Annu. Rev. Pharmacol. Toxicol. 2004. 44:325–47
doi: 10.1146/annurev.pharmtox.44.101802.121735

First published online as a Review in Advance on November 10, 2003

# PROTEIN SULFENIC ACIDS IN REDOX SIGNALING

Leslie B. Poole,[1] P. Andrew Karplus,[2] and Al Claiborne[1]
[1]*Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157; email: lbpoole@wfubmc.edu, alc@invader.biochem.bgsm.edu*
[2]*Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331; email: karplusp@ucs.orst.edu*

**Key Words** cell signaling, cysteine, redox center, oxidation, sulfinic acid

■ **Abstract** Reactive (low $pK_a$) cysteine residues in proteins are critical components in redox signaling. A particularly reactive and versatile reversibly oxidized form of cysteine, the sulfenic acid (Cys-SOH), has important roles as a catalytic center in enzymes and as a sensor of oxidative and nitrosative stress in enzymes and transcriptional regulators. Depending on environment, sometimes the sulfenic acid provides a metastable oxidized form, and other times it is a fleeting intermediate giving rise to more stable disulfide, sulfinic acid, or sulfenyl-amide forms.

## INTRODUCTION

The description of the *oxyR* regulatory network by Christman et al. (1) in 1985 represents one of the true hallmarks of the principle of redox signaling. In the nearly two decades that have passed since that report, we have followed the developments in bacterial physiology, genetics, and biochemistry (see Reference 2 for a recent review), and more recently in yeast (3), describing in greater detail the molecular mechanisms involved in cellular adaptations to $H_2O_2$-mediated oxidative stress. The *oxyR* regulons in *Salmonella typhimurium* and *Escherichia coli* are now complemented by the $H_2O_2$ "stimulon" in *Saccharomyces cervisiae*, and one focus of this review is on the roles of cysteine sulfenic acids (Cys-SOH) in the regulation of specific transcription factors (OxyR, OhrR, and Yap1, respectively) in response to $H_2O_2$-mediated redox challenges. Rhee and others (4, 5) have extended this interpretation of $H_2O_2$ as a cellular threat to one in which $H_2O_2$ may represent a key signaling molecule in mammalian systems; protein phosphorylation appears to be modulated through $H_2O_2$-mediated cysteine thiol (Cys-SH) oxidation. Stamler & Hausladen (6) have proposed a continuum of NO- and $H_2O_2$-mediated Cys-SH modifications (primarily oxidations) that constitute important biological signaling events on the one hand and irreversible hallmarks of oxidative stress on the other. The highly reactive and reversible nature of the Cys-SOH modification gives it a unique suitability to $H_2O_2$ and other redox signaling pathways; its susceptibility, unlike protein disulfides, to irreversible oxidation (to cysteine sulfinic and

cysteine sulfonic acids, Cys-$SO_2H$ and Cys-$SO_3H$, respectively) also renders it labile to "oxidative stress."

In addition to well-defined (as well as controversial in some instances) roles in redox regulation of gene expression, Cys-SOH also serve as a novel class of protein-derived redox cofactors in specific antioxidant enzymes, such as the bacterial NADH peroxidase and the peroxiredoxins (Prxs) that have been characterized in bacterial, yeast, and mammalian systems (7). The AhpC Prx from *S. typhimurium* is regulated transcriptionally by OxyR and thus gives us a multidimensional view of Cys-SOH function: first, at the level of the $H_2O_2$-sensing transcription factor and, second, at the level of the $H_2O_2$-scavenging enzyme. Recent structural studies of the Prxs (8), which are another major focus of this review, suggest a molecular basis for their further functional evolution as "scavenging" versus signaling proteins. The yeast Yap1-Gpx3 system represents yet another exciting development in which a catalytic scavenging enzyme plays a direct role in transcriptional regulation of the $H_2O_2$ stimulon (9).

The chemical properties of sulfenic acids have been covered extensively in earlier reviews (10–12), as has the biochemistry of Cys-SOH (12–14). This review places an increased emphasis on recent and/or current developments and questions, focusing on enzymes that utilize Cys-SOH cofactors in catalyzing reductions of $H_2O_2$, ROOH, peroxynitrite and methionine sulfoxide, and on three specific transcription factors that clearly involve Cys-SH oxidations in mediating the respective cellular response to oxidative stress. Overall, there is an emphasis on relating the various biochemical, biological, and genetic lines of evidence for Cys-SOH involvement to the theme of redox signaling.

## SULFENIC ACID FORMATION AND REACTIVITY

Sulfenic acid formation in proteins is chiefly recognized to ensue as the direct product of the reaction of cysteine thiol(ate)s with hydrogen peroxide ($H_2O_2$), although alkyl hydroperoxides, peroxynitrite, and hypochlorous acid may all play a role in cellular sulfenic acid formation (15–17). Reactivity of particular protein thiols toward peroxides and other oxidants is strongly dependent on their ionization state, as thiolates are far more nucleophilic than their protonated counterparts. Still, additional features are important, as documented reaction rates of $H_2O_2$ with thiolate anions range from $\sim 10\ M^{-1}\ s^{-1}$ for small molecule thiols and protein tyrosine phosphatases (18, 19) to $\sim 10^6\ M^{-1}\ s^{-1}$ for cysteine-dependent peroxidases (20–22) and the transcription factor OxyR (23). This protein environment effect contributes to the specificity required for the participation of specific cysteine residues in cell signaling.

Four other less common reactions may also lead to protein sulfenic acid formation. The first example is the hydrolysis of S-nitrosothiols (R-SNO) (Figure 1) (6, 24–26). Second, protein disulfide bonds may be hydrolyzed through an enzyme-facilitated mechanism (to generate one thiol and one sulfenic acid), as has been

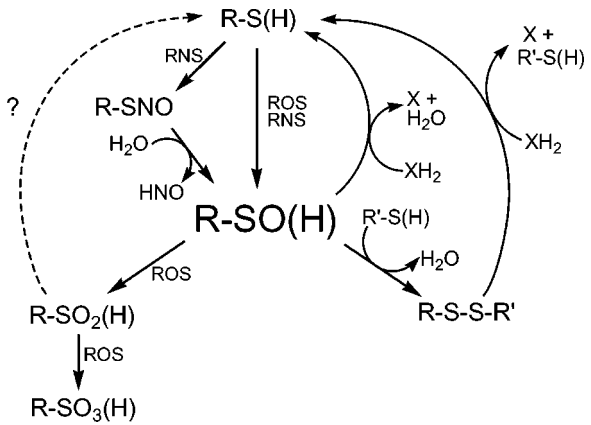



**Figure 1** Biological fates of cysteine sulfenic acids. $XH_2$ refers to chemical or biological reductants such as dithiothreitol, glutathione, or reduced, thiol-containing proteins such as thioredoxin and glutaredoxin. Note that R′-SH could also represent glutathione.

proposed to account for the production of angiostatin fragments (27). Third, protein disulfide bonds can be oxidized in vitro to the mono-oxide (thiosulfinate), which can react with a thiol group to generate a new disulfide bond and a protein sulfenic acid (28). Finally, thiyl radicals of protein cysteines formed in the presence of hydroxyl radicals can produce Cys-SOH, although the biological significance of this is unclear.

Once formed, Cys-SOH can go on to generate other forms of reversibly or irreversibly modified cysteinyl groups, or can be stabilized within the protein environment and recycled, usually via disulfide-bonded intermediates, back to the thiol state by cellular reductants such as Trx, Grx, and/or reduced glutathione (GSH). Thus, the propensity of sulfenic acids to condense with proximal thiol groups to form intra- or intermolecular disulfide bonds is the most important determinant of their fate. In this context, it is relevant to note that sulfenic acids may play an important role in glutathionylation, the covalent attachment of glutathione to protein cysteines through a mixed disulfide bond. Although its in vivo function is not well understood, protein glutathionylation is observed to a small degree in "normal" cells and to a greater extent in oxidatively challenged cells (29, 30). In principle, to generate glutathionylated proteins, either an oxidized glutathione (GSSG) can react with a protein thiol(ate) or an oxidized protein cysteine can react with GSH. Because glutathionylation in oxidative stress precedes any major change in the GSH/GSSG ratio, and GSH itself is relatively unreactive toward $H_2O_2$, the second pathway may be the more relevant (29–31). In this scenario, the buildup of the glutathionylated protein would be preferred when the environment of a protein sulfenic acid (i.e., steric and electronic factors) is such that it reacts readily with GSH to generate a mixed disulfide bond, but that this disulfide is then relatively stable toward reduction by a second GSH.

Besides disulfide bond formation followed by reduction, sulfenic acids may be further oxidized to the sulfinic ($R\text{-}SO_2H$) or sulfonic ($R\text{-}SO_3H$) acid states by action of some of the same oxidants. This "overoxidation" of the thiol group has been considered biologically irreversible, and in the case of the sulfonic acid (also known as cysteic acid), this is clearly true. This may yet be generally true for sulfinic acids; reduction of these species by chemical reductants requires a very low pH (<4) (32), and neither GSH nor thioredoxin can reduce them. Nonetheless, recent evidence suggests that rereduction of the sulfinic acid form of some human Prxs can occur in vivo (33a–33c). Details of the specificity and mechanism of this process remain unclear, however (see below).

