitric oxide synthase (NOS) catalyzes the synthesis of nitric oxide (NO) and L-citrulline via oxidation of the guanidine group of L-arginine (Alderton et al 2001). NO, like carbon monoxide (CO) and hydrogen sulfide (H\(_2\)S), is a small
gaseous signaling molecule implicated in numerous physiological processes (Cary et al. 2006). NO is a free radical species (\(N=O\)) with a relative long half-life in comparison to other ROS/RNS (\(t_{1/2} = 1\) s, Pacher et al. 2007) and can freely diffuse across membranes. Although an S-nitrosothiol could be directly generated by reaction with thiyld radicals (Jourd’heuil et al. 2003), most of its activity is mediated by development of intermediary nitrosating agents (Keszler et al. 2010). These nitrosating species are generated via a series of oxidation reactions. NO forms nitrous anhydride (\(N_2O_3\)) in the presence of oxygen (Eq. 11-12), and this unstable species can easily be hydrolyzed (Eq. 13) or undergo two-electron nucleophilic substitution by thiols (Goldstein et al. 1996) and amines (Caulfield et al. 1998). \(N_2O_3\) is generally accepted as the major nitrosylating agent \textit{in vivo}, although other pathways are proposed to explain S-nitrosothiol formation in cells (see section 4.2).

\[
\begin{align*}
\text{NO} + \text{O}_2 & \rightarrow \text{NO}_2 \\
\text{NO} + \text{NO}_2 & \rightarrow \text{O}_2\text{N-N}=\text{O} \\
\text{O}_2\text{N-N}=\text{O} + 2 \text{OH}^- & \rightarrow 2 \text{NO}_2^- + \text{H}_2\text{O} \\
\text{O}_2\text{N-N}=\text{O} + \text{R-S}^- & \rightarrow \text{R-S-NO} + \text{NO}_2^- \\
\text{O}_2\text{N-N}=\text{O} + \text{R-NH}_2 & \rightarrow \text{R-NH-NO} + \text{HNO}_2
\end{align*}
\]

\(\text{O}_2^-\) transforms the relatively unreactive NO into peroxynitrous acid (ONOOH), a strong oxidant (Eq. 16). This diffusion-controlled reaction has the largest rate constant known for NO (1 \(\times\) \(10^{10}\) \(\text{M}^{-1} \text{ s}^{-1}\)) and, \textit{in vivo}, is spatially associated with the sources of superoxide (Radi et al. 2001). ONOOH is a weak acid with a \(pK_a\) value of 6.8 (Kissner et al. 1997) and is in equilibrium with its deprotonated form (peroxynitrite, ONOO\(^-\)) at physiological pH. Both species are strong oxidants and can participate in one- and two-electron oxidation reactions, although HONOHO shows a faster reactivity (Moro et al. 1994) and ONOO\(^-\) appears to be a powerful nucleophile as well. Despite its short half-life, ONOOH appears able to diffuse across the membranes (Ferrer-Sueta & Radi 2009) where it can react with a wide group of substrates (Marla & Groves 1997). Typically, thiols are oxidized to disulfides by ONOOH (Squadrito & Pryor 1998), but it is still uncertain which intermediate species are involved between the sulfinic acid (R-SOH)
and S-nitrothiol (R-SNO₂). Nevertheless, kinetic analysis clearly shows that this reaction involves the pairing of the thiolate/peroxynitrous acid. Other studies have shown that ONOOH can also mediate the S-nitrosylation of GSH with elimination of H₂O₂ (van der Vliet et al. 1998). In any case, considering that ONOOH is very unstable and can undergo hemolytic fission to generate the one-electron oxidant, hydroxyl (’OH), as well as ’NO₂ radicals (Szabo et al. 2007), it is difficult to assess which reaction ONOOH participates in directly. ONOOH decomposition is also facilitated by bicarbonate (HCO₃⁻). CO₂ and readily undergoes nucleophilic addition by ONOO⁻ (with a constant rate of 5.8 x 10⁴ M⁻¹ s⁻¹) to yield ’NO₂ and CO₃⁻ (Eq. 18-19). CO₂ also mediates the generation of other oxidant species (Eq. 20) and is considered to contribute to the nitration of tyrosine side chains (Squadrito & Pryor 1998).

\[ \text{NO} + \text{O}_2^- \rightarrow \text{ONOOH} \]  (16)
\[ \text{ONOOH} \rightarrow \text{’NO}_2 + \text{’OH} \]  (17)
\[ \text{ONOO}^- + \text{CO}_2 \rightarrow \text{ONO-OCO}_2^- \]  (18)
\[ \text{ONO-OCO}_2^- \rightarrow \text{’NO}_2 + \text{CO}_3^- \]  (19)
\[ \text{ONO-OCO}_2^- \rightarrow \text{O}_2\text{N-OCO}_2^- \]  (20)

The development of powerful tools to detect NO signaling remains an active area of research. The first approach developed to detect NO was based on the o-phenylenediamine scaffold which, in the presence of NO and O₂ (Uppu & Pryor 1999), yields the corresponding aryl triazole (Fig. 4a). The electronic difference between the electron-rich diamine (19a) and the electron-poor triazole (19b) leads to a robust fluorescent switch (Kojima et al. 1998). Obviously, this is an indirect approach for NO detection. Specifically, the aryl diamine reacts with N₂O₃ to form N-nitrosylamine, which evolves to triazole via intramolecular nucleophilic displacement. Recently, Xu’s research group has developed an elegant new probe based on the fluorescein scaffold; in this case, the diamine blocks the fluorophore in spirolactame form; reaction with NO leads to the formation of a triazole ring and concomitant fluorescence response (Zheng et al. 2008).

Parallel approaches for direct detection of NO have also been developed. It is well known that NO has strong affinity towards transition metals, including the ability to bind the heme-group. Taking advantage of this property, several probes have been designed
conjugating transition metals to a fluorescent core (Lim & Lippard 2007). This approach was based upon the release of a fluorophore, initially quenched by electron or energy transfer through coordination to a paramagnetic transition-metal center such as Fe(II), Co(II), or Cu(II). The presence of NO causes the displacement of the metal-complex with concomitant fluorescence activation. Taken together, the two approaches described are perfectly complementary for chemospecific NO detection in living systems.

Largely because of the ongoing difficulty of direct and unambiguous detection, the biological relevance of ONOOH remains controversial. Selective probes for peroxynitrous acid have been inspired based on the similar chemical behavior of ONOO⁻ and peroxymonosulfate (HOOSO₃⁻). In fact, ONOO⁻ can react with ketones to yield dioxiranes (Yang et al. 1999). Furthermore, it is known that internal dioxiranes can intramolecularly oxidize phenol derivatives to quinones and that this reaction is facilitated when the dioxiranes are generated from ketones with electron-withdrawing groups (Yang et al. 2000). Yang’s group has demonstrated that ONOO⁻ can also oxidize anisole-derivative ketones via dioxirane in situ formation (Fig. 4b). Based upon this reaction, several fluorescent probes have been developed in which an anisole-derivative is conjugated with a fluorophore and the peroxynitrite-mediated oxidation selectively allows the release of the fluorescent molecule (e.g., 20a; Dan et al. 2006; Peng & Dan 2010).

3.4 Hydrogen sulfide

For many years, hydrogen sulfide (H₂S) was solely considered as a toxic gas, but in the last decade its involvement in mammalian cell signaling has become apparent (Lefer 2009). H₂S is synthesized by two pyridoxal 5'-phosphate-dependent enzymes involved in cysteine metabolism (Chiku et al. 2009): cystathione β-synthase (CBS) and cystathione γ-lyase (CSE). H₂S can also be generated from L-methionine via transsulfuration (Zhao et al. 2007) or release from a persulfide (RS-SH) by thiol-disulfide exchange. Like other gaseous signaling molecules, such as NO and CO, H₂S rapidly crosses cell membranes with no specific transporter (Mathai et al. 2007). In fact, although
H₂S shows structural similarities with H₂O; it does not form strong hydrogen bonds and, with a dipole moment of 0.9 D, H₂S can freely diffuse through the hydrophobic core of biological membranes.

