Reduction of Cysteine Sulfinic Acid in Eukaryotic, Typical 2-Cys Peroxiredoxins by Sulfiredoxin

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Abstract

The eukaryotic, typical 2-Cys peroxiredoxins (Prxs) are inactivated by hyperoxidation of one of their active-site cysteine residues to cysteine sulfinic acid. This covalent modification is thought to enable hydrogen peroxide-mediated cell signaling and to act as a functional switch between a peroxidase and a high-molecular-weight chaperone. Moreover, hyperoxidation has been implicated in a variety of disease states associated with oxidative stress, including cancer and aging-associated pathologies. A repair enzyme, sulfiredoxin (Srx), reduces the sulfinic acid moiety by using an unusual ATP-dependent mechanism. In this process, the Prx molecule undergoes dramatic structural rearrangements to facilitate repair. Structural, kinetic, mutational, and mass spectrometry–based approaches have been used to dissect the molecular basis for Srx catalysis. The available data support the direct formation of Cys sulfinic acid phosphoryl ester and protein-based thiosulfinate intermediates. This review discusses the role of Srx in the reversal of Prx hyperoxidation, the questions raised concerning the reductant required for human Srx regeneration, and the deglutathionylating activity of Srx. The complex interplay between Prx hyperoxidation, other forms of Prx covalent modification, and the oligomeric state also are discussed. *Antioxid. Redox Signal.* 15, 99–109.

Introduction

THE PEROXIREDOXINS (Prxs) function as cysteine-dependent thiol peroxidases that detoxify hydrogen peroxide (H_2O_2), lipid peroxides, and peroxynitrate in a variety of biologic contexts and disease states. Given their high abundance within cells and reactivity with H_2O_2 (10^5–10^7 $M^{-1}s^{-1}$), Prxs are also ideally suited to regulate H_2O_2-mediated intracellular signaling (20, 68). Prxs are categorized by the number and location of Cys residues, and whether inter- or intramolecular disulfide bonds are formed with the adjacent monomer of the dimer during the normal catalytic cycle (21). The "peroxidatic" Cys (Cys-S_PH) of the typical 2-Cys or Prx1 subclass attacks a H_2O_2 molecule (Fig. 1) to form a Cys sulfenic acid (Cys-S_POH) intermediate. An intermolecular disulfide bond is then formed with the "resolving" Cys (Cys-S_RH), located at the C-terminus of the adjacent monomer, and ultimately reduced by thioredoxin (Trx). In addition to the large structural changes associated with disulfide bond formation, the Prx molecules predominantly cycle between dimeric and decameric (*i.e.,* five dimers) oligomeric states. The reduced decamer is the most active form (51, 73, 75). Other oligomeric states have been observed, but the physiological significance for the majority of these remains to be determined.

In contrast to prokaryotic, typical 2-Cys Prxs, the eukaryotic enzymes possess two architectural elements: an internal GGLG-containing loop and C-terminal YF motifs (74). The interaction between these motifs is thought to restrict the ability of the Cys-S_RH residue to approach the Cys-SOH moiety, and therefore decreases the rate of disulfide bond formation. As a result, the Cys-S_POH can react with a second H_2O_2 molecule and become hyperoxidized to the Cys sulfinic acid (Cys-$S_PO_2^-$) (70, 77). Under conditions of extreme oxidative stress, this latter species can be further oxidized to the Cys sulfonic acid (Cys-$S_PO_3^{2-}$). The hyperoxidation of 2-Cys Prxs can lead to the formation of spherical aggregates (Fig. 1) of very high molecular mass (>2,000 kDa), resulting in a switch in the enzymatic activity from a peroxidase to a molecular chaperone that can prevent the unfolding and precipitation of model proteins (4, 25, 26, 37). This alternative function is thought to be an important protection against oxidative stress; and in one study, it was shown to block the initiation of apoptosis (43). Hyperoxidation of human PrxII (hPrxII) has also resulted in the formation of filamentous aggregates and cell-cycle arrest (52).