An important theme to note is that some proteins follow more than one of the chemical pathways introduced above. The protein tyrosine phosphatases (PTPs) are a good example of this phenomenon. Although not discussed in detail in this review, there is evidence for inhibitory sulfenic acid formation at the catalytic cysteine of PTPs by $H_2O_2$ added exogenously or generated by mitogen activation of cells, and the reactivation of PTPs can occur through reduction by either GSH or reduced thioredoxin (4, 34). In an interesting twist, a low-molecular-weight PTP possesses a second cysteine proximal to the active-site thiol (35a). This cysteine thiol rapidly attacks the putative Cys-SOH to generate an intrasubunit disulfide bond as the oxidatively (reversibly) inactivated form of the enzyme. Thus, sulfenic acids in PTPs (*a*) are highly stabilized, (*b*) can be reduced via a glutathionylated intermediate, or (*c*) can form a protein disulfide bond prior to reductive reactivation. A novel fate for sulfenic acids discovered recently by crystallographic studies of reversibly oxidized PTPs is their conversion to a stable sulfenyl-amide (Cys-S-N-R), in this case formed on reaction of the nascent Cys-SOH with the main chain amide nitrogen of an adjacent Ser residue (35b, 35c). Formation of this species triggers conformational changes in the PTP catalytic site and is considered to both stabilize the oxidized cysteine toward overoxidation and facilitate its reduction by thiols. Adding still more diversity to the possible fates of protein sulfenic acids, another recent report suggests sulfinamide [Cys-S(O)-N-R] bond formation between a nascent Cys-SOH and a lysine residue in the phagocyte protein S100A2 (17).

## GLUTATHIONE REDUCTASE AND NADH PEROXIDASE: PRINCIPLES OF PROTEIN-SOH STABILIZATION

The flavoprotein NAD(P)H:disulfide reductase family currently consists of at least 13 functionally distinct FAD-dependent enzymes that constitute four structure-function classes (12, 36). These are represented by glutathione reductase (GR), mercuric reductase (MR), NADH peroxidase (Npx), and *E. coli* thioredoxin reductase. The members of the Npx class [Npx, NADH oxidase (Nox), coenzyme A-disulfide reductase (CoADR), and other homologues (37–39)] differ primarily in the presence of a single redox-active half-cystine, as contrasted with the redox-active cystine disulfides common to the other three disulfide reductase classes. In Npx and Nox, the catalytically essential cysteinyl redox center has been

identified as a stabilized Cys-SOH (12, 14). Aside from the crystal structure of native, oxidized Npx (Figure 2*a*), the active-site Cys42-SOH has also been characterized by $^{13}C$ nuclear magnetic resonance (NMR) methods as well as by analyses of the ultraviolet (UV)-visible, fluorescence, and redox properties of wild-type and mutant Npx forms. These approaches have been described in detail in recent reviews (12, 14). Here, we review structural and functional comparisons between GR and Npx that give valuable insights into the stabilization of protein sulfenic acids and mechanisms of thiol-dependent redox catalysis and regulation. Although limited solvent access for the Cys-SOH side chain, active-site hydrogen-bonding interactions, and/or ionization of Cys-SOH have been discussed as important parameters, the absence of proximal cysteine thiol (Cys-SH) groups is the dominant factor in Cys-SOH stabilization.

In GR, reduction of the catalytic disulfide yields two functionally distinct Cys-SH (the $EH_2$ form) (36), somewhat akin to the "peroxidatic" and "resolving" Cys-SH designations in the 2-Cys Prxs, as described below. Cys58-SH is solvent accessible, reacts with GSSG in the catalytic cycle to form the Cys58-SSG mixed-disulfide intermediate, and also reacts with iodoacetamide to give the inactive EHR (enzyme alkylated at Cys58) species. Cys63-SH has a low $pK_a$ (3.7 and 4.8 in yeast GR EHR and $EH_2$ forms, respectively), reacts only with the proximal Cys58-SSG disulfide in the catalytic cycle, and does not react with iodoacetamide. The Cys63-thiolate serves as a charge-transfer donor to the FAD, giving rise to the 540 nm absorbance band seen in the $EH_2$ and EHR spectra. This interaction and hydrogen-bonding interactions with the Thr339 and FAD side chains are thought to stabilize the Cys63-$S^-$ (40). Although Cys63 in GR and Cys42 in Npx are structurally equivalent (41) and share several properties (e.g., charge-transfer interactions with the respective FADs and low $pK_a$s), they are totally different in their reactivities toward $H_2O_2$. The Npx $EH_2$ form reacts with $H_2O_2$ with a second-order rate constant of $18 \times 10^7$ $M^{-1}$ $min^{-1}$ at 5°C (42); the yeast GR EHR form (which can only react at Cys63) reacts with $H_2O_2$ at only 0.3 $M^{-1}$ $min^{-1}$ (43). The comparison of the $H_2O_2$ oxidation of the GR $EH_2$ and EHR forms nonetheless gives an excellent experimental platform for testing the effect of the proximal Cys58-SH on Cys63-SOH stabilization. There is no evidence for any spectral intermediate (e.g., Cys63-SOH or Cys58-SOH) in the reaction of the GR $EH_2$ form with $H_2O_2$, but for the same reaction with the GR EHR form, a clearly detectable Cys63-SOH intermediate is observed, spectrally distinct from both EHR and the final EHR-Cys63-$SO_3H$ product. Thus, elimination of the proximal Cys58-SH (by alkylation) stabilizes Cys63-SOH.

These results with GR took on added biological significance in subsequent studies from other laboratories (44, 45), focusing on inhibition of human erythrocyte GR by two NO donors, S-nitrosoglutathione (GSNO) and diglutathionyl-dinitrosoiron (DNIC-$[GSH]_2$). Given its central role in intracellular defense against oxidative stress, combined with evidence that GR is not essential for normal erythrocyte function, the human enzyme has been considered a promising target for rational design of new inhibitors potentially useful in combating malaria and in cancer therapy. In solution studies, GSNO (1 mM, 3 h) reacted with the GR $EH_2$ form

to inhibit the enzyme. Spectral analyzes showed loss of the $EH_2$ charge-transfer interaction, and S-nitrosation of Cys63-SH (and/or Cys58-SH) was suggested as the basis for inhibition. Surprisingly however, the GSNO-inhibited GR crystal structure (Figure 2*b*) (46) revealed that Cys58 had been glutathionylated (Cys58-SSG) and Cys63-SH had been oxidized to the stable Cys63-SOH. Apparently, the Cys58-SSG modification has the same effect of eliminating the proximal Cys-SH as did alkylation in the $H_2O_2$ reactivity studies described above, thus fulfilling the primary criterion for Cys63-SOH stabilization. Furthermore, Cys63 is located in a fully buried active-site pocket, and $O_\delta$ of Cys63-SOH is hydrogen bonded to its main-chain N and to the FAD side chain (Figure 2*b*). Given that the active-site environment offers dramatic stabilization of the $EH_2$ Cys63-$S^-$, similar considerations would support the conclusion that Cys63-SOH is present as Cys63-sulfenate (Cys-$SO^-$). This conclusion is also supported by the charge-transfer interaction observed for the EHR-Cys63-SO(H) intermediate, which should require the electron-rich sulfenate as the donor to FAD.

While the GSNO inhibition was not easily reversible, prolonged (24 h) crystal soaks with 10-mM dithiothreitol (DTT), followed by removal of the reductant, restored the native Cys58-SS-Cys63 disulfide, and treatments in solution (5-mM DTT, 24 h) led to the full return of GR activity (46). The chemical mechanism for the inhibition of the GR by GSNO has been proposed to involve initial GR-Cys-SNO intermediates (e.g., Figure 1), although the formation of the Cys58-SSG disulfide could involve direct S-thiolation (47).

Interestingly, when GR was treated with the NO carrier DNIC-$[GSH]_2$ (36 $\mu$M, 30 min) (45), enzyme activity was irreversibly lost. The 1.7 Å crystal structure of the inactivated GR form (46) demonstrated the same Cys58-SSG structure as described previously, but Cys63-SOH had been further oxidized to the Cys63-$SO_2H$ derivative in the presence of the iron complex and oxygen.

A mutagenesis study in Npx also shows the importance of proximal thiols to sulfenic acid stability. Trying to generate a proximal Cys-SH that could react with Cys42-SOH, Miller et al. (48) replaced four nearby residues (Ser38, Phe39, Leu40, and Ser41) with cysteine, and the L40C mutant yielded the anticipated active-site disulfide form of the holoenzyme. Formation of the disulfide with Cys40 requires movement of Cys42-S$\gamma$ to a new position almost 6 Å from the FAD coenzyme, and the mutant is catalytically inactive.

## ENZYME CATALYSIS AND REGULATION VIA CYSTEINE SULFENIC ACIDS: ENZYMES INVOLVED IN THE REDUCTION OF PEROXIDES AND METHIONINE SULFOXIDE

### Cysteine-Based Peroxidases

We discuss below several families of nonheme, cysteine-requiring peroxidases from both bacterial and eukaryotic sources in terms of their mechanistic (sulfenic acid) characterization and their emerging roles in redox signaling.

BACTERIAL PEROXIDASES: CHEMICAL MODIFICATION METHODS FOR SOH DETECTION IN THE C165S MUTANT OF AhpC Although the peroxide-reducing enzymes most familiar to researchers, catalase and glutathione peroxidase, have been studied for many years, another family of cysteine-dependent peroxidases that relies on a chemistry similar to that of Npx for peroxide reduction has more recently been identified. This new family of peroxidases, collectively referred to as Prxs, is found in all branches of life (7, 21). Prokaryotic and eukaryotic Prxs generally reduce a wide variety of peroxides, including $H_2O_2$, organic hydroperoxides, and peroxynitrite, and are expressed at high levels; they are among the top ten most abundant *E. coli* proteins (49) and typically compose ~0.1% to 0.8% of the total cellular proteins in mammalian cells (7, 21, 50). The main Prx of *E. coli*, AhpC, was found to be the primary enzyme responsible for the reduction of endogenously generated $H_2O_2$, with catalase only protecting against intracellular $H_2O_2$ levels around 5 $\mu$M or higher (51).