H₂S is a diprotic acid with a pKₐ₁ of 6.9 and pKₐ₂ of 11.96; consequently, H₂S is in equilibrium with its deprotonated form (with a H₂S/HS⁻ of 1:3) at physiological pH (Liu et al. 2011). In many respects, H₂S exhibits a reactivity profile akin to cysteine. It is a powerful nucleophile and, like thiols, can react with electrophiles as well as oxidants. With two-electron oxidants (Eq. 21), H₂S forms hydrogen thioperoxide (HSOH – Carballal et al. 2011). HSOH is a very reactive sulfur species and can be oxidized rapidly to SO₄²⁻ or undergo reaction with thiols. In vitro, the reaction of H₂S with HSOH leads exclusively to the formation of a complex mixture of polysulfides (HSₓ⁻ with x = 2-8) (Eq. 22), which have been proposed to mediate the S-sulfhydration of proteins (Nagy & Winterbourn 2010).

\[
\begin{align*}
\text{HS}^- + \text{H}_2\text{O}_2 & \rightarrow \text{HSOH} + \text{H}_2\text{O} \\
\text{HSOH} + n \text{HS}^- & \rightarrow \text{HS}^-\text{S}_n^- + \text{H}_2\text{O}
\end{align*}
\]

The biological functions of H₂S are still unclear, but growing evidence suggests a role for H₂S in the regulation of cardiovascular (Elsey et al. 2010) and gastrointestinal (Wang 2010) systems. Moreover, its biologically relevant levels are still debated, and a wide range of concentrations has been reported (Kabil & Banerjee 2010). To investigate such issues, a number of research groups have focused their efforts on the development of small-molecule probes for H₂S detection (Fig. 5). H₂S can be considered a strong reducing agent and this property has been widely exploited in organic synthesis to reduce aromatic nitro (Lin & Lang Jr. 1980) and azido (N₃) (Pang et al. 2009) groups to aniline. Chang’s research group developed a selective probe for H₂S detection by installing an N₃ group in the 6’ position of a rhodamine core (21a), which locks the fluorophore in the spirocyclic non-fluorescent form. Reduction to the amine releases the fluorescence signal (21b) and such probes display high selectivity for H₂S over other biologically relevant reactive sulfur, oxygen, and nitrogen species (Lippert et al. 2011). Subsequently, Wang’s research group used a similar approach to design a sulfonylazide dansyl derivative.
whereby the difference in electronegativity between the azide and amine group triggers a change in electronic and, thus, fluorescent properties (Liu et al. 2011). Both probes appear quite promising given that azido group is quite inert and has shown considerable compatibility for application in living systems.

As previously mentioned, H$_2$S exhibits a similar reactivity as compared to cysteine. Theoretically, H$_2$S can be considered a non-substituted thiol and can give nucleophilic attack twice, whereas other thiols, such as cysteine, can participate in just one nucleophilic attack. Exploring this property, Xian’s research group hypothesized that a probe with two electrophilic centers could be selective for H$_2$S detection and developed a thiosalicylic acid ester of fluorescein in which the thiol group is functionalized as thiopyridyl disulfide (22a). Both thiols and H$_2$S readily yield disulfide exchange with activated disulfide, but only the disulfide intermediate from H$_2$S (22b) can rearrange, via nucleophilic attack on the intramolecular phenyl ester, leading to benzodithiolone (22c) and fluorescein (22d) products (Liu et al. 2011). Along these lines, Chuan’s research group has developed a selective probe for H$_2$S using a Michael acceptor and an aldehyde group as two electrophilic centers (23a). H$_2$S forms a thioacetal with the aldehyde group (23b) followed by an intramolecular Michael addition with the internal α,β-unsaturated compound (23c). Such H$_2$S-specific rearrangement leads to a strong increase in fluorescence. Using this probe, Chuan’s group has succeeded for the first time in imaging enzymatic H$_2$S production in living cells (Quian et al. 2011).

4. Oxidative modification of protein cysteine residues

The cysteine side chain, with its high nucleophilic capacity, appears to be the principal target of ROS/RNS in cells. The sulfur atom of cysteine may assume a wide range of oxidation states (i.e., -2 to +4, Fig. 6) and each form exhibits a distinct chemical reactivity. In the thiolate form, sulfur undergoes oxidation to generate a sulfenic acid, and this oxoform can be considered as a central species among thiol modifications. Sulfenic acid may be reduced to a disulfide by reaction with intra- and inter-molecular thiols or further oxidized to sulfinic acid at high ROS/RNS concentrations. In some cases, the
sulfenic acid can lead to the formation of sulfenamide and thiosulfinate ester groups. The cysteine reactivity landscape becomes more complex given that the thiolate may react with RNS and RSS to form S-nitrosothiol and persulfide, respectively. Moreover, depending upon the nature of the protein microenvironment, many of these modifications are reactive and can interconvert with one another. In order to highlight the possible role and significance of each modification, their distinct physical and chemical properties are outlined below.

4.1 Sulfenic acid

Sulfenic acids (RSOH) are directly formed in vivo by the oxidation of thiols with two-electron oxidants such as H₂O₂, ONOOH, or alkyl peroxides and many enzymes form SOH intermediates during their catalytic cycles (Lim et al. 2011). In addition, RSOH can be generated from the hydrolysis of sulfonyl-halides (Nagy & Ashby 2007a), sulfonyl-thiocyanates (Ashby & Aneetha 2004), and thiosulfimates (Nagy & Ashby 2007b). Hydrolysis of S-nitrosothiols can also yield RSOH (Percival et al. 1999), but the reaction has a high activation barrier (Moran et al. 2011). The thyl radical (RS•) can also yield sulfenic acid in the presence of hydroxyl radicals (’OH); however, this reaction is not likely to be widespread in a biological setting. Otherwise, sulfenic acids are usually obtained in organic synthesis from the thermolysis of sulfoxides (Sivaramakrishnan et al. 2005) or thiosulfimates (Block & O’Conner 1974).

The S-O bond length in RSOH is distinctly longer than that of sulfoxides, indicating that the tautomeric structure, R-S(O)H, can be excluded (Goto et al. 1997). RSOH is a weak acid but, because of their high reactivity, their pKa has been difficult to measure. In the case of some stable small-molecule sulfenic acids, an acidity of two to three orders of magnitude lower than the corresponding thiols has been found (Heckel & Pfleiderer 1983). This trend could quite realistically offer an estimation of pKa value for other sulfenic acids. For example, the pKa value of a high-hindered triptycenyl sulfenic species was recently reported to be 12.5 (McGrath et al. 2010), about three times higher than a typical thiol. Although they are usually defined as transient species, intramolecular
hydrogen bonds (Heckel & Pfleiderer 1983) and steric hindrance (Nakaruma 1983) can play major roles in stabilizing protein RSOH. More generally, hydrogen bonds in apolar protein microenvironments, and the absence of vicinal thiols can all stabilize sulfenic acids (Claiborne et al. 1993).

Sulfenic acids exhibit potent electrophilic (Fig. 7) and relative weak nucleophilic (Fig. 8) reactivity. In small-molecule RSOHs, this dual behavior can lead to self-condensation in which one sulfur atom functions as a nucleophile and the second as an electrophile to yield a thiosulfinate ester (Fig. 7a). In this reaction, intermolecular hydrogen bonding mediates the self-condensation of RSOH, thereby reducing the free energy of activation for thiosulfinate formation thermodynamically preferred over the acid (Davies et al. 1986). On the other hand, self-condensation of RSOH only competes with thiol-based reduction of sulfenic acid at high pH (Nagy & Ashby 2007) and is otherwise considered negligible.