Importantly, the Cys-$S_PO_2^-$ moiety can be reduced, and the peroxidase activity restored by an enzyme known as sulfiredoxin (Srx) (6, 69, 71). Another enzyme called sestrin was
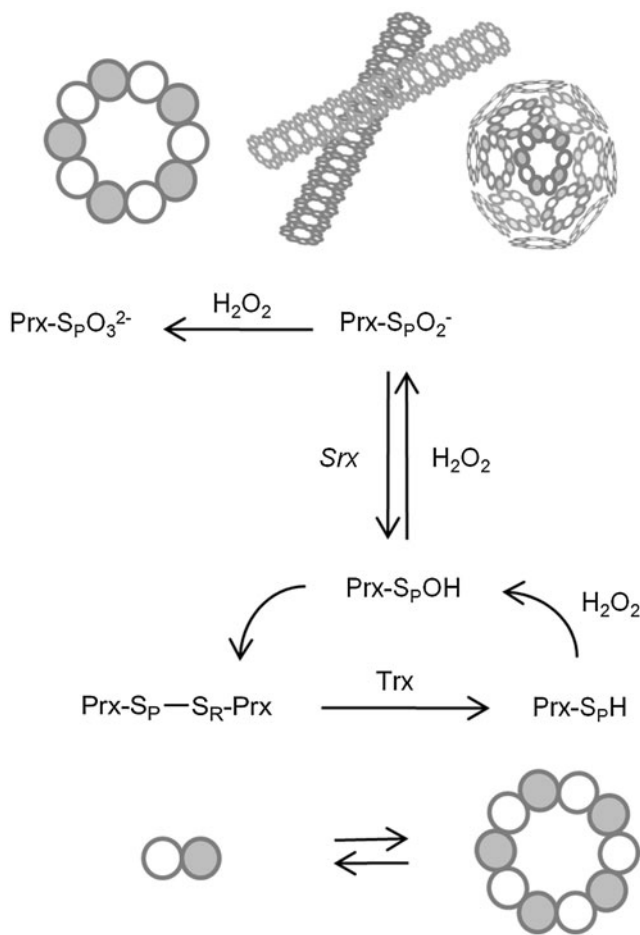
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FIG. 1. **Typical 2-Cys peroxiredoxin catalytic cycle and hyperoxidation.** Low levels of H_2O_2 are reduced by Prx through a pair of essential Cys residues, Cys-S_PH and Cys-S_RH. The sulfenic acid intermediate (Cys-S_POH) reacts with the Cys-S_RH residue to form an intermolecular disulfide bond, which is subsequently reduced by thioredoxin. During this process, the Prx molecules alternate between dimeric and decameric states. The reduced, decameric form of the protein is the most reactive with H_2O_2 (51, 73, 75). As the level of H_2O_2 increases, eukaryotic Prxs can react with a second H_2O_2 molecule to form the sulfinic acid form (Cy-s$S_PO_2^-$) and, as a result, are inactivated. This hyperoxidation stabilizes the decameric state of the Prx molecule and can lead to the formation of filamentous and spherical, high-molecular-weight species; depicted schematically here. The molecular details of these interactions are unknown. Further oxidation of the Prx molecule to the Cys sulfonic acid form (Cys$S_PO_3^{2-}$) can occur. Srx, however, can only reduce the Cys$S_PO_2^-$ moiety.

also initially thought to have sulfinic acid reductase activity, but this claim has recently been challenged by a careful analysis of the recombinant protein, transgenic expression in a variety of cells, and the knockout mouse (8, 69). Reversible Prx inactivation is an essential element of the floodgate hypothesis whereby H_2O_2 levels can increase in a localized manner, leading to downstream signaling events (21, 74). In addition, studies in yeast indicate that hyperoxidized Prx molecules can themselves function as a peroxide dosimeter and cellular stress signal (14, 20, 66). Thus, Srx-mediated repair of Prxs

represents a physiologically important process that can allow cells to return to homeostasis by turning off peroxide-based signaling and chaperone activity.