Prxs can be structurally and functionally divided into three classes, known as the typical 2-Cys Prxs, the atypical 2-Cys Prxs, and the 1-Cys Prxs (7). Whereas Prxs in all three classes appear to catalyze the reduction of hydroperoxides to yield a sulfenic acid at the conserved active-site cysteine (the peroxidatic cysteine), the mechanism for recycling of the sulfenic acid to regenerate the activated thiolate differs among the three classes. In 1-Cys Prxs, the sulfenic acid likely reacts with a small-molecule thiol prior to reduction with a second molecule of the reductant, although the identity of the electron donor is unclear. In 2-Cys Prxs, the thiol that condenses with the nascent sulfenic acid comes from another cysteine group in the protein (termed the resolving cysteine), either on the same subunit as the peroxidatic cysteine (atypical 2-Cys Prxs) or, more commonly, from the other subunit of a homodimer (typical 2-Cys Prxs). The electron donor required for recycling of 2-Cys Prxs generally includes a reduced pyridine nucleotide (NADH or NADPH), a flavoprotein disulfide reductase (thioredoxin reductase, AhpF, trypanothione reductase, Cp34, or lipoamide dehydrogenase), and an additional CXXC-containing protein or module (thioredoxin, the N-terminal domain of AhpF, tryparedoxin, Cp9, or AhpD) (7, 21, 52).

Prior to 1998, when the crystal structure of a sulfenic acid–containing 1-Cys Prx was published (Figure 2*c*), the strongest evidence for Cys-SOH involvement in Prx-catalyzed peroxide reduction came from the studies of a mutant of *S. typhimurium* AhpC, C165S, in which the resolving cysteine had been removed by mutagenesis, leaving only the peroxidatic Cys46. This enzyme exhibited full peroxidatic activity toward cumene hydroperoxide in the presence of excess NADH and AhpF, showing that it had effectively been converted to a 1-Cys Prx. Like Npx, the sulfenic acid at the active site of the peroxide-oxidized protein reacted stoichiometrically with 2-nitro-5-thiobenzoate (TNB) to form the mixed disulfide, required a single equivalent of NADH (and a catalytic amount of the reductase, AhpF) for regeneration of the thiol group, and was oxidatively inactivated upon treatment with excess peroxide via sulfinic and/or sulfonic acid formation (53). Reactivity of this species with dimedone (5,5-dimethyl-1,3-cyclohexanedione), as shown by mass spectrometry, was also demonstrated for oxidized C165S AhpC (15).

The unique reactivity of sulfenic acids toward nucleophiles, such as TNB, dimedone, and benzylamine, was the chief criterion established by Allison and others in the 1970s to confirm the presence of these groups in proteins such as glyceraldehyde-3-phosphate dehydrogenase and papain (13).

In an effort to establish additional criteria that could be used to convincingly and more directly demonstrate the presence of Cys-SOH in C165S AhpC, a method that used the electrophilic agent NBD chloride (7-chloro-2-nitrobenzo-2-oxa-1,3-diazole) was developed. Both the reduced (Cys-$S^-$) and oxidized (Cys-$SO^-$) forms of C165S AhpC reacted with NBD, but the adducts formed could be distinguished by their characteristic UV-visible and fluorescence properties (Figure 3) and their masses were distinguished by electrospray ionization mass spectrometry (ESI-MS). Whether the NBD adduct formed with the sulfenic acid is a sulfoxide or sulfenate ester was not established by these studies, although arguments supporting sulfoxide formation have been made based partly on the acid stability of the product (12). As described for a number of examples below, this reagent has subsequently been used for the identification of stable sulfenic acids in such proteins as PTPs, OxyR, and OhrR (19, 54, 55).

Although AhpC-like enzymes are the best studied and most abundant Prxs in a wide range of Gram-positive and Gram-negative bacteria, at least two other distant relatives of the Prx family are also present in many bacteria (56). First, a periplasmic peroxidase in *E. coli*, designated thiol peroxidase (Tpx) (57), has an active-site cysteine residue (Cys61) equivalent to the peroxidatic cysteine of *S. typhimurium* AhpC and an analogous catalytic mechanism (20). *E. coli* Tpx exhibits a much greater catalytic efficiency with organic hydroperoxides than with $H_2O_2$, however ($k_{cat}/K_m = 8 \times 10^6\ M^{-1}\ s^{-1}$ versus $4 \times 10^4\ M^{-1}\ s^{-1}$), and the disulfide bond formed is between cysteine residues (Cys61 and Cys95) on the same subunit, making this a member of the atypical 2-Cys Prx group. Second, the bacterioferritin comigratory protein (BCP), also expressed in *E. coli*, reduces $H_2O_2$ and organic hydroperoxides through an active-site cysteine residue (Cys45) corresponding to Cys46 in AhpC (58). Reactivity of the oxidized protein toward NBD chloride indicated the presence of sulfenic acid in the oxidized protein. This result plus the lack of any other conserved or "essential" cysteines in the protein suggest that BCP operates as a 1-Cys Prx.

For completeness, two other enzymes distinct from the Prx family should also be mentioned, although data proving sulfenic acid formation in each case is not yet available. Although bacteria do not possess conventional glutathione peroxidases, at least some, including *E. coli*, do have a homologue with a cysteine in place of the active-site selenocysteine of higher organisms. The *E. coli* protein, designated BtuE, is encoded in an operon associated with vitamin B12 transport (GenBank P06610). Genetic studies have established antioxidant functions for several of these bacterial Gpx homologues (59, 60), although preliminary investigations suggest that thioredoxin, rather than GSH, is the reductant for these peroxidases (L.M.S. Baker & L.B. Poole, unpublished observations). This is similar, then, to the thioredoxin-dependent activity observed for the "Gpx3" homologue of yeast

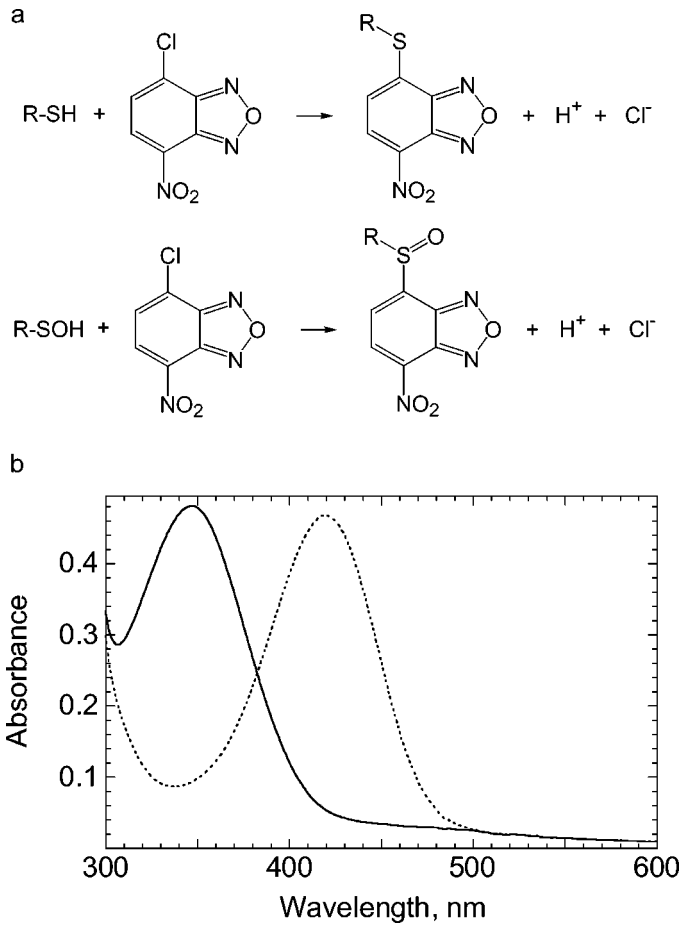



**Figure 3** NBD adducts of cysteine thiols and sulfenic acids. The reduced (R-SH) and oxidized (R-SOH) forms of the C165S mutant of AhpC (*panel a*) give rise to distinctive spectra (*panel b*) with maxima at 420 nm (R-S-NBD, *dotted line*) and 347 nm [R-S(O)-NBD, *solid line*]; unlike R-S(O)-NBD, R-S-NBD is also fluorescent. Identity of these adducts was also confirmed by electrospray ionization mass spectrometry [20,765.2 and 20,781.5 atomic mass units for R-S-NBD and R-S(O)-NBD, respectively]. Reprinted with permission from Reference 15. Copyright © 1997 American Chemical Society.

(9). Interestingly, this latter protein also functions in redox regulation of the Yap1 transcription factor; a discussion of this recently discovered system is therefore included with the transcriptional regulators (below).

A novel organic hydroperoxide resistance gene, *ohr*, was discovered in 1998 in the plant pathogen *Xanthomonas campestris* (61) and was subsequently shown to exist in a variety of Gram-positive and Gram-negative organisms, but not in *E. coli*

(62). Ohr was postulated to act as a cysteine-based peroxidase structurally unrelated to the Prx family, and recent studies have borne this out. The overall fold of the dimeric protein is quite distinct from that of the Prxs, and the peroxidatic cysteine (Cys60) appears to form an intrasubunit disulfide bond with Cys124 following reaction with organic peroxides or, more slowly, with $H_2O_2$ (63, 64). Although intermediate sulfenic acid formation has not been directly demonstrated, precedent from *S. typhimurium* AhpC and other Prxs strongly favors such a mechanism for Ohr as well.

PEROXIREDOXINS AND SIGNALING: THE CATALYTIC SOH AS A PEROXIDE-SENSITIVE SWITCH Many organisms produce more than one Prx; at least six different isoforms expressed from different genes have been identified in mammalian cells (7, 21, 65). In eukaryotic cells, Prxs are important not only as antioxidant proteins, keeping toxic $H_2O_2$ and peroxynitrite levels low, but also as players in such processes as apoptosis, differentiation, and proliferation. At least one Prx, Prx I, also known as heme-binding protein 23 (HBP23) and proliferation-associated gene (PAG), interacts directly with and inhibits *c*-Abl, a nonreceptor tyrosine kinase (66). Evidence has been accumulating that eukaryotic Prxs act as regulators of $H_2O_2$-mediated cell signaling and are implicated in such disease states as cancer and neurodegenerative diseases (8, 67). Given their function in controlling $H_2O_2$ levels, alterations in Prx activity can clearly modulate redox-dependent signaling pathways.