As indicated above, thiols can reduce sulfenic acid to form a disulfide (Fig. 8a) and this condensation represents an important biological reaction. Moreover, this reaction allows the recycling of sulfenic acid in as much as the disulfide can be reduced to thiol through the action of cellular-reducing agents such as glutathione (GSH), glutaredoxin (Grx) and thioredoxin (Trx). Dithiols (such as DTT) and Trx can also directly reduce RSOH back to the thiol form (Poole et al. 2004). Thiols are also used as trapping agents to demonstrate the formation of aleatory sulfenic acid in the thermolysis of sulfoxides (Kamiya et al 1973). Several inorganic molecules, such as NaAsO₂ (Saurin et al. 2004), hydrazine, and NaN₃ (Allison 1976), have been reported to reduce RSOH to the thiol; however, their selectivity has not been sufficiently explored. Although sodium ascorbate (Fig. 9a) is generally considered a specific reducing agent for S-nitrosothiols (Turell et al. 2008), it can also reduce RSOH through an addition/elimination mechanism (You et al. 1975; Fig. 8b); recently, the ability of sodium ascorbate to reduce sulfenic acid in vivo has also been proposed (Carvhalo et al. 2007).

Sulfenic acids can also be oxidized by two-electron oxidants (Fig. 7b) or by O₂ in the presence of trace metal ions. The oxidation of non-hindered thiols leads only to the disulfide. Considering that sulfenic acids can be intermediates in disulfide formation, it is worth noting that oxidation of sulfenic acid is slower, compared to its condensation rate.
with thiols (Luo et al. 2004). Kinetic studies have showed that the rate constant for oxidation of cysteine sulfenic acid is approximately two to three orders of magnitude slower than cysteine (Hugo et al. 2009). Two pathways may be hypothesized for H_2O_2 oxidation of RSOH: first, a concerted mechanism mediated by formation of hydrogen bond and, second, the direct participation of a sulfenate anion (RSO`). The pH profile of H_2O_2-mediated oxidation of RSOH indicates that the sulfenate is the reacting species and therefore, RSOH generated from a very low pKa cysteine should be more susceptible to irreversible oxidation to sulfinic acid.

Reactive cysteine residues in specific proteins may readily oxidize to sulfenic acid. This reversible thiol modification plays a significant role in protein regulation and cell signaling (Paulsen & Carroll 2010). As the first (and sometimes only) oxidation product, RSOH is a key “marker” for ROS/RNS-sensitive cysteine residues and, as such, there is clear evidence of its role in cell signaling (Klomsiri et al. 2011; Roos & Messens 2011). As a result, interest in the RSOH detection has grown rapidly in the last decade and the reactivity of protein sulfenic acids continues to be explored. Sulfenic acids react as weak nucleophiles with alkyl or aryl halides, alkenes, and alkynes (Fig. 7c-e). Although sulfenate ions (RSO`) should be an ambident nucleophile, alkylation with halides always yields the sulfoxide (Hogg et al. 1979). This reaction should be taken in account when blocking protein thiols with iodoacetamide since alkylation of sulfenic acid (Fig. 7e, 24) becomes relevant at reagent concentrations typically employed in such procedures. The aromatic nucleophilic substitution of halonitroarene has been employed to detect the sulfenic acid group in proteins (Fig. 7f). For example, the electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) reacts with both sulfenic acids and thiols to yield covalent adducts (25 and 7b) that are distinguishable from each other with respect to mass and their UV-Vis absorption profiles (Ellis & Poole 1997). Conversely, alkenes (Kingsbury & Cram 1960) and electron-deficient alkynes (Goto et al. 1997) have long been used in organic synthesis in organic solvent at high temperature for to trap transient sulfenic acid species (Fig. 7c-d). Such nucleophilic addition reactions occur through a concerted, cyclic process – the reverse of the elimination of sulfenic acids from sulfoxides – and should therefore proceed through the same transition state via regiospecific Markovnikov addition (Fig. 7c).
The sulfur atom in sulfenic acid also functions as an electrophile (Fig. 8a-e). Primary and secondary amines react with RSOH to yield sulfenamides (Fig. 8c, Allison et al. 1973). This reaction is quite slow and, in a biological context, only makes sense when this reaction takes place intramolecularly. In fact, in some proteins sulfenic acid can react with the nitrogen backbone of the neighboring residue to yield a five-member cyclic sulfenamide (see Section 4.4). Although RSOH could also react with intramolecular lysine residues, this species has been rarely detected (Raftery et al. 2001) and its formation in vivo remains unclear. Sulfenic acids also react with phosphorous III compounds to yield trialkylphosphine oxide and the corresponding thiol via hydrolysis of the intermediate phosphonium (Fig. 8d, Goto et al. 2003). To date, coupling of RSOH with cyclic 1,3-diketones is the only reaction that has demonstrated high selectivity for this cysteine oxoform. Cyclic 1,3-diketones react with sulfenic acid in their enol-form, likely through hydrogen bond-mediated direct substitution (Fig. 8e).

5,5-dimethyl-1,3-cyclohexandione, commonly known as dimedone, is often used trapping agent for detecting protein sulfenic acid modifications (Benitez, Allison 1974). However, this reagent is not ideal for proteomic studies since it lacks an affinity and/or detection handle. In recent years, several advantageous derivatives of dimedone have been developed. The first approach was direct conjugation of 1,3-cyclohexandione with a biotin or a fluorophore (Fig. 8, 25) moiety (Poole et al. 2005). More recently, Carroll’s research group has functionalized the 1,3-cyclohexandione scaffold with an azide (26a) (Leonard et al. 2009) or alkyne (26b) (Paulsen et al. 2011) chemical reporter, known as DAz-2 and DYn-2, respectively. These small probes are membrane-permeable and enable detection and trapping of protein sulfenic acids directly in cells. After cellular tagging, lysates are generated and coupled to detection reagents via Staudinger ligation or Huisgen azide-alkyne 1,3-dipolar cycloaddition (also known as click chemistry). Carroll’s group has also reported the development of a heavy-isotope coded dimedone analogue, which in combination with 10 (see Section 2) represents a powerful new approach to quantify the extent of sulfenic acid modification at individual protein cysteine residues (Seo & Carroll 2011). Furdui’s research group has reported a new effective probe for sulfenic acids based on a related scaffold, 1,3-cyclopentadione (27, Quian et al. 2011). Finally, Carroll’s group has recently reported tri-functional probes consisting of a dimedone-like
warhead, chemical reporter, and binding module (28) that enhances the detection of sulfenic acid in specific classes of signaling proteins (e.g., PTPs, Leonard et al. 2011). In parallel research, antibodies that recognize the protein-dimedone adduct have also been developed (Seo & Carroll 2009; Maller et al. 2011). Carroll and coworkers have applied such antibodies to visualize sulfenic acid modifications in tumor cells and profile growth factor-dependent changes in thiol oxidation among breast cancer cell lines (Seo & Carroll 2009).

4.2 Nitrosothiol

NO and its metabolites may react with the side chain of cysteine to yield S-nitrosothiols (Fig. 9, RSNO). This modification can lead to significant changes in the structure and function of proteins (Denninger & Marletta 1999). S-nitrosylation represents an important signaling mechanism and many proteins have been identified as S-nitrosylation targets (Jaffrey et al. 2001). S-NOs may be formed through the direct reaction of thiols with N₂O₃ and less commonly with ONOOH (see Section 3.3) or by trans-nitrosylation with an NO-donor (Fig. 9a). Trans-nitrosylation is also the most important reaction in which RS-NOs are involved in biology and appears to be the principal mechanism upon which NO-signaling is based (Tsikas et al. 1999). RS-NOs are exceptionally labile (Kashiba-Iwatsuki et al. 1997) and easy react with other thiols by trans-nitrosylation or, in some cases, by disulfide formation. These two possible pathways are due to the concomitant presence of two electrophilic centers on the S-nitrosothiol: one is the sulfur atom and the second is the nitrogen atom of the NO group. Sodium ascorbate (Fig. 9b) or Cu I (Dicks & Williams 1996) can also restore the thiol group from RS-NOs. Although the mechanism of ascorbate reduction is not entirely clear, the formation of O-nitrosoascorbic acid (Holmes & Williams 2000) appears quite accepted. The ascorbate-mediated reaction is used in the biotin switch assay. This technique is the most popular method for detecting S-nitrosothiols in proteins from cell extracts (Jaffrey et al. 2001) and involves three steps: 1) free thiols are blocked with a “thiol-specific reagent” (see Section 2) such as MMTS; 2) nitrosothiols are reduced by sodium ascorbate to thiols; and 3) nascent thiols are then conjugated to biotin with N-[6-
(biotinamido)hexyl]-30-(20-pyridyldithio)-propionamide (biotin-HPDP, 1) and analyzed by Western blot (Fig. 9).