Humans have four typical 2-Cys Prx isoforms with different cellular compartmentalization and susceptibilities to hyperoxidation and inactivation (13, 21, 42, 54, 75). This inactivation can have serious systemic consequences, as evidenced by the increased oxidative stress found in the knock-out mice of PrxI, PrxII, and PrxIII and their development of anemia, splenomegaly, hypersensitivity to lipopolysaccharide challenge, and arterial thickening (12, 20, 40, 46). More-over, the hyperoxidation of Prxs is a biomarker for oxidative stress associated with doxorubicin (Adriamycin) treatment, leading to "chemobrain," Alzheimer disease, Parkinson disease, normal aging, and ischemia/reperfusion injury to transplanted liver and heart (3, 9,18, 34, 44, 57, 65, 78). The importance for the repair of 2-Cys Prxs is further underscored by the upregulation of Srx gene (*SRXN1*), an AP-1 and Nrf2 target, in skin cancer, immunostimulated macrophages, synaptic NMDA-receptor activity, cigarette-induced emphysema, and cardiac dysfunction (15, 60–62, 64, 67).

This review focuses on the current state of knowledge and open questions concerning the molecular basis for human Srx action and the complex interplay between Prx hyperoxidation, other forms of covalent modification, and the oligomeric state. The reader is directed to the following articles for insight into the roles Prxs and Srx play in chloroplast protection (24, 41, 45).

Sulfiredoxin, a Specific 2-Cys Prx Repair Enzyme

Srx was first identified in *Saccharomyces cerevisiae* as a gene induced by H_2O_2 treatment (6). The isolation of disulfide bond–mediated complexes between Srx and the yeast 2-Cys Prx, Tsa1, suggested that Srx may be involved in modulating the redox state of Prxs. Further analysis showed that Srx was able to reduce the hyperoxidized form of Tsa1 in a process dependent on the addition of ATP-Mg^{2+}, the presence of a conserved Cys residue, and an exogenous reductant (*i.e.,* dithiothreitol, Trx, or glutathione). Subsequent studies with rat, human, yeast, and plant Srxs have confirmed these requirements and determined the affinity for ATP to be ~ 6–$30\,\mu M$ (11, 27, 32, 71). GTP, dATP, and dGTP also support the reaction, but the relevance of these nucleotide forms has not been investigated (11). The K_M values for human Trx1 and glutathione (GSH) ($1.2\,\mu M$ and $1.8\,\text{m}M$, respectively) suggest that either could be the physiological reductant for the Srx reaction (11). As described in more detail later, however, questions remain as to the role of the exogenous reductant in the overall mechanistic scheme. Interestingly, the k_{cat} values for the rat, human, and *Arabidopsis thaliana* Srx range from 0.1 to $1.8\,\text{min}^{-1}$ (11, 24, 28, 56). Thus, Srx is an inefficient enzyme. It is thought that this low activity is of physiological relevance, as the Prx molecules may require slow repair so that downstream, H_2O_2-mediated signaling events can be potentiated.