Several mechanisms have been proposed to regulate Prx activity in vivo, and it is likely that there is considerable overlap between these regulatory features. Oligomerization of several mammalian and bacterial 2-Cys Prxs has been shown to depend on redox state; reduced (or overoxidized) Prxs favor $(\alpha_2)_5$ decamer formation, giving a toroid-shaped molecule, whereas disulfide bond formation favors dissociation of the decamer to generate homodimers (7, 68). There have been some reports suggesting that dimeric forms of Prxs are less active (69–71). Phosphorylation at Thr90 in Prx I (and at the equivalent position of Prxs II through IV) has been shown to be mediated by cyclin-dependent kinases (72); Wood et al. (7) have postulated that the resulting decrease in activity may result from the disruption of the decamer that likely would ensue if two adjacent dimers in the toroid became phosphorylated, putting the two negatively charged phosphate groups in close proximity. Proteolysis resulting in the removal of C-terminal residues beyond the resolving cysteine of several eukaryotic Prxs has been observed (73–75), and disruption or removal of C-terminal residues was shown to decrease the sensitivity of a yeast Prx to substrate-induced inactivation (75). This latter phenomenon, resulting from overoxidation of the active-site Cys-SOH, has been observed in a number of eukaryotic systems (76–78) and has recently been proposed as the primary mechanism by which Prxs regulate $H_2O_2$-mediated cell signaling (8).

As shown previously for Npx and the C165S mutant of AhpC, sulfenic acids at peroxidatic active sites can undergo further oxidation by excess hydroperoxides,

thereby forming sulfinic and/or sulfonic acids. In Prxs, the sulfinic acid formed at the active site seems to be particularly stabilized toward further oxidation owing to the formation of a strong salt bridge between the two oxygens of the sulfinic acid and two nitrogens of the active-site Arg, as observed in the overoxidized Prx II structure (79). The formation of the cysteine sulfinic acid in 2-Cys Prxs seems surprising given the presence of the resolving cysteine, which should react quickly to generate a disulfide bond at the active site. However, it has been noted that the sulfur atoms of the peroxidatic and resolving cysteines are ~13 Å apart in the reduced Prxs, and that local structural rearrangements, including the unraveling of a turn of $\alpha$-helix, are required for disulfide bond formation (8, 80). The opportunity exists, then, for the sulfenic acid to be further oxidized by peroxides as an alternative to disulfide bond formation, and this overoxidation can be detected as a shift toward a more acidic pI for these proteins as analyzed by two-dimensional (2-D) gel electrophoresis.

The phenomenon of Prx overoxidation seems counterintuitive; why should these abundant antioxidant proteins be so sensitive toward inactivation by their own substrate? In fact, bacterial AhpCs exhibit far less sensitivity toward peroxide-mediated inactivation, requiring the presence of 110-fold more $H_2O_2$ to observe inactivation comparable to that detected in Prx I studies (8, 78). This difference highlights the fact that the sensitivity of eukaryotic Prxs toward inactivation by peroxides is an acquired characteristic, rather than an unavoidable consequence of the peroxidatic mechanism.

The benefit to the cell of having an apparently "poorer" peroxidase may lie in the role $H_2O_2$ plays in eukaryotic cell signaling pathways (8). The balance between efficient peroxide removal and inactivation of Prxs by peroxide may have been fine tuned during evolution so that the Prxs can act as a floodgate, preventing the buildup of toxic $H_2O_2$ levels during normal cellular metabolism, but responding to a rapidly rising burst of signaling levels of $H_2O_2$ through inactivation. In fact, eukaryotic Prxs contain two structural motifs, including a GGLG motif adjacent to a 3–10 helix and a YF motif within an extended C-terminal helix, that are absent from the resistant bacterial AhpCs (Figure 4*a*) (8). These two structural features in eukaryotic Prxs cover the catalytic cysteine residue, making the conformational changes required for disulfide bond formation more difficult (Figure 4*b*). In comparison, regions around the peroxidatic cysteine and the C terminus (including the resolving cysteine) of bacterial AhpC are much more flexible, likely facilitating the rearrangements required for efficient disulfide bond formation in these cases (Figure 4*c*). Thus, the structural features in eukaryotic Prxs that impart their sensitivity toward inactivation by peroxide are likely to be acquired features allowing these Prxs to take on the dual roles of antioxidant and regulator of peroxide signaling, in contrast to the primary role of Prxs as antioxidants in bacteria. In this model, fine tuning of the peroxide signal strength could be achieved by balancing the size of a peroxide burst with the expression levels of the floodgate Prxs. Consistent with the importance of this balance, the overexpression of Prxs interferes with some known peroxide signaling pathways (21, 81).

Regulation of Prx activity by irreversible overoxidation seems wasteful to a cell that has expended energy to synthesize high levels of these antioxidants. Whether this control mechanism comes into play frequently in the life of a cell or is invoked primarily by cells destined for apoptosis is not yet known. However, several new studies suggest that the generation of the sulfinic acid form of Prxs is reversible in some cell types. In the first study (33a), using pulse-chase experiments, acidic forms of Prx I and Prx II that were generated following exogenous treatment of cells with $H_2O_2$ reappeared at the higher pI position of the unmodified proteins over minutes to hours, apparently without requiring new protein synthesis or the removal of the sulfur atom. A subsequent study showed that the lower pI spot, often associated with the sulfinic acid form of Prxs, can also be attributed to other posttranslational modifications (e.g., phosphorylation), although this work also confirmed what was termed the retroreduction of overoxidized forms of Prx II and perhaps Prx I (33b). Interestingly, the four different Prxs expressed in HeLa cells exhibited a wide range of regeneration rates, with Prx VI and Prx III likely not regenerated by this process. Very recently, Toledano and colleagues have identified sulfiredoxin, a eukaryotic enzyme that catalyzes the ATP-dependent reduction of the cysteine sulfinic acid of sensitive yeast 2-Cys Prxs (33c). This enzyme is absent in prokaryotes, corroborating the idea that Prx inactivation by overoxidation and its reversion by sulfiredoxin were acquired during evolution. This apparent reversal of sulfinic acid formation lends credence to the proposal that Prx overoxidation is a regulator of $H_2O_2$ signaling, as the inactivation process may actually be followed by regeneration of function in these Prxs.

## Methionine Sulfoxide Reductases (Msrs)

One hallmark of oxidative damage to proteins by reactive oxygen and nitrogen species is the oxidation of methionine residues to asymmetric methionine sulfoxides (epimeric R and S forms). Recently, enzymes that can repair this damage using reduced thioredoxin as an electron source have been identified from both prokaryotes and eukaryotes and include both S- and R-specific reductases [(82–84) reviewed in 85]. The facts that (*a*) methionine oxidation at specific sites can interfere with normal protein function and (*b*) the generation of methionine sulfoxide can be reversed by enzymes that may exhibit specificity toward their targets have led to the proposal that reversible methionine oxidation may be another regulatory mechanism involved in redox signaling (85, 86). There is a growing list of proteins and peptides wherein methionine oxidation, reversed by Msr treatment, affects biological function. These include calmodulin, a voltage-gated potassium channel; $\alpha$-1 proteinase inhibitor; and human immunodeficiency virus type-2 proteins (85, 86).

The catalytic cycle of most Msrs includes the generation of a Cys-SOH in the reduction of methionine sulfoxide; in some cases, the critical cysteine residue is replaced by a selenocysteine, and the reaction likely proceeds through the analogous selenenic acid intermediate (83, 84, 87). During turnover, an essential

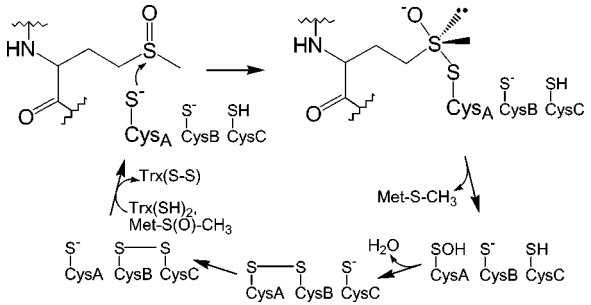



**Figure 5** Catalytic mechanism of methionine sulfoxide reductases (Msrs). Attack of the active-site cysteine (CysA) on the sulfur of the sulfoxide substrate generates a covalent intermediate, then methionine is released as the Cys-SOH is formed at the active site. The enzyme is regenerated by a series of thiol-disulfide interchange reactions (85).

conserved cysteine at the active site (CysA) acts as the nucleophile and attacks the sulfur of the methionine sulfoxide, leading to the formation of a trigonal-bipyramidal intermediate (Figure 5) (83, 85). Collapse of the intermediate generates the restored methionine product and a sulfenic acid at the active-site cysteine. Condensation with CysB to form a disulfide bond and a series of thiol-disulfide exchange reactions with CysA, -B, and -C in Msrs and the two cysteines of the thioredoxin electron donor results in reactivation of the enzyme for another turnover.

In order to prove the generation of sulfenic acid during catalysis by MsrA, removal of CysB and CysC was undertaken to prevent subsequent disulfide bond formation with the CysA-sulfenic acid (CysA $=$ Cys51) (83). Under these conditions, the Cys-SOH formed on breakdown of the covalent intermediate was stabilized sufficiently to allow direct observation of the addition of 16 amu by ESI-MS. This species was also reactive toward nucleophilic reagents such as dimedone or TNB, which do not react with thiol groups. Some sulfenic acid formation was detected even in the wild-type enzyme, demonstrating that the subsequent generation of the disulfide bond is relatively slow in this enzyme. The mutant also retained Msr activity when DTT, but not thioredoxin, was used as the reductant. This, therefore, represents another case where sulfenic acid intermediacy is sufficient for the protein to be functional, although subsequent disulfide bond formation provides additional functionality, in this case permitting thioredoxin to act as a direct reductant of the enzyme. It should be noted that there is recent evidence for the oxidative modification of cysteine residues in cytosolic MsrA from rat liver, as indicated in part by the appearance of additional acidic forms detected during 2-D gel electrophoresis (88). The specific cysteine residues undergoing modification have not been identified, and it is not yet clear whether such cysteine oxidation is involved in the redox regulation of some Msrs.