Along with issues inherent to indirect or subtractive methods of detection, another challenge for this technique is the selective reduction of RSNOs with sodium ascorbate. It is well known that sodium ascorbate can give false-positive signals for S-nitrosothiols when detected by the biotin switch assay (Huang & Chen 2006). In fact, ascorbate can also reduce sulfenic acids (see Section 4.1) and disulfides, albeit less efficiently. Furthermore, the ascorbate-mediated reduction of RS-NOs is quite slow and some nitrosothiols cannot be reduced during the incubation time. The reaction rate of reduction can be increased using catalytic amounts of Cu⁺ (Kirsch et al. 2009). Copper promotes the direct de-nitrosylation of RS-NOs while ascorbate maintains the minimum concentration of Cu(I) necessary to catalyze the reaction, reducing Cu²⁺ to Cu⁺ (Eq. 23-24). However, it is critical to note that Cu⁺ may also lead to oxidation of protein thiols.

\[
\text{RS-NO + Cu}^+ \rightarrow \text{R-S}^- + \cdot\text{NO} + \text{Cu}^{2+} \quad (23)
\]
\[
\text{Cu}^{2+} + \text{Asc}^- \rightarrow \text{Cu}^+ + \text{Asc}^- \quad (24)
\]

In light of these limitations the biotin switch assay has been superseded by direct and selective bioorthogonal reactions for RS-NOs in recent years. It is known that triaryl phosphines react with RS-NOs to yield an aza-ylide intermediate, which is hydrolyzed to phosphine oxide in water (Fig. 9c, Haake 1972). Xian’s lab has employed such phosphine-mediated reactions to selectively modify S-nitrosothiols. In these studies, an aza-ylide nucleophile is proposed, and in the presence of an intramolecular electrophilic group (such as an ester), the S-nitrosothiols can be converted into a sulfenamide by intramolecular acyl transfer (Wang & Xian 2008). Although the resulting sulfenamide is more stable than the S-nitrosothiol, it is not suitable for RS-NO detection in proteins. In particular, the sulfenamide is reduced to the thiol in the presence of excess phosphine reagent. Consequently, Xian’s research group has developed triarylphosphines (30a) with a thioester as a trap for the aza-ylide intermediate (30b). This kind of reagent reacts with RS-NOs to generate a sulfenamide (30c), which immediately undergoes nucleophilic attack of the thiol leaving-group (formed after the intramolecular acyl transfer) to yield a
disulfide (30d), which is still prone to reduction, but is more stable than an S-N bond. This approach appears promising and has been used to detect protein RS-NOs in cell extracts (Zhang et al. 2010). However, the relative instability of the disulfide link remains as a significant challenge for robust application of this approach in vitro and especially in cells.

Several other phosphine reagents have been developed to detect RS-NOs (see the review: Wang & Xian 2011). King’s group reported the first water-soluble phosphine capable of direct labeling of S-nitrosothiols (Bechtold et al. 2010). Tris(4,6-dimethyl-3-sulfonatophenyl)-phosphine trisodium salt hydrate (TXPTS) appears to form a stable sterically hindered S-alkylphosphonium adduct, which can be detected by $^{31}$P-NMR. However, the selectivity of all these reagents must be carefully investigated. In fact, disulfides (see Section 4.3) and sulfenic acids (see Section 4.1) are also reduced by phosphines. On the other hand, triarylphosphines do not appear to be fast disulfide-reducing agents (Saxon & Bertozzi 2000) and the presence of electron-withdrawing substituents should further reduce their reactivity (Wang & Xian 2008). In contrast, Davis’s group has shown that a disulfide formed between a short thio-peptide and methyl 4-thio-2-nitrobenzoate reacts very rapidly (~15 min) with several phosphines, including triphenylphosphine (Chalker et al. 2011). This reaction can be explained by the low pKa of methyl 4-thio-2-nitrobenzoate, which works as an excellent leaving group during the phosphine attack. Along these lines, some disulfides could exhibit cross-reactivity with the phosphine reagents developed for RS-NOs detection (Bechtold et al. 2010). Finally, cross-reactivity with sulfenic acids remains poorly understood. Although the selectivity of triarylphosphine probes for RS-NOs detection over sulfenic acids was recently suggested (Li et al. 2012), however, the use of PTB1B as a model appears contradictory. In fact, when PTB1B is exposed to $\text{H}_2\text{O}_2$ the catalytic cysteine residue forms a stable, cyclic sulfenamide (Salmeen et al. 2003), which is predicted to be less reactive toward phosphines.

4.3 Disulfides
The formation of a disulfide bond between two cysteine residues or between a cysteine residue and a low-molecular-weight thiol may also have a significant impact on the structure and function of the macromolecule (Fan et al. 2009; Wouters et al. 2010). Protein disulfide generation is generally mediated by sulfenic acid formation (Eq. 25, see Section 4.1) or by thiol-disulfide exchange (Eq. 26). Thiols can also react with sulfenyl halides (Eq. 27), sulfenyl thiocyanates (Eq. 28), and thiosulfinate esters (Eq. 29) to form disulfide. Moreover, the reaction of S-nitrosothiols (Eq. 30) with thiols can sometimes proceed with release of nitroxyl (HNO) and disulfide formation (Hogg 2002).

\[
\begin{align*}
R-S-OH + R_1-S^- & \rightarrow R-S-S-R_1 + OH^- \\
R-S-SR_1 + R_2-S^- & \rightarrow R-S-S-R_2 + R_1-S^- \\
R-S-X + R_1-S^- & \rightarrow R-S-S-R_1 + X^- \\
R-S-SCN + R_1-S^- & \rightarrow R-S-S-R_1 + SCN^- \\
R-S-S(O)R_1 + R_2-S^- & \rightarrow R-S-S-R_2 + R_1-S-O^- \\
R-S-NO + R_1-S^- & \rightarrow R-S-S-R_1 + NO^- 
\end{align*}
\]

Thiol-disulfide exchange is one of the most important sulfur-based reactions in biology and can regulate the structure and activity of proteins that contain regulatory cysteines (Ilbert et al. 2007). Although this reaction is spontaneous, it can also be catalyzed by transition metals (Arisawa & Yamaguchi 2008). The driving force of the reaction is the relatively low activation energy required for thiols to break disulfide bonds (Fernandes & Ramos 2004). The thiol–disulfide exchange reaction is a bimolecular nucleophilic substitution reaction (SN2) in which the attacking nucleophile is the thiolate. The reaction proceeds with a linear transition state and this geometry has a significant impact on the reaction rate. Consequently, in proteins, cysteines that are situated in a position in which a linear transition state is difficult to achieve will be much less reactive.