Srx is highly conserved between species (Fig. 2) and found only in eukaryotic organisms, with *Caenorhabditis elegans* as a notable exception, currently without explanation (30). Bacteria apparently do not need Srx, as their Prxs are not readily hyperoxidized (74). Human Srx exhibits a ubiquitous tissue distribution, although the expression level varies greatly (11). Srx is localized predominantly in the cytosol and can repair
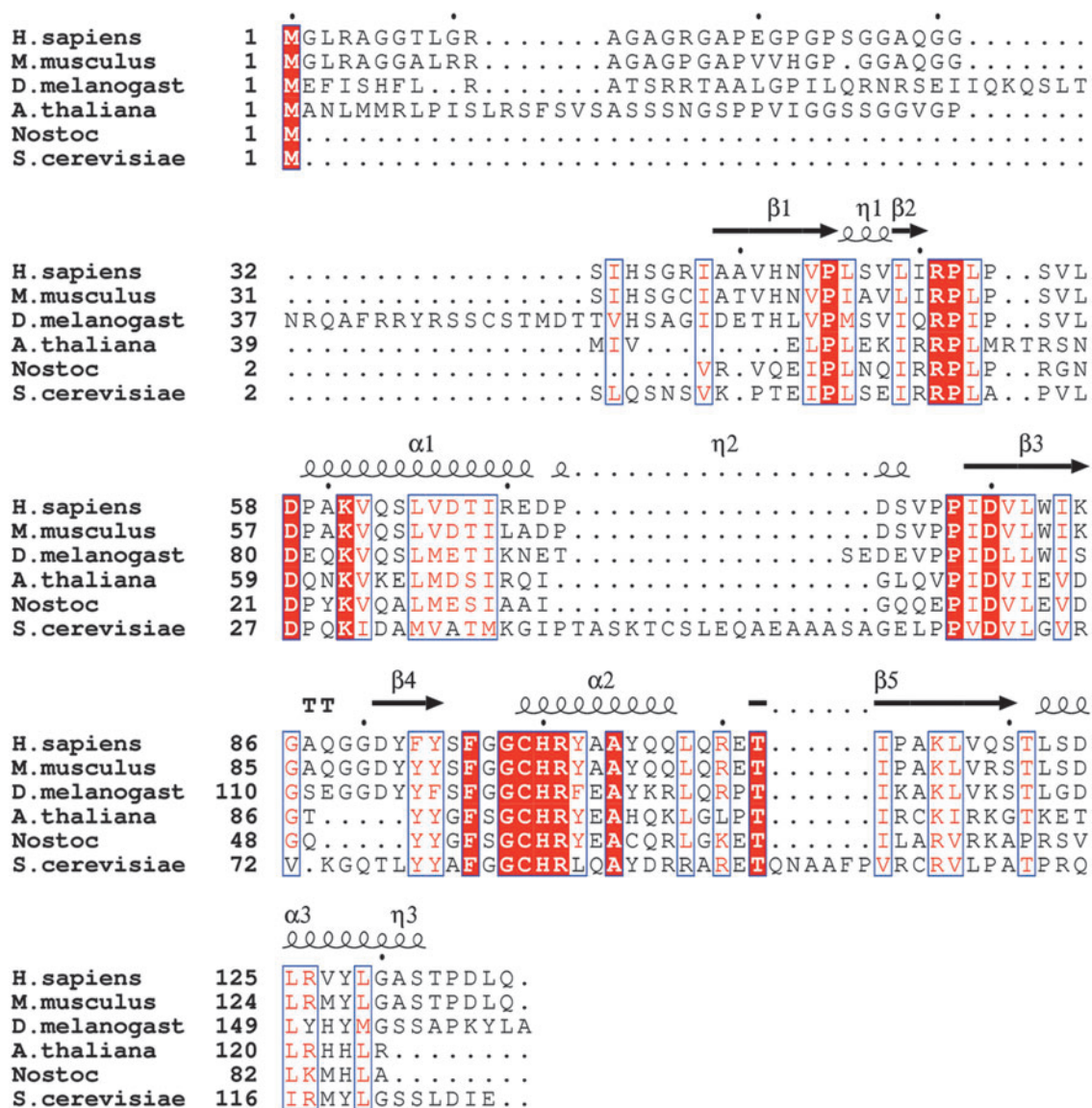
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                              β1        η1 β2
H.sapiens     1  MGLRAGGTLGR.......AGAGRGAPEGPGPSGGAQGG.......
M.musculus    1  MGLRAGGALRR.......AGAGPGAPVVHGP.GGAQGG.......
D.melanogast  1  MEFISHFL..R.......ATSRRTAALGPILQRNRSEIIQKQSLT
A.thaliana    1  MANLMMRLPISLRSFSVSASSSNGSPPVIGGSSGGVGP.......
Nostoc        1  M............................................
S.cerevisiae  1  M............................................

H.sapiens    32  .................SIHSGRIAAVHNVPLSVLIRPLP..SVL
M.musculus   31  .................SIHSGCIATVHNVPIAVLIRPLP..SVL
D.melanogast 37  NRQAFRRYRSSCSTMDTTVHSAGIDETHLVPMSVIQRPIP..SVL
A.thaliana   39  ..................MIV.......ELPLEKIRRPLMRTRSN
Nostoc        2  ................VR.VQEIPLNQIRRPLP..RGN
S.cerevisiae  2  ................SLQSNSVK.PTEIPLSEIRRPLA..PVL

                    α1                    η2              β3
H.sapiens    58  DPAKVQSLVDTIREDP.................DSVPPIDVLWIK
M.musculus   57  DPAKVQSLVDTILADP.................DSVPPIDVLWIK
D.melanogast 80  DEQKVQSLMETIKNET.................SEDEVPPIDLLWIS
A.thaliana   59  DQNKVKELMDSIRQI.................GLQVPIDVIEVD
Nostoc       21  DPYKVQALMESIAAI.................GQQEPIDVLEVD
S.cerevisiae 27  DPQKIDAMVATMKGIPTASKTCSLEQAEAAASAGELPPVDVLGVR