## TRANSCRIPTIONAL REGULATORS OPERATING THROUGH CYSTEINE SULFENIC ACID INTERMEDIATES

An excellent review, "Thiol-Based Regulatory Switches," has recently appeared (2), focusing on several different examples of transcription factors and other proteins that transduce changes in the redox environment into altered patterns of gene expression, protein folding, and/or enzyme catalysis. Although not elaborated on here, Cys-SOH redox cofactors have also been implicated (Reference 14 and references contained therein) as key intermediates in the redox regulation of such transcription factors as Fos and Jun (the activator protein-1 complex), bovine papillomavirus-1 E2 protein, and the NF-$\kappa$B p50 subunit (89, 90). In keeping with the focus of the present review on protein sulfenic acids and their involvement in thiol-dependent redox catalysis and regulation, we discuss the bacterial OxyR and OhrR transcription factors and the yeast Yap1-Gpx3 system, perhaps from a more biochemical perspective than other recent reviews.

### OxyR: A Tale of Two Cysteines

*Escherichia coli* OxyR, which in response to $H_2O_2$-mediated oxidative stress activates the *oxyR* regulon, has developed into a paradigm for cysteine-based redox regulation of gene expression. Of six cysteines in the protein, only two (Cys199 and Cys208) influence activity, and many studies have been done using mutants with the remaining cysteines converted to alanines. Cys199 is more crucial than Cys208, as the C199S mutant completely fails to promote *oxyS* expression, whereas the C208S mutant retains a slight ability to activate expression. Although early studies (91, 92) suggested that a stable Cys199-SOH might be the active DNA-binding form, an elegant study by Zheng et al. (93) established the formation of a Cys199-SS-Cys208 intramolecular disulfide within the tetrameric protein. The identification rested on mass spectrometric analyzes and protein thiol/disulfide content measurements of the oxidized and reduced full-length OxyR4C $\rightarrow$ A mutant.

In 2001, Choi et al. (94) published the three-dimensional structures for the reduced (mimicked by a C199S mutation) (92) and oxidized forms of an OxyR4C$\rightarrow$A mutant containing just the regulatory and oligomerization domain (ROD; residues 80–305 of OxyR, lacking the DNA-binding domain). In the reduced structure, residue 199 is in a surface pocket near Arg266, which may play a role in peroxide reactivity, and residues 200–215 form a highly mobile surface loop that places Cys208 $\sim$17 Å away from residue 199. In the oxidized structure, the Cys199-Cys208 disulfide is visible, and residues 195–215 are rearranged and much better ordered. These substantial local structural changes are coupled with a change in the subunit association geometry, and this geometry is in turn proposed to be the key to modulating DNA binding. Choi et al. coined the term "fold editing" to describe how the disulfide formation impacts the structure and

suggested such fold editing might be important for a wider range of redox-regulated proteins.

This now orthodox picture of OxyR function has been challenged recently by Stamler and colleagues (55). Kim et al. report that there are multiple oxidized forms of OxyR that have graded levels of DNA-binding activity in the order SNO (nitroso) < SOH (sulfenic) < SSG (glutathionylated), and, surprisingly, that the Cys199-Cys208 disulfide form is not formed significantly and thus is not a part of this series. The broader point of this work is that OxyR is not an all-or-none switch but a rheostat responsive to various levels and types of oxidative or nitrosative stress.

Although both studies agree that a sulfenic acid form of OxyR plays a key role in its function, there is disagreement as to which oxidized form(s) of OxyR stably bind the DNA to activate transcription. Kim et al. (55) suggest that the discrepancy could be partly because the earlier studies used mutated and/or truncated forms of OxyR, and that the analyses of reactive functional groups were done on protein digests rather than the intact protein. Kim et al., in contrast, worked with full-length wild-type OxyR, but their work also has some inconsistencies. The major weakness with their work seems to be that, although they claim to have studied each of the OxyR forms (SH, SNO, SOH, and SSG) in pure form, the mass spectrometry and quantitative chemical analyses do not conclusively support the claims of purity. Some of the observed molecular masses reported agree poorly with the expected masses, and those that do agree are often not the major peak in the spectrum. With regard to the quantitative analyzes, the range of "0.15–0.8 free thiols" does not inspire confidence in the oxidative homogeneity of the purified protein, and in general many of the analyses are not consistent with the existence of pure, stable, fully modified forms. For instance, using the millimolar extinction coefficients for Cys-S-NBD and Cys-S(O)-NBD (13.0 $mM^{-1}$ $cm^{-1}$ and $\sim$13.4 $mM^{-1}$ $cm^{-1}$, respectively (15), the spectral data given by Kim et al. for NBD adducts of OxyR-SOH and OxyR-SH indicate that the OxyR-SOH adduct spectrum has an OxyR-SH absorbance component (at 420 nm) equivalent to 50% of the OxyR-SH thiol titer itself. In an analysis such as this, involving the study of interconverting forms that might have different activities, quantification is crucial. For instance, to use an extreme example, the results could be explained if the C199-C208 disulfide form were the only active form and it were present at various levels of impurity in each of the other forms.

From a structural perspective, two points are worth clarifying. First, Kim et al. suggest that the 17-Å separation of Cys199 and Cys208 in the reduced OxyR structure is evidence against a disulfide being formed, but many precedents (including the Prxs mentioned in this review and the structure of oxidized OxyR itself) make this argument absurd. Second, Kim et al. suggest that the nonfunctional disulfide they report as existing between Cys180 and Cys259 in wild-type OxyR is consistent with the crystal structure. However, although the two $\alpha$ carbons are only approximately 6 Å apart, the side chains point in opposite directions, so such a disulfide could not form without a structural rearrangement.

The current controversy with regard to the structural basis, as well as the functional implications, of OxyR-mediated redox signaling has been addressed in several recent commentaries and reviews (2, 95–97) by noted authorities in the areas of gene expression, redox regulation, as well as biomolecular engineering. This review has attempted to enhance the biochemical and structural component of OxyR understanding. Charles Dickens wrote, ". . .it was the epoch of belief, it was the epoch of incredulity (98)," and in that sense, we await further studies that will sort out the truth of the matter.

## OhrR

OhrR is the best evidence to date for a functional sulfenic acid generated by the peroxide modification of a transcriptional regulator (54, 99). OhrR in its reduced form binds to the promoter region and represses the expression of Ohr, a cysteine-dependent peroxidase mechanistically, but not structurally, related to the Prx family (61, 63, 64). Upon treatment with organic peroxides, such as cumene hydroperoxide or *tert*-butyl hydroperoxide, the OhrR repressor dissociates from its target DNA, strongly inducing Ohr expression (54, 100). While Ohr and OhrR have been identified in a number of Gram-positive and Gram-negative organisms, the repressor proteins from *Xanthomonas campestris* pv. *phaseoli* (XcOhrR) and from *B. subtilis* (BsOhrR) have been best characterized (99).

In both XcOhrR and BsOhrR, when the redox-sensing cysteine (Cys15 in BsOhrR) is mutated to serine or glycine, OhrR still binds its cognate operator, but has lost its sensitivity toward peroxides (54, 100). Evidence for sulfenic acid formation in BsOhrR consists of the DTT reversibility of the peroxide-mediated oxidation; the lack of intersubunit disulfide bond formation (BsOhrR only has one cysteine per monomer); and, more directly, reaction with NBD chloride (54). Furthermore, ESI-MS analysis demonstrated the addition of one, two, or three oxygen atoms after brief in vitro treatment with excess cumene hydroperoxide, indicating that overoxidation (to sulfinic and sulfonic acids) can occur. Although reversible Cys-SOH formation likely accounts for the peroxide-sensing redox cycle of BsOhrR, the presence of two additional cysteine residues in XcOhrR allows for a mechanism more akin to that of OxyR, wherein peroxide-mediated sulfenic acid generation is followed by disulfide bond formation before rereduction to produce the activated repressor. Preliminary work with XcOhrR (W. Panmanee, L.B. Poole & S. Mongkolsuk, unpublished) indicates that a mutant possessing only the peroxide-sensitive Cys22 of XcOhrR functions via sulfenic acid formation, but in wild-type XcOhrR, the stable protein form involved in derepression has a disulfide bond, presumably resulting from the attack of one of the two additional Cys residues on the Cys22 sulfenate. This result again illustrates that, within a family of proteins, sulfenic acid formation can have the same or similar functional effect(s) as does subsequent disulfide bond formation; which occurs depends on whether the protein has additional protein thiols proximal to the reactive cysteine.

## *Saccharomyces Cerevisiae* Yap1-Gpx3 (Orp1)

Recently, glutathione peroxidase (Gpx) homologs have been identified in bacteria and yeast in which the typical active-site SeCys residue is replaced by a functional Cys (or cystine disulfide). One of these proteins, *E. coli* BtuE (GenBank P06610), has been mentioned earlier. Another, *S. cerevisiae* Gpx3, which reportedly exhibited GSH-dependent peroxidase activity based on assays of crude extracts (101), was subsequently shown to catalyze, instead, the thioredoxin-dependent reduction of $H_2O_2$ (9). Of the three cysteines per Gpx3 monomer, Cys36 aligns with the peroxidatic SeCys52 of the bovine erythrocyte enzyme, and it and Cys82 are essential for the peroxidatic activity (9). The proposed peroxidase mechanism of Gpx3 involves initial $H_2O_2$ reactivity at Cys36, yielding the Cys36-SOH intermediate, followed by Cys82-SH condensation with Cys36-SOH to give the intramolecular protein disulfide. There is no crystal structure for Gpx3, but alignment with the bovine Gpx structure (102) places Cys82 approximately 13 Å from Cys36, meaning that a conformational change would have to occur to form the Cys36-Cys82 disulfide bond. The catalytic cycle is completed as $Trx(SH)_2$ reduces the Gpx3 disulfide to the dithiol form.