The rate of protein thiol–disulfide exchange reactions is influenced by several additional factors. These include the pKa of the thiolate and the leaving-group thiol, nearby charged amino acid residues, and steric factors. When a thiolate anion attacks an asymmetrical disulfide (R1S-SR2), the best leaving group will be the thiol with lowest
pKa (Jensen et al. 2009). Moreover, since thiol-disulfide exchange involves negatively charged species, the reaction rate can be influenced by electrostatic factors, such as negative charges adjacent to the reaction center (Bulaj et al. 1998). The formation of an intramolecular disulfide is typically more efficient than the formation of intermolecular disulfides. Consequently, the incubation of disulfide with dithiothreitol (DTT) always leads to the oxidation of DTT. In biology, thiol-disulfide exchange is the basis of the mechanism of action of many enzymes. Another thiol/disulfide exchange process that deserves special consideration is S-glutathionylation of cysteine in proteins. Although S-glutathionylation was thought to protect cysteine from irreversible oxidation (Thomas et al. 1995), it was later shown that this modification affects the catalytic activity for several enzymes, suggesting a regulatory role (Demai et al. 2003).

Disulfides also react with strong oxidants to yield thiosulfinate and thiosulfinate esters. However, this reaction is significantly slower than oxidation of a thiol or sulfenic acid and thus, would take place only at extremely elevated, cytotoxic concentrations of ROS/RNS. Disulfides are reduced to thiols by a number of inorganic compounds, including sodium borohydride (NaBH₄) and sodium cyanoborohydride (NaBH₃CN). In biochemistry, dithiols and phosphite esters are the most common reducing agents. It has been demonstrated that sodium ascorbate can also reduce a disulfide (Holmes & Williams 2000), but this reaction appears to be catalyzed by Copper(II). Chemical approaches to the detection of disulfides are largely indirect. The initial step consists of blocking free thiols with an irreversible alkylating agent (see Section 2), followed by a disulfide reduction (e.g., with tris(2-carboxyethyl)phosphine - TCEP). Finally, the nascent thiols are trapped with the usual reagents (see Section 2). Two important considerations are: 1) owing to a pKa of ~8 for most thiols, the blocking step must be carried out at neutral pH or higher for this reaction to be efficient; 2) commonly used reducing agents also reduce other reversible cysteine oxoforms, such as sulfenic acids, nitrosothiols, sulfenamides, and thiosulfinates. Direct detection methods exist only for S-glutathionylation (RS-SG), which can be monitored through reduction of this bond by glutaredoxin (Lind et al. 2002), radiolabeled with ³⁵S-GSH (Fratelli et al. 2002) or biotinylated glutathione ethyl ester (BioGEE, Sullivan et al. 2000). Alternately, an immunochemical approach using
specific antibodies against the protein-glutathione adduct may be employed (Dalle-Donne et al. 2003).

4.4 Sulfenamide

A sulfenamide is a compound containing a trivalent nitrogen atom bonded to divalent sulfur and is formally derived from the condensation of sulfenic acid with an amine (Allison et al. 1973). Although a sulfenic acid and sulfenyl halide may react with any nucleophilic amine to generate a sulfenamide, in general, this reaction is several orders of magnitude slower than the equivalent reaction with a thiol. Nonetheless, interest in such modifications has grown since the discovery of cyclic sulfenamide formation in the active site of protein tyrosine phosphatase 1B (PTP1B) (Salmeen et al. 2003). The sulfenic acid intermediate produced by oxidation of the PTP1B catalytic cysteine is rapidly converted into the sulfonamide form. The crystalline structure of oxidized PTP1B reveals that the sulfenamide is characterized by an isothiazolidinone ring formed by the binding of sulfur of the cysteine to the backbone nitrogen of the adjacent serine residue (van Montfort et al. 2003). This species appears to be generated by nucleophilic attack of the main-chain amide nitrogen on the electrophilic sulfur in sulfenic acid with elimination of water. Beyond PTP1B, few other proteins have been found to form cyclic sulfonamide modifications (Lee et al. 2007) and, as a result, it is difficult to say whether such modification has a wider role in biology.

Sulfenamides appear to have similar a reactivity profile akin to sulfenic acid and undergo attack by several nucleophilic species. For example they react with thiols to yield disulfides (Fig. 10a) and, consequently sulfenamide formation may mediate the S-glutathionylation of proteins such as PTP1B (den Hertog et al. 2005). Sulfenamides can also undergo trans-amination reactions (Craine & Raban 1989); considering that the only biologically relevant sulfenamide known to date is cyclic, however, this reaction is unlikely occur in cells because the equilibrium of the reaction is always shifted toward the cyclic form. Sulfenamides can be reduced to thiols by phosphite esters, which also react with disulfides, sulfenic acids, and S-nitrosothiols. The cyclic sulfonamide within
PTP1B is often cited for its ability to protect the reactive thiol against irreversible oxidation to sulfinic and sulfonic acid. This statement can be rationalized by the lesser nucleophilic character of the sulfur atom in the cyclic sulfonamide as compared to the sulfinic acid. In aqueous basic solutions of H₂O₂, the sulfinamide is readily oxidized to sulfinamide and sulfonamide (Kharasch et al. 1946), but at physiological pH such oxidation is negligible. In fact, although sulfinamide formation has been reported in short peptides exposed to ROS (Shetty et al. 2009), no evidence of sulfinamide or sulfonamide formation has been observed in PTP1B. This is true even in the presence of high H₂O₂ concentrations, as PTP1B exclusively undergoes sulfinic and sulfonic acid modification, ascribed to the direct oxidation of the cysteine sulfinic acid intermediate (Held et al. 2010).

The development of specific probes for sulfinamides is made more difficult by the similarity with sulfinic acid reactivity (Fig. 10b). Shiau et al. have used a series of relatively weak nucleophilic thiols to identify compounds that react with sulfinamides, but not disulfides (Fig. 10). They identified a coumarin thioacid (32), which reacts with a model sulfinamide (31) to generate the fluorescent disulfide (33) and exhibits no cross-reactivity with disulfides (Shiau et al. 2006). Unfortunately, this study provided no data regarding reactivity of 32 with sulfinic acid; nonetheless, it is almost certain that the coumarin-derivative should also react with sulfinic acids and related thiosulfinites.

4.4 Thiosulfinate ester

Thiosulfinate esters can be formed by the condensation of two sulfinic acids (see Chapter 4.1) or by oxidation of disulfides (Eq. 31). In a biological context, the auto-condensation of sulfinic acids appears to have poor relevance in proteins owing to steric hindrance, while it should be quite likely for small molecules such as cysteine or glutathione. The direct oxidation of disulfide to the thiosulfinate would require very high, localized concentrations of ROS/RNS and only biologically relevant for glutathione in its oxidized state (Giles et al. 2002). Interestingly, the thiosulfinate group forms as a reaction intermediate during sulfiredoxin (Srx)-mediated reduction of cysteine sulfinic acid in
Prxs; however, this modification is generated through a completely different mechanism (see Section 4.5).