         β4            α2                  β5
      TT
H.sapiens    86  GAQGGDYFYSFGGCHRYAAYQQLQRET......IPAKLVQSTLSD
M.musculus   85  GAQGGDYYYSFGGCHRYAAYQQLQRET......IPAKLVRSTLSD
D.melanogast 110 GSEGGDYYFSFGGCHRFEAYKRLQRPT......IKAKLVKSTLGD
A.thaliana   86  GT.....YYGFSGCHRYEAHQKLGLPT......IRCKIRKGTKET
Nostoc       48  GQ.....YYGFSGCHRYEACQRLGKET......ILARVRKAPRSV
S.cerevisiae 72  V.KGQTLYYAFGGCHRLQAYDRRARETQNAAFPVRCRVLPATPRQ

         α3       η3
H.sapiens    125 LRVYLGASTPDLQ.
M.musculus   124 LRMYLGASTPDLQ.
D.melanogast 149 LYHYMGSSAPKYLA
A.thaliana   120 LRHHLR........
Nostoc        82 LKMHLA........
S.cerevisiae 116 IRMYLGSSLDIE..
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FIG. 2. Sequence alignment of representative sulfiredoxins. Murine, *Drosophila*, *Arabidopsis*, *Nostoc* species PCC7120 (a cyanobacterium), and *S. cerevisiae* Srxs show 91%, 60%, 43%, 41%, and 33% sequence identity to human Srx, respectively. The secondary structural elements for hSrx are shown above the alignment: α, α-helices; β, β-strands; η, 3_{10} helices. The residues highlighted by the red background and white lettering are strictly conserved. Residues that are either conserved in the majority of the proteins or have conservative substitutions are boxed in blue and colored red. The black dots above the alignment indicate every tenth residue of human Srx. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

PrxI and PrxII. Srx can also be imported into the mitochondria to repair PrxIII during stress conditions, despite not having a canonical mitochondrial targeting signal (47). Human PrxIV within the ER is also repaired by Srx *in vitro*, but whether this occurs *in vivo* is unclear. Therefore, Srx can bind to and repair all of the 2-Cys subclass of human Prxs, PrxI-IV (71). In contrast, Srx is not able to bind to or reduce the Cys sulfinic acid within the atypical 2-Cys PrxV, which uses an intramolecular disulfide bond during catalysis, and the 1-Cys PrxVI. Srx also cannot repair glyceraldehyde-3-phosphate dehydrogenase. As described later, the specificity of Srx for 2-Cys Prxs makes sense, given the unique interaction and chemical reaction between the two molecules.