Interestingly, Delaunay et al. (9) recently demonstrated a second, more important role for the Gpx3 Cys36-SOH in the cellular defense against $H_2O_2$-mediated oxidative stress. Cys36-SOH, instead of forming the intramolecular disulfide, can alternatively form an intermolecular protein disulfide with Yap1 Cys598. This Gpx3-SS-Yap1 intermediate undergoes a subsequent intramolecular thiol-disulfide interchange involving Yap1 Cys303. The intramolecular disulfide is in turn thought to interfere with the conformation of the Yap1 nuclear export signal, leading to nuclear accumulation of the transcription factor and activation of genes encoding both antioxidants and components of thiol-reducing pathways in response to $H_2O_2$ stress. As is the case in the Gpx3 scavenging cycle, $Trx(SH)_2$ also appears to serve as the cellular reductant of the Yap1 disulfide. It is notable that the Yap1 disulfide bond formed as a result of Gpx3 interaction differs from those formed upon treatment with diamide, suggesting that different stresses may yield differentially activated forms of Yap1.

The principle of transcriptional activation of $H_2O_2$-mediated stress response genes in yeast involving the Cys303-Cys598 disulfide in Yap1 is, of course, similar to the prevailing view of OxyR activation in *E. coli* (93). $Trx(SH)_2$ reduces oxidized Yap1 to quench the response, whereas $Grx1(SH)_2$ serves the same function with OxyR. The major distinction between these views of the two systems is the obligatory role of the Gpx3 "accessory protein" in the Yap1 system, which contrasts with the direct $H_2O_2$ reaction of OxyR Cys199 ($k \sim 2.3 \times 10^5\ M^{-1}\ s^{-1}$) (23). The long, Cys36-SH-to-Cys82-SH distance predicted in Gpx3, and the corresponding need for a conformational change, might provide for a crucial kinetic "pause" that would allow for formation of the Gpx3-Cys36-Yap1-Cys598 intermolecular disulfide. Interestingly, exactly this kind of kinetic pause is a crucial feature of the floodgate control of peroxiredoxin activity (see above), and is a

feature of redox regulated proteins that could be selected for as a timing switch, much like the way the rate of GTP hydrolysis times the signaling activities of G-proteins.

## ACKNOWLEDGMENTS

The authors thank Dr. Zachary A. Wood for preparing Figure 2 and many colleagues for their input during the preparation of this review. Grant support from the National Institutes of Health (GM50389 to L.B.P. and P.A.K. and GM35394 to A.C.) and from the American Heart Association (Established Investigatorship to L.B.P.) is also acknowledged.

**The *Annual Review of Pharmacology and Toxicology* is online at http://pharmtox.annualreviews.org**

## LITERATURE CITED

1. Christman MF, Morgan RW, Jacobson FS, Ames BN. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in *Salmonella typhimurium*. *Cell* 41:753–62
2. Paget MSB, Buttner MJ. 2003. Thiol-based regulatory switches. *Annu. Rev. Genet.* 37:91–121
3. Godon C, Lagniel G, Lee J, Buhler JM, Kieffer S, et al. 1998. The $H_2O_2$ stimulon in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273:22480–89
4. Rhee SG, Bae YS, Lee S-R, Kwon J. 2000. Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Science's STKE.* www.stke.org/cgi/contentfull/OC_sigtrans;2000/53/pel
5. Finkel T. 2000. Redox-dependent signal transduction. *FEBS Lett.* 476:52–54
6. Stamler JS, Hausladen A. 1998. Oxidative modifications in nitrosative stress. *Nat. Struct. Biol.* 5:247–49
7. Wood ZA, Schröder E, Harris JR, Poole LB. 2003. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.* 28:32–40
8. Wood ZA, Poole LB, Karplus PA. 2003. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300:650–53
9. Delaunay A, Pflieger D, Barrault M-B, Vinh J, Toledano MB. 2002. A thiol peroxidase is an $H_2O_2$ receptor and redox-transducer in gene activation. *Cell* 111: 471–81
10. Kice JL. 1980. Mechanisms and reactivity in reactions of organic oxyacids of sulfur and their anhydrides. *Adv. Phys. Org. Chem.* 17:65–181
11. Hogg DR. 1990. Chemistry of sulphenic acids and esters. In *The Chemistry of Sulphenic Acids and Their Derivatives*, ed. S Patai, pp. 361–402. New York: Wiley
12. Claiborne A, Mallett TC, Yeh JI, Luba J, Parsonage D. 2001. Structural, redox, and mechanistic parameters for cysteine-sulfenic acid function in catalysis and regulation. *Adv. Protein Chem.* 58:215–76
13. Allison WS. 1976. Formation and reactions of sulfenic acids in proteins. *Acc. Chem. Res.* 9:293–99
14. Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ III, et al. 1999. Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 38:15407–16

15. Ellis HR, Poole LB. 1997. Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. *Biochemistry* 36:15013–18
16. Bryk R, Griffin P, Nathan C. 2000. Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 407:211–15
17. Raftery MJ, Yang Z, Valenzuela SM, Geczy CL. 2001. Novel intra- and intermolecular sulfinamide bonds in S100A8 produced by hypochlorite oxidation. *J. Biol. Chem.* 276:33393–401
18. Winterbourn CC, Metodiewa D. 1999. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic. Biol. Med.* 27:322–28
19. Denu JM, Tanner KG. 1998. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37:5633–42
20. Baker LMS, Poole LB. 2003. Catalytic mechanism of thiol peroxidase from *Escherichia coli*: sulfenic acid formation and overoxidation of essential Cys61. *J. Biol. Chem.* 278:9203–11
21. Hofmann B, Hecht H-J, Flohé L. 2002. Peroxiredoxins. *Biol. Chem.* 383:347–64
22. Crane EJ III, Parsonage D, Poole LB, Claiborne A. 1995. Analysis of the kinetic mechanism of enterococcal NADH peroxidase reveals catalytic roles for NADH complexes with both oxidized and two-electron-reduced enzyme forms. *Biochemistry* 34:14114–24
23. Åslund F, Zheng M, Beckwith J, Storz G. 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. *Proc. Natl. Acad. Sci. USA* 96:6161–65
24. Gu ZZ, Kaul M, Yan BX, Kridel SJ, Cui JK, et al. 2002. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297:1186–90
25. Ishii T, Sunami O, Nakajima H, Nishio H, Takeuchi T, Hata F. 1999. Critical role of sulfenic acid formation of thiols in the inactivation of glyceraldehyde-3-phosphate dehydrogenase by nitric oxide. *Biochem. Pharmacol.* 58:133–43
26. Percival MD, Ouellet M, Campagnolo C, Claveau D, Li C. 1999. Inhibition of cathepsin K by nitric oxide donors: evidence for the formation of mixed disulfides and a sulfenic acid. *Biochemistry* 38:13574–83
27. Hogg PJ. 2003. Disulfide bonds as switches for protein function. *Trends Biochem. Sci.* 28:210–14
28. Giles GI, Tasker KM, Jacob C. 2001. Hypothesis: the role of reactive sulfur species in oxidative stress. *Free Radic. Biol. Med.* 31:1279–83
29. Dickinson DA, Forman HJ. 2002. Glutathione in defense and signaling: lessons from a small thiol. *Ann. NY Acad. Sci.* 973:488–504
30. Klatt P, Lamas S. 2000. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur. J. Biochem.* 267:4928–44
31. Thomas JA, Poland B, Honzatko R. 1995. Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Arch. Biochem. Biophys.* 319:1–9
32. Finlayson AJ, MacKenzie SL, Finley FW. 1979. Reaction of alanine-3-sulfinic acid with 2-mercaptoethanol. *Can. J. Chem.* 57:2073–77

33a. Woo HA, Chae HZ, Hwang SC, Yang K-S, Kang SW, et al. 2003. Reversible oxidation of the catalytic site cysteine of peroxiredoxins to cysteine sulfinic acid in mammalian cells. *Science* 300:653–56

33b. Chevallet M, Wagner E, Luche S, Van Dorsselaer A, Leize-Wagner E, Rabilloud T. 2003. Regeneration of peroxiredoxins during recovery after oxidative stress: only some overoxidized peroxiredoxins can be reduced during recovery after oxidative stress. *J. Biol. Chem.* In press

33c. Biteau B, Labarre J, Toledano M. 2003.

ATP-dependent reduction of cysteine sulfinic acid by *S. cerevisae* sulfiredoxin. *Nature* 425:980–84

34. Denu J, Tanner KG. 2002. Redox regulation of protein tyrosin phosphatases by hydrogen peroxide: detecting sulfenic acid intermediates and examining reversible inactivation. *Methods Enzymol.* 348:297–305

35a. Caselli A, Marzocchini R, Gamici G, Manao G, Moneti G, et al. 1998. The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by $H_2O_2$. *J. Biol. Chem.* 273: 32554–60

35b. Salmeen A, Andersen JN, Myers MP, Meng TC, Hinks JA, et al. 2003. Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* 423:769–73

35c. Van Montfort RL, Congreve M, Tisi D, Carr R, Jhoti H. 2003. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* 423:773–7

36. Williams CH Jr. 1992. Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase, and mercuric ion reductase—a family of flavoenzyme transhydrogenases. In *Chemistry and Biochemistry of Flavoenzymes*, ed. F Müller, 3:121–211. Boca Raton, FL: CRC Press

37. Crane EJ III, Ward DE, van der Oost J, She Q, Garrett R. 1999. Sequence analysis and overexpression of putative NADH oxidases from the hyperthermophilic arecheaons *Sulfolobus solfataricus* and *Pyrococcus horikoshii*. In *Flavins and Flavoproteins 1999*, ed. S Ghisla, P Kroneck, P Macheroux, H Sund, pp. 281–84. Berlin: Agency Sci. Publ.