The thiosulfinate ester is a highly reactive species and can readily undergo the nucleophilic attack by thiols to yield a disulfide and a sulfinic acid (Nagy et al. 2007). Glutathione disulfide S-monoxide has consequently been proposed as a mediator in S-glutathionylation (Huang et al. 2007). Thiosulfimates can also undergo hydrolysis (Eq. 32), a reaction that is in equilibrium with self-condensation of sulfinic acids (Nagy & Ashby 2007). Formally, thiosulfimates can be oxidized to sulfonate esters (Eq. 33), but this should take place only in the presence of high concentrations of very strong oxidants such as those found in the phagosome. Finally, disproportionation of aromatic thiosulfinate esters has been reported to yield a disulfide and a thiosulfonate ester via a complex radical mechanism (Eq. 34, Poudrel & Cole 2001).

$$R\text{-}S\text{-}SR + H_2O_2 \rightleftharpoons R\text{-}S\text{-}S(O)R + H_2O$$ (31)

$$R\text{-}S\text{-}S(O)R + H_2O \rightleftharpoons 2R\text{-}S\text{-}OH$$ (32)

$$R\text{-}S\text{-}S(O)R + H_2O_2 \rightarrow R\text{-}S\text{-}S(O)_2R + H_2O$$ (33)

$$2R\text{-}S\text{-}S(O)R \rightarrow RS\text{-}S\text{-}R + R\text{-}S\text{-}S(O)_2R$$ (34)

Except for the Prx-thiosulfinate intermediate, no other thiosulfimates are known in proteins. This may be due, at least in part, to the difficulty of finding a selective strategy for detecting this thiol modification. The thiosulfinate ester, like a thiosulfonate ester (Schank et al. 2007) may react with dimedone (and its derivatives) to yield a dimedone-thioether and a sulfinic acid, which in turn reacts with a second molecule of dimedone. Rabinkov’s group has developed a simple spectrophotometric assay to determine the presence of allicin (a natural thiosulfinate ester present in garlic) and of allinase activity by the reaction between 4-mercaptopypyridine (4-MP) and allicin (Miron et al. 2002). The selectivity of this technique is based on the high reactivity of the thiosulfinate in comparison to the disulfide, but its application with proteins in complex biological samples appears since 4-MP should also react with the more abundant sulfinic acid.
4.6 Sulfinic acid

Sulfinic acid can be further oxidized to sulfinic acid (RSO$_2$H) in the presence of excess ROS/RNS (see Section 4.1). RSO$_2$H is a relatively stable species as compared to RSOH, and with a pK$_a$ value of ~2, is always deprotonated at physiological pH (Burkhard et al. 1959). Sulfinic acids can be also generated by disproportionation of sulfenic acid (Eq. 35, Abraham et al. 1983) or by nucleophilic displacement of thiosulfonate esters (Eq. 36-37, Harpp et al. 1979).

\[
2 \text{R-S-OH} \rightarrow \text{R-SH} + \text{RS}_2\text{OH} \quad (35)
\]
\[
\text{R-S-S(O)R}_1 + \text{HO}^- \rightarrow \text{RSO}_2^- + \text{R}_1\text{-S-OH} \quad (36)
\]
\[
\text{R-S-S(O)R}_1 + \text{R}_2\text{-S}^- \rightarrow \text{RSO}_2^- + \text{R}_1\text{-S-S-R}_1 \quad (37)
\]

In the deprotonated form, the sulfinate group (RSO$_2^-$) is an ambident anion and the negative charge is delocalized between the sulfur and the two oxygen atoms. RSO$_2^-$ behaves mainly as a soft nucleophile (Reddie & Carroll 2008) and can react with many electrophilic species such as halides (Fig. 11a) (Thamis et al. 2010) and $\alpha,\beta$-unsaturated compounds (Fig. 11b) (Ogata et al. 1970). Sulfinic acids can therefore be alkylated by iodoacetamide as well as ethylmaleimide, although these reactions are slower in comparison to thiols. In all these reactions, S-attack is generally favored and leads to the thermodynamically more stable sulfone. In the presence of strong electrophiles, the sulfonyle ester can be kinetically generated (Baidya et al. 2010), but this unstable species slowly rearranges to a sulfone (Fig. 11a). Sulfinic acids can oxidize further to sulfonic acid, but neither of these oxoforms can be reduced directly by thiols, hence the term “irreversible”. In recent years, a biological role for RSO$_2$H has emerged, for example, in both Parkinson’s disease protein DJ-1 (Blackinton et al. 2009) and matrilysin (MMP-7) activation (Fu et al. 2001), although the best-known cysteine sulfinyl modification is occurs within the Prx family (Wood et al. 2003). Finally, the discovery of sulfiredoxin, an ATP-dependent protein that specifically reduces sulfinic acid in Prx, has opened the
door to an additional layer of redox regulation (Jönsson et al. 2008) and increased interest in detecting this specific modification.

Antibodies against individual proteins with sulfinyl/sulfonyl modifications have been developed (see Section 4.7). At the moment, however, no selective chemical probes are available to detect sulfinic acid formation in proteins. On the basis of early physical-organic studies (Ritchie et al. 1961), the use of diazonium salts (Fig. 11c, 34) as a trapping agent for protein sulfinic acids was recently proposed (Jacob & Ba 2011). Although this approach has proven to be effective for colorimetric detection of methane sulfinic acid (Babbs & Gale 1987), several complications are present in this system. Diazonium salts are highly unstable species that readily decompose under neutral aqueous conditions. Moreover, considering the potential cross-reactivity with tyrosine (Hooker et al. 2004), as well as with cysteine (Patt & Patt 2002), future application of this technique with proteins may be limited. Whereas avoiding basic conditions can minimize cross-reactivity with tyrosine, cysteine appears to react with diazonium salts under a wide range of pHs (Eq. 38) to yield a stable sulfenyldiazenic species (Lo Conte & Carroll, unpublished data).

$$\text{R-SH} + \text{Ar-N}_2^+ \rightarrow \text{R-S=Ar} + \text{H}^+$$ (38)

As an alternative, the Carroll research group has been working to developing a new selective ligation strategy using C-nitroso compounds, which condense with sulfinic acids to yield N-sulfonyl hydroxylamines (Fig. 11d). N-sulfonyl hydroxylamines are unstable at neutral or basic pH (Darchen & Moinet 1976). As a result, in order to convert the product into a stable adduct, we can take advantage of the nucleophilic behavior of hydroxylamine. In the presence of an electrophilic center (e.g., a carboxylic ester) on the C-nitroso compound (35a), N-sulfonyl hydroxylamine (35b) undergoes intramolecular cyclization to form a stable N-sulfonyl benzisoxazolone adduct (35c). Although thiols can also target the C-nitroso group, selectivity for sulfinic acid is ensured by the fact that reaction with the thiol leads to an unstable N-sulfenyl hydroxylamine linkage, which is readily cleaved in the presence of additional thiols; this chemical approach is currently
under being adapted for detection of protein sulfinic acids *in vitro* (Lo Conte & Carroll, unpublished data).

4.7 Sulfonic acid

Sulfonic acid represents the highest oxidation state for the cysteine sulfur atom and no biological pathway is known to reduce this cysteine oxoform. Sulfinic acid can be further oxidized to sulfonic acid by strong oxidizing species such as peroxynitrous acid and hydrogen peroxide (Eq. 39, see Section 4.6). Sulfonic acid can also be generated by the disproportionation of sulfinic acid (Eq. 40). As alluded to above, when an essential enzyme active site cysteine is oxidized to sulfonic acid the catalyst is irreversibly inhibited. However, it has also been proposed that sulfonic acid modification of Prx could enhance its proposed chaperone activity (Lim *et al.* 2008). This modification may also target proteins for degradation. For example, the oxidation of N-terminal cysteine residues to sulfonic acid can mediate arginylation and degradation of GTPase-activating proteins (Tasaki & Kwon 2007).

\[
R-\text{SO}_2\text{H} + \text{H}_2\text{O}_2 \rightarrow R-\text{SO}_3\text{H} + \text{H}_2\text{O} \quad (39)
\]
\[
4 R-\text{SO}_2\text{H} + \text{H}_2\text{O} \rightarrow \text{RSO}_3\text{H} + R-\text{S(O)}_2\text{S}-R \quad (40)
\]

Sulfonic acid is one of the strongest organic acids. Cysteic acid, with a pKa of –3, is always present in the deprotonated form as sulfonate at physiological pH (Chang *et al.* 2010). Sulfonic acid is a very poor nucleophile and its derivatives are usually obtained by activation as the sulfonyl chloride. No facile chemical methods are available to trap and tag the sulfonic acid modification in proteins. Although base-mediated β-elimination of cysteine sulfonic acid to dehydroalanine and sulfite could provide a starting point for assay design (Dai *et al.* 2005), the concomitant β-elimination of phosphoserine and phosphothreonine (McLachlin & Chait 2003) represents a serious limitation of such an approach. To date, antibodies are the most common technique for detecting the sulfonic acid form of a specific protein (Woo *et al.* 2003). Unfortunately, this approach is not
easily applied to all proteins, and the resulting antibodies typically exhibit the same affinity for sulfinic and sulfonic acid cysteine oxoforms. Recently, an innovative mass spectroscopy assay was developed to selective enrich and identify peptides containing cysteine sulfonic acid. This approach is based on ionic affinity capture using polyarginine-coated nanodiamonds that exhibit good specificity even in the presence of phosphopeptides (Chang et al. 2010). The technique was applied to selectively enriched sulfopeptides obtained from tryptic digests of over-oxidized BSA.

4.4 Sulphydration

The process by which H₂S mediates S-sulphydration of proteins is still unclear. For example, the direct reaction of cysteine side chains with H₂S proposed by Snyder’s research group (Mustafa et al. 2011) appears quite unlikely without any intermediary oxidant species. Persulfides can be generated by the direct reaction of sulfenic acids (Kabil & Banerjee 2010), sulfenyl halides, sulfenyl-thiocyanates, or thiosulfenyl esters with H₂S (Eq. 41-44). Although less reactive, disulfides can also generate persulfides via disulfide exchange with H₂S (Eq. 45). Finally hydrogen persulfide (HSSH) has recently been proposed as the physiological sulphydration agent in cells (Nagy & Winterbourn 2010). HSSH is generated by reaction of H₂S with two-electron oxidants and can easy react with low pKa thiols (Eq. 46).

\[
\begin{align*}
R\text{-SOH} + HS^- & \rightarrow R\text{-S}^+ + H_2O & \text{(41)} \\
R\text{-S-X} + HS^- & \rightarrow R\text{-S-S}^- + HX & \text{(42)} \\
R\text{-S-SCN} + HS^- & \rightarrow R\text{-S-S}^- + HSCN & \text{(43)} \\
R\text{-S-S(O)R}_1 + 2 HS^- & \rightarrow 2 R\text{-S-S}^- + H_2O & \text{(44)} \\
R\text{-S-S-R}_1 + HS^- & \rightarrow R\text{-S-S}^- + R_1S^- & \text{(45)} \\
R\text{-S}^- + HS\text{-SH} & \rightarrow R\text{-S-S}^- + HS^- & \text{(46)}
\end{align*}
\]

Persulfides show intermediate behavior between thiol and disulfide reactivity. In fact, the terminal sulfur is ambiphilic and can behave as an electrophile or a nucleophile. For example, persulfides can undergo oxidation reactions to yield a wide range of
products or may also undergo disulfide exchange (Eq. 47-48), following the same rules described in Section 4.3. If the internal sulfur atom of a persulfide group has a pKa lower than 6.9 (corresponding to the pKa of hydrogen sulfide), the reaction may proceed via trans-sulfhydration; alternately, the result is disulfide formation and the release of hydrogen sulfide. The terminal sulfur, however, can also directly attack a disulfide or react with a sulfenic acid to generate a trisulfide (Eq. 49-50). Trisulfides are widely distributed in the biological world, although they are not a common post-translational modification, and the number of proteins in which a trisulfide has been unambiguously identified is small (Nielsen et al. 2011).

\[
\begin{align*}
R-S-SH + R_1S^- & \rightarrow R-S-S-R_1 + HS^- \quad (47) \\
R-S-SH + R_1S^- & \rightarrow R_1-S-S^- + RS^- \quad (48) \\
R-S-S^- + R_1-S-S-R_1 & \rightarrow R-S-S-S-R_1 + R_1S^- \quad (49) \\
R-S-S^- + R_1-S-OH & \rightarrow R-S-S-S-R_1 + OH^- \quad (50)
\end{align*}
\]

In the presence of transition metals, persulfides can readily decompose to generate ROS, and such reactions could mediate the anti-microbial and anti-cancer properties of some natural products (Chatterji et al. 2005). The terminal sulfur is a strong nucleophile and can react with a huge range of electrophiles. The richness of its chemistry makes the persulfide group a versatile reagent for the incorporation of sulfur along many metabolic pathways (Mueller 2006).

In the last decade, interest in H$_2$S signaling has grown rapidly, but relatively few proteins have been found to be sulphydrates (Krishnan et al. 2011). This is due to the difficulty of monitoring such modifications uniquely. The Snyder research group has reported an assay for proteins persulfides, which is a modification of the classic biotin switch assay (Mustafa et al. 2009). In this method, MMTS is employed to block free thiols and any persulfides are reacted with biotin-HPDP (possibly forming a disulfide or trisulfide linkage) and analyzed by Western blot. Applying this approach to complex cell lysates, several proteins were reported as targets of H$_2$S signaling. A key feature of this approach requires that the MMTS reagent only modify thiols and not persulfides. Unfortunately, no rationale or data was provided by Mustafa et al. to validate this claim.
Therefore, considering that the chemical mechanism and selectivity remain unknown, results obtained using this approach should be interpreted with caution.

5. Conclusions

In the last decade, awareness of the number of proteins containing redox-sensitive cysteine has grown significantly. Cysteine can assume a wide range of oxidative states in response to dynamic changes in intracellular redox potentials. To understand the role of such protein modifications, methods have been developed to distinguish between different cysteine oxidation states. The role of the biologist is not simply to choose the most appropriate technique, but also to maintain a critical view of possible artifacts. Opting for an in vitro approach can prove unsatisfactory because many cysteine oxoforms are reactive and unstable outside of the cellular milieu. The use of indirect methods, by which all free thiols are trapped, must be preceded by the awareness that these thiol-trapping agents can react inefficiently or be incompletely selective, resulting in under- or over-estimation. The same considerations must be taken into account when using reducing agents to identify specific modifications. Indeed, the selectivity and efficiency of such reagents requires careful evaluation. Furthermore, in many cases, interconversion of cysteine redox states dramatically increases the challenges of studying an individual modification. Finally, given that the local microenvironment can exert a remarkable influence on protein thiolate reactivity, the exploration of probes and chemical methods exclusively in low-molecular-weight model systems may prove unsatisfactory. Although more complex, a recommended course for future research is the identification of selective reactions that can be employed to monitor cysteine oxidation in situ, directly in cells. Obviously, this presents a formidable challenge, but given the vast number of biological processes that thiol oxidation plays a significant regulatory role, it should be well worth the effort.

6. References


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Figure 1. Chemical reactivity and detection of thiols.

**Thiol reactivity**

(a) Disulfide exchange

\[ R-S^- + R'-S \xrightleftharpoons{catalyst} R-SR' + R'-S^- \]

(b) Reaction with thiosulfonate esters

\[ R-S^- + R'-S \xrightleftharpoons{catalyst} R-SR' + O^\cdot \]

(c) Reaction with halo-compounds

\[ R^- + X \xrightarrow{halo-compound} R^- + X^- \]

(d) Reaction with Halonitrobenzene derivatives

\[ \text{Halonitrobenzene} \xrightarrow{halo-compound} \text{product} \]

(e) Michael addition

\[ R-S^- + \text{Michael acceptor} \xrightarrow{catalyst} R-SR' \]

(f) Reaction with 2-halo-1,3-diketone

\[ R-S^- + \text{2-halo-1,3-diketone} \xrightarrow{catalyst} \text{product} + X^- \]

**Probes for thiol trapping and imaging**

- **Biotin-HPDP**
- **2**
- **3**
- **4** \( R = H \)
- **5** \( R = (\text{CH}_3)_3\text{N} \)
- **7a** \( \lambda_{\text{max}} 347 \text{ nm} \)
- **8**
- **9**
- **10** \( R = \text{N}_3 \)
- **11** \( R = \cdot \text{CH} \)
Figure 2. Probes for the detection of hydrogen peroxide.