38. Ward DE, Donnelly CJ, Mullendore ME, van der Oost J, de Vos WM, Crane EJ III. 2001. The NADH oxidase from *Pyrococcus furiosus*. Implications for the protection of anaerobic hyperthermophiles against oxidative stress. *Eur. J. Biochem.* 268:5816–23

39. Reed DW, Millstein J, Hartzell PL. 2001. $H_2O_2$-forming NADH oxidase with diaphorase (cytochrome) activity from *Archaeoglobus fulgidus*. *J. Bacteriol.* 183:7007–16

40. Karplus PA, Schulz GE. 1989. Substrate binding and catalysis by glutathione reductase as derived from refined enzyme: substrate crystal structures at 2 Å resolution. *J. Mol. Biol.* 210:163–80

41. Stehle T, Ahmed SA, Claiborne A, Schulz GE. 1991. Structure of NADH peroxidase from *Streptococcus faecalis* 10C1. *J. Mol. Biol.* 221:1325–55

42. Crane EJ III, Parsonage D, Claiborne A. 1996. The active-site histidine-10 of enterococcal NADH peroxidase is not essential for catalytic activity. *Biochemistry* 35:2380–87

43. Miller H, Claiborne A. 1991. Peroxide modification of monoalkylated glutathione reductase. Stabilization of an active-site cysteine-sulfenic acid. *J. Biol. Chem.* 266:19342–50

44. Becker K, Gui M, Schirmer RH. 1995. Inhibition of human glutathione reductase by S-nitrosoglutathione. *Eur. J. Biochem.* 234:472–78

45. Keese MA, Bose M, Mulsch A, Schirmer RH, Becker K. 1997. Dinitrosyl-dithiol-iron complexes, nitric oxide (NO) carriers in vivo, as potent inhibitors of human glutathione reductase and glutathione-S-transferase. *Biochem. Pharmacol.* 54: 1307–13

46. Becker K, Savvides SN, Keese M, Schirmer RH, Karplus PA. 1998. Enzyme inactivation through sulfhydryl oxidation by physiologic NO-carriers. *Nat. Struct. Biol.* 5:267–71

47. Hogg N. 2002. The biochemistry and physiology of S-nitrosothiols. *Annu. Rev. Pharmacol. Toxicol.* 42:585–600

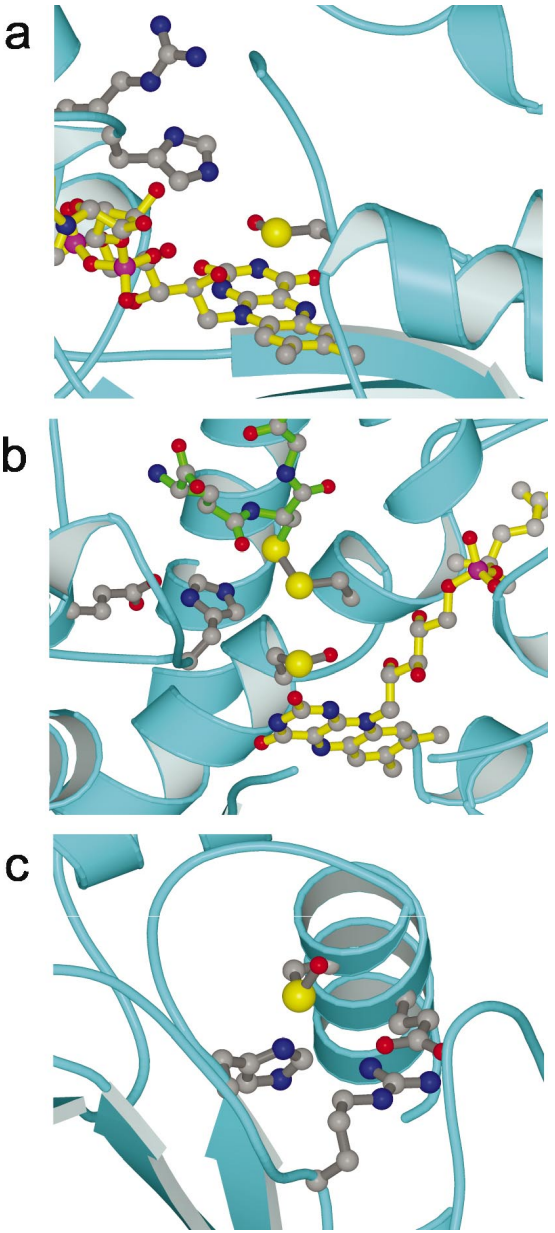
48. Miller H, Mande SS, Parsonage D, Sarfaty SH, Hol WG, Claiborne A. 1995. An L40C mutation converts the cysteine-sulfenic acid redox center in enterococcal NADH peroxidase to a disulfide. *Biochemistry* 34:5180–90

49. Link AJ, Robison K, Church GM. 1997. Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. *Electrophoresis* 18:1259–313
50. Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG. 1994. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc. Natl. Acad. Sci. USA* 91:7017–21
51. Seaver LC, Imlay JA. 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J. Bacteriol.* 183:7173–81
52. Poole LB, Reynolds CM, Wood ZA, Karplus PA, Ellis HR, Calzi ML. 2000. AhpF and other NADH:peroxiredoxin oxidoreductases, homologues of low Mr thioredoxin reductase. *Eur. J. Biochem.* 267:6126–33
53. Ellis HR, Poole LB. 1997. Roles for the two cysteine residues of AhpC in catalysis of peroxide reduction by alkyl hydroperoxide reductase from *Salmonella typhimurium*. *Biochemistry* 36:13349–56
54. Fuangthong M, Helmann JD. 2002. The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine-sulfenic acid derivative. *Proc. Natl. Acad. Sci. USA* 99:6690–95
55. Kim SO, Merchant K, Nudelman R, Beyer WF, Keng T, et al. 2002. OxyR: a molecular code for redox-related signaling. *Cell* 109:383–96
56. Poole LB. 2003. Bacterial peroxiredoxins. In *Signal Transduction by Reactive Oxygen and Nitrogen Species: Pathways and Chemical Principles*, ed. M Torres, JM Fukuto, HJ Forman, pp. 80–101. Dordrecht, The Netherlands: Kluwer Acad.
57. Cha MK, Kim HK, Kim IH. 1995. Thioredoxin-linked 'thiol peroxidase' from periplasmic space of *Escherichia coli*. *J. Biol. Chem.* 270:28635–41
58. Jeong W, Cha MK, Kim IH. 2000. Thioredoxin-dependent hydroperoxide peroxidase activity of bacterioferritin comigratory protein (BCP) as a new member of the thiol-specific antioxidant protein (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family. *J. Biol. Chem.* 275:2924–30
59. Moore TDE, Sparling PF. 1996. Interruption of the *gpxA* gene increases the sensitivity of *Neisseria meningitidis* to paraquat. *J. Bacteriol.* 178:4301–5
60. King KY, Horenstein JA, Caparon MG. 2000. Aerotolerance and peroxide resistance in peroxidase and PerR mutants of *Streptococcus pyogenes*. *J. Bacteriol.* 182:5290–99
61. Mongkolsuk S, Praituan W, Loprasert S, Fuangthong M, Chamnongpol S. 1998. Identification and characterization of a new organic hydroperoxide resistance (*ohr*) gene with a novel pattern of oxidative stress regulation from *Xanthomonas campestris* pv. *phaseoli*. *J. Bacteriol.* 180:2636–43
62. Atichartpongkul S, Loprasert S, Vattanaviboon P, Whangsuk W, Helmann JD, Mongkolsuk S. 2001. Bacterial Ohr and OsmC paralogues define two protein families with distinct functions and patterns of expression. *Microbiology* 147:1775–82
63. Lesniak J, Barton WA, Nikolov DB. 2002. Structural and functional characterization of Ohr, an organic hydroperoxide resistance protein from *Pseudomonas aeruginosa*. *EMBO J.* 21:6649–59
64. Renato J, Cussiol JR, Alves SV, Oliveira MA, Netto LES. 2003. Organic hydroperoxide resistance gene encodes a thiol-dependent peroxidase. *J. Biol. Chem.* 278: 11570–78
65. Jin D-Y, Jeang K-T. 2000. Peroxiredoxins in cell signaling and HIV infection. In *Antioxidant and Redox Regulation of Genes*, ed. CK Sen, H Sies, PA Baeuerle, pp. 381–407. San Diego: Academic
66. Wen ST, Van Etten RA. 1997. The PAG gene product, a stress-induced protein