Oxidation reactions of $\text{H}_2\text{O}_2$

(a) Boronate oxidation

(b) Decarboxylation of $\alpha$-ketoacids

(c) Baeyer-Villiger benzyl oxidation

Selective probes for $\text{H}_2\text{O}_2$ imaging

13a Non-fluorescent

13b Fluorescent

14a $\text{H}_2\text{O}_2$

14b Different NMR spectral properties

15a Non-fluorescent

15b Fluorescent
Figure 3. Probes for the detection of hypochlorous acid.

**Oxidation reactions of HOCl**

(a) Oxidation of $p$-methoxyphenol

\[
\text{HOCl} + \text{H}_2\text{CO} \rightarrow \text{CH}_2\text{OH} + \text{HCl}
\]

(b) Oxidation of hydroxamic acid

\[
\text{H}_2\text{O} \rightarrow \text{RNO}_2 + \text{H}_2\text{O} + \text{HCl}
\]

(c) Oxidation of thioether to sulfone

\[
\text{R-S-R} \rightarrow \text{R-S-O} + \text{HCl}
\]

**Selective probe for HOCl imaging**

16a Non-fluorescent

16b Fluorescent

17a Non-fluorescent

17b Fluorescent

17c Fluorescent

18a Non-fluorescent

18b Fluorescent

18c Fluorescent
Figure 4. Probes for the detection of nitric oxide and peroxynitrite.

**Bioorthogonal reactions for NO and HONO<sub>2</sub>**

(a) Nitrosation of 1,2-diphenylenediamine

\[
\begin{align*}
\text{NH}_2\text{NH}_2 & \xrightarrow{\text{NO}} \text{NH}_2\text{NH}_2^+ \\
\text{NH}_2\text{NH}_2^+ & \xrightarrow{\text{H}_2\text{O}} \text{NH}_2\text{NH}_2
\end{align*}
\]

(b) Peroxynitrite-mediated oxidation of phenol-derived ketone

**Selective probes for NO and HONO<sub>2</sub> imaging**

- **19a**
  - Non-fluorescent
  
- **19b**
  - Fluorescent

- **20a**
  - Non-fluorescent

- **20b**
  - Fluorescent
Figure 5. Probes for the detection of hydrogen sulfide.

### Bioorthogonal reactions for H$_2$S

(a) Reduction of azide

\[ R-N_3 + H_2S \rightarrow R-NH_2 + N_2 \]

(b) Disulfide exchange

\[ R-S-S-R' + H_2S \rightarrow R-SH + R'SH \]

(c) Michael addition

\[ \text{acyl} + H_2S \rightarrow \text{acyl-SH} \]

### Selective probes for H$_2$S

- **21a** Non-fluorescent
- **21b** Fluorescent
- **22a** Non-fluorescent
- **22b**
- **22c** Fluorescent
- **23a** Non-fluorescent
- **23b**
- **23c** Fluorescent
Figure 6. Biologically relevant cysteine oxidation states. Oxidation number correspond to sulfur atom denoted in red.
Figure 7. Sulfenic acid reactivity: S atom as a nucleophile.

(a) Self-condensation
\[ \text{R}S\text{SOH} \xrightarrow{\Delta} \text{R-S}^\text{O} \text{H} \]

(b) Oxidation to sulfinic acid
\[ \text{R-S}^\text{O} \text{H} \xrightarrow{\Delta} \text{R-S}^\text{O} \text{OH} + \text{H}_2\text{O} \]

(c) Addition to alkenes
\[ \text{R-S}^\text{O} \text{H} \xrightarrow{\Delta} \text{R-S}^\text{O} \text{H} \]
(organic solvent)

(d) Addition to activated alkynes
\[ \text{R-S}^\text{O} \text{H} \xrightarrow{\Delta} \text{R-S}^\text{O} \text{OMe} \]
(organic solvent)

(e) Reaction with halo-compounds
\[ \text{R}^\text{X} \xrightarrow{\Delta} \text{R-S}^\text{O} \text{H} + \text{HX} \]

(f) Reaction with Halonitrobenzene derivatives
\[ \text{R-S}^\text{O} \text{H} \xrightarrow{\Delta} \text{R-S}^\text{O} \text{NH}_2 + \text{HX} \]

4 \hspace{1cm} 24

7a \hspace{1cm} 25, \lambda_{\text{max}} 420 \text{ nm}
Figure 8. Sulfenic acid reactivity: S atom as an electrophile.

(a) Disulfide formation

\[
\text{R}_S\text{O}_H \xrightarrow{\text{SR}'} \text{R}^\cdot \text{S} \cdot \text{R}^\cdot + \text{H}_2\text{O}
\]

(b) Ascorbate reduction (postulated mechanism)

\[
\text{R}\text{S}^\cdot \text{O}_H + \text{HO}_3\text{O}_H \xrightarrow{\text{H}_2\text{O}} \text{R}_3\text{O}_H \xrightarrow{\text{H}_2\text{O}} \text{R}_3\text{O}_H^\cdot + \text{RSH}
\]

(c) Sulfenamide formation

\[
\text{R}_S\text{O}_H + \text{NH}_2\text{R}^\cdot \xrightarrow{\text{H}_2\text{O}} \text{R}^\cdot \text{S} \cdot \text{R}^\cdot + \text{H}_2\text{O}
\]

(d) Reaction with phophines

\[
\text{R}_S\text{O}_H + \text{PR}^\cdot \text{R}^\cdot \text{H}_2\text{O} \xrightarrow{\text{H}_2\text{O}} \text{R}_S\text{O}_H \xrightarrow{\text{H}_2\text{O}} \text{R}^\cdot \text{S} \cdot \text{R}^\cdot + \text{RSH}
\]

Probes for the detection of sulfenic acids

26a \( R = N_2 \) (DAz-2)
26b \( R = C\text{CCH} \) (DYn-2)
26c

27

28
Figure 9. Chemical reactivity and detection of S-nitrosothiols.

Reactions of RSNO: N as an electrophile

(a) Transnitrosylation

\[ \text{RSN}^\text{-} \rightarrow \text{RSH} + \text{RSN}^\text{O} \]  

(b) Ascorbate reduction

\[ \text{RSN}^\text{-} + \text{H}_2\text{O} \rightarrow \text{RSH} + \text{RSNO} \]

(c) Phospine reduction

\[ \text{RSN}^\text{-} + 2\text{PR}^\text{III} \rightarrow \text{RSN}^\text{P}^\text{III}\text{PR}^\text{III} + \text{PR}^\text{II}_2 \text{H}_2 \]

Methods for the detection of RSNO

Biotin switch assay

1) MMTS

2) ascorbate

S-alkylphosphonium adduct generation

\[ \text{RSNO} + \text{NaO}_{3}\text{S}_{3}\text{SO}_3\text{Na} \rightarrow \text{RS-P}^+ \]

Reductive ligation

\[ \text{RSNO} + \text{Ph} \rightarrow \text{RSNR}^\text{-} \]

\[ \text{HN} \rightarrow \text{NH} \]

\[ \text{HS}\rightarrow \text{SS} \]
Figure 10. Chemical reactivity and detection of sulfenamides.

(a) Disulfide formation

\[ R + R' \xrightarrow{S \cdot N \cdot R} SR' \rightarrow R'\cdot S\cdot R' + H \cdot R' \]

Methods for sulfenamide detection

\[ \text{BocHN} \cdot \text{N} \cdot \text{CO}_{2}\text{CH}_{3} \rightarrow \text{R} \cdot \text{S} \cdot \text{R'} \]

\[ \text{BocHN} \cdot \text{N} \cdot \text{CO}_{2}\text{CH}_{3} \rightarrow \text{R} \cdot \text{S} \cdot \text{R'} \]
Figure 11. Chemical reactivity and detection of sulfinic acids.

Reactions of sulfinic acids

(a) Addition to halo-compounds

(b) Michael addition

(c) Diazo-coupling

(d) Reaction with nitroso-compounds

Methods for RSO₂H detection

34a also forms a stable product with thioles
(see Eq. 38)

34b intensely colored