with antioxidant properties, is an Abl SH3-binding protein and a physiological inhibitor of c-Abl tyrosine kinase activity. *Genes Dev.* 11:2456–67
67. Georgiou G, Masip L. 2003. An overoxidation journey with a return ticket. *Science* 300:592–94
68. Wood ZA, Poole LB, Hantgan RR, Karplus PA. 2002. Dimers to doughnuts: redox-sensitive oligomerization of 2-cysteine peroxiredoxins. *Biochemistry* 41: 5493–504
69. Kitano K, Niimura Y, Nishiyama Y, Miki K. 1999. Stimulation of peroxidase activity by decamerization related to ionic strength: AhpC protein from *Amphibacillus xylanus*. *J. Biochem* 126:313–19
70. Chauhan R, Mande SC. 2001. Characterization of the *Mycobacterium tuberculosis* H37Rv alkyl hydroperoxidase AhpC points to the importance of ionic interactions in oligomerization and activity. *Biochem. J.* 354:209–15
71. König J, Lotte K, Plessow R, Brockhinke A, Baier M, Dietz KJ. 2003. Reaction mechanism of plant 2-Cys peroxiredoxin: role of the C-terminus and the quarternary structure. *J. Biol. Chem.* 278:24409–20
72. Chang TS, Jeong W, Choi SY, Yu S, Kang SW, Rhee SG. 2002. Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. *J. Biol. Chem.* 277:25370–76
73. Schröder E, Willis AC, Ponting CP. 1998. Porcine natural-killer-enhancing factor-B: oligomerisation and identification as a calpain substrate in vitro. *Biochim. Biophys. Acta* 1383:279–91
74. Cha M-K, Yun C-H, Kim IH. 2000. Interaction of human thiol-specific antioxidant protein 1 with erythrocyte plasma membrane. *Biochemistry* 39:6944–50
75. Koo KH, Lee S, Jeong SY, Kim ET, Kim HJ, et al. 2002. Regulation of thioredoxin peroxidase activity by C-terminal truncation. *Arch. Biochem. Biophys.* 397:312–18
76. Mitsumoto A, Takanezawa Y, Okawa K, Iwamatsu A, Nakagawa Y. 2001. Variants of peroxiredoxins expression in response to hydroperoxide stress. *Free Radic. Biol. Med.* 30:625–35
77. Rabilloud T, Heller M, Gasnier F, Luche S, Rey C, et al. 2002. Proteomics analysis of cellular response to oxidative stress: evidence for in vivo over-oxidation of peroxiredoxins at their active site. *J. Biol. Chem.* 277:19396–401
78. Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, et al. 2002. Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J. Biol. Chem.* 277:38029–36
79. Schröder E, Littlechild JA, Lebedev AA, Errington N, Vagin AA, Isupov MN. 2000. Crystal structure of decameric 2-Cys peroxiredoxin from human erythrocytes at 1.7 Å resolution. *Structure* 8:605–15
80. Alphey MS, Bond CS, Tetaud E, Fairlamb AH, Hunter WN. 2000. The structure of reduced tryparedoxin peroxidase reveals a decamer and insight into reactivity of 2Cys-peroxiredoxins. *J. Mol. Biol.* 300:903–16
81. Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. 1998. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J. Biol. Chem.* 273:6297–302
82. Moskovitz J, Rahman MA, Strassman J, Yancey SO, Kushner SR, et al. 1995. *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. *J. Bacteriol.* 177:502–7
83. Boschi-Muller S, Azza S, Sanglier-Cianferani S, Talfournier F, Van Dorsselear A, Branlant G. 2000. A sulfenic acid enzyme intermediate is involved in the catalytic mechanism of peptide methionine sulfoxide reductase from *Escherichia coli*. *J. Biol. Chem.* 275:35908–13
84. Lowther WT, Weissbach H, Etienne F,

Brot N, Matthews BW. 2002. The mirrored methionine sulfoxide reductases of *Neisseria gonorrhoeae pilB*. *Nat. Struct. Biol.* 9:348–52

85. Weissbach H, Etienne F, Hoshi T, Heinemann SH, Lowther WT, et al. 2002. Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. *Arch. Biochem. Biophys.* 397:172–78
86. Davis DA, Newcomb FM, Moskovitz J, Fales HM, Levine RL, Yarchoan R. 2002. Reversible oxidaiton of HIV-2 protease. *Methods Enzymol.* 348:249–59
87. Olry A, Boschi-Muller S, Marraud M, Sanglier-Cianferani S, Van Dorsselear A, Branlant G. 2002. Characterization of the methionine sulfoxide reductase activities of PILB, probably a virulence factor from *Neisseria meningitidis*. *J. Biol. Chem.* 277:12016–22
88. Vougier S, Mary J, Friguet B. 2003. Subcellular localisation of methionine sulphoxide reductase (MsrA): evidence for mitochondrial and cytosolic isoforms in rat liver cells. *Biochem. J.* 373:531–37
89. Pineda-Molina E, Klatt P, Vázquez J, Marina A, García de Lacoba M, et al. 2001. Glutathionylation of the p50 subunit of NF-kappaB: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 40:14134–42
90. Pineda-Molina E, Lamas S. 2002. S-glutathionylation of NF-kappaB subunit p50. *Methods Enzymol.* 359:268–79
91. Storz G, Tartaglia LA, Ames BN. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* 248:189–94
92. Kullik I, Toledano MB, Tartaglia LA, Storz G. 1995. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. *J. Bacteriol.* 177:1275–84
93. Zheng M, Åslund F, Storz G. 1998. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279:1718–21
94. Choi H, Kim S, Mukhopadhyay P, Cho S, Woo J, et al. 2001. Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105:103–13
95. Helmann JD. 2002. OxyR: a molecular code for redox sensing? *Science's STKE*. www.stke.org/cgi/content/full/sigtra ns; 2002/157/pe46
96. Georgiou G. 2002. How to flip the (redox) switch. *Cell* 111:607–10
97. Cooper CE, Patel RP, Brookes PS, Darley-Usmar VM. 2002. Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. *Trends Biochem. Sci.* 27:489–92
98. Dickens C. 1859. *A Tale of Two Cities*. London: Chapman Hall
99. Mongkolsuk S, Helmann JD. 2002. Regulation of inducible peroxide stress responses. *Mol. Microbiol.* 45:9–15
100. Panmanee W, Vattanaviboon P, Eiamphungporn W, Whangsuk W, Sallabhan R, Mongkolsuk S. 2002. OhrR, a transcription repressor that senses and responds to changes in organic peroxide levels in *Xanthomonas campestris* pv. phaseoli. *Mol. Microbiol.* 45:1647–54
101. Inoue Y, Matsuda T, Sugiyama K, Izawa S, Kimura A. 1999. Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 274:27002–9
102. Flohé L. 1989. The selenoprotein glutathione peroxidase. In *Glutathione. Chemical, Biochemical and Medical Aspects, Part A*, pp. 643–731. New York: Wiley




See legend on next page

**Figure 2** Cysteine sulfenic acids at the active sites of (*a*) enterococcal NADH peroxidase (2.1 Å version of 1joa), (*b*) S-nitrosoglutathione-inhibited glutathione reductase (1gsn at 1.7 Å), and (*c*) human peroxiredoxin VI (1prx at 2.0 Å). Shown are X-ray crystallographic structures depicted as ribbon diagrams for the protein backbones, and sidechain and cofactor atom colors of yellow = S, red = O, blue = N, gray = C, and pink = P. Yellow bonds highlight the flavin cofactors in panels (*a*) and (*b*), and green bonds indicate the position of the covalently bound glutathione in panel (*b*).

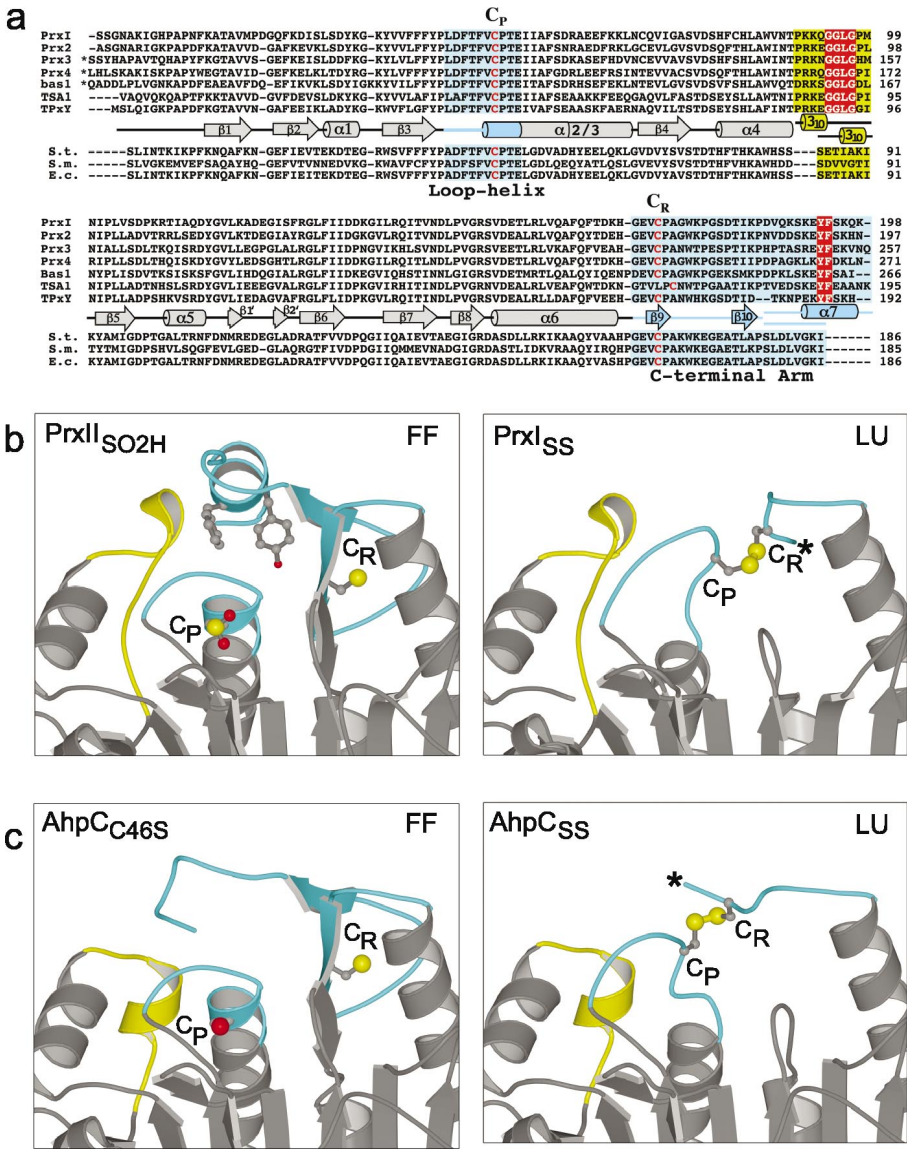



**Figure 4** Structural differences between robust and insensitive 2-Cys Prxs. (*a*) The sensitive Prxs (*top group of sequences*) contain several unique motifs (*red*) compared to robust bacterial Prxs (*lower group*). (*b,c*) Regions that change structure during catalysis (*blue*) are more ordered in the fully folded (FF) forms than in the disulfide-bonded, locally unfolded (LU) forms, and the blue regions of C46S AhpC are more mobile than those of Prx II $SO_2H$, facilitating rearrangement. Reprinted with permission from Reference 8. Copyright ©2003 American Association for the Advancement of Science.