Molecular Basis for Srx Action

In the first step of the original mechanism proposed by the Toledano laboratory (Fig. 3, gray shaded region), the Cys-$S_PO_2^-$ moiety (Cys52 in human PrxI, hPrxI) is phosphorylated by the γ-phosphate of ATP to form the sulfinic phosphoryl ester (Cys-$S_PO_2PO_3^{2-}$) (6). This type of ATP-mediated activation is reminiscent of the activation of carboxyl groups in a variety of biologic processes, but is novel for sulfur chemistry (7, 16, 17). A thiosulfinate intermediate (Prx-S_PO-S-Srx) is then formed, after the attack of a conserved Cys residue in Srx (Cys[99] in hSrx). GSH or Trx could then facilitate the collapse of the thiosulfinate to release the repaired Prx molecule in the
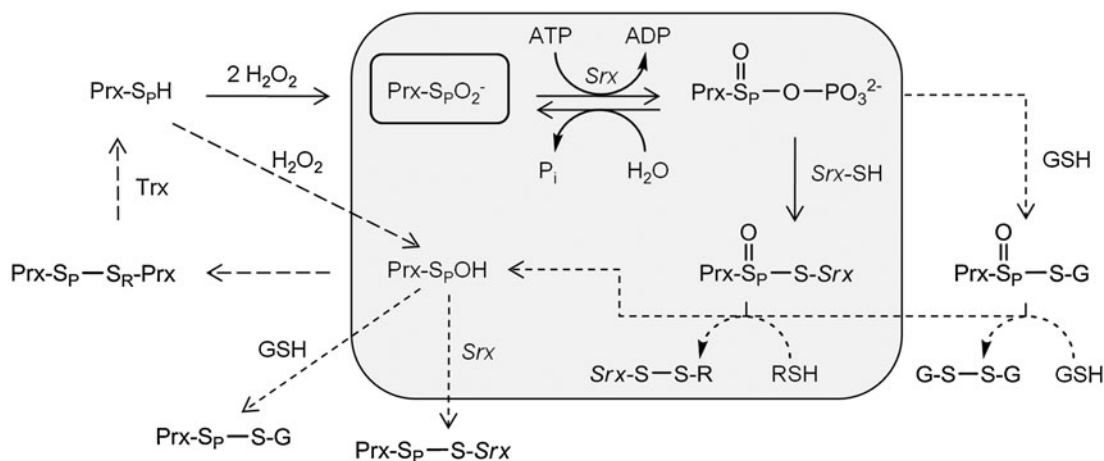
FIG. 3. Sulfiredoxin reaction mechanism and intermediates. The original mechanism, based on the analysis of *S. cerevisiae* Srx (gray shading), relies on the formation of sulfinic phosphoryl ester (Cys-$S_PO_2PO_3^{2-}$) and a thiosulfinate intermediate (Prx-S_PO-S-Srx) between the Srx and Prx molecules (6). Structural and biochemical data support the direct formation of the former intermediate (see text for details). The Srx-Prx thiosulfinate intermediate has been confirmed for the yeast and human enzyme systems (33, 55). On reduction of this thiosulfinate with GSH or Trx (R-SH), the repaired Prx molecule (Prx-S_POH) can return to the Prx catalytic cycle (*long dashed lines*). A recent study showed that yeast Srx, which contains an additional Cys residue within a loop insertion (Fig. 2; also see the regions highlighted in green in Fig. 4), can resolve the Srx-Prx thiosulfinate through the formation of an intramolecular disulfide bond [Srx-(S-S)] (56). Alternative reaction paths and intermediates between Srx, Prx, and GSH (*short dashed lines and arrows*) remain to be investigated.

Cys-S_POH state, which can return to the Prx catalytic cycle. Subsequent studies by several laboratories have used structural, kinetic, mutational, and mass spectrometry–based approaches to dissect this mechanistic proposal and to understand the molecular basis for Srx catalysis. Along the way, alternative scenarios have been proposed and tested. New questions have also been raised, particularly with regard to the identity and role of the reductant in the regeneration of Srx for another round of catalysis.

Novel structural features of Srx

The structures of human Srx alone and in complex with different ligands have been determined by x-ray crystallography and NMR (PDB codes 1XW3, 1XW4, 3CYI, and 1YZS) (31, 32, 38). Structures of Srx from other organisms are currently not available. Srx exhibits a novel three-dimensional fold with some sequence similarity to the parB domain fold, the chromosomal segregation protein Spo0J, and a protein of unknown function (5, 38). The latter two proteins contain an additional domain, and it is not known whether these proteins bind ATP or have reductase activity. The ATP•Mg^{2+} and ADP complexes of Srx (Fig. 4) reveal a unique nucleotide-binding motif that is generated by the following residues: Lys[61], Ser[64], Thr[68], His[100] and Arg[101]. Cys[99] interacts with Arg[51] (not shown) at the bottom of the pocket and exhibits a pK_a of ~ 7.3 (11). Mutational analyses have confirmed the importance of these residues to ATP binding and catalysis (6, 24, 27, 32, 55). The Mg^{2+} ion interacts with all three phosphate groups of ATP, resulting in the projection of the γ-phosphate away from the protein toward solvent. A large, predominantly hydrophobic pocket is located adjacent to the ATP-binding site (Fig. 4B), which, at this stage of the investigation, was proposed to be a key element of the Srx-Prx interface (32).

The Srx nucleotide motif does show some resemblance to the phospho-Tyr binding site of the protein tyrosine phosphatase PTP1B (48). The phosphate-binding motif of PTP1B, however, replaces His[100] and Arg[101] of Srx with several main-chain amide groups. Importantly, the Cys residue of PTP1B is positioned for a direct attack of the phosphate moiety. In contrast, the sulfur atom of Cys[99] of Srx is ~ 5 Å directly below the γ-phosphate of ATP (Fig. 4B) and positioned incorrectly for phosphate transfer, suggesting that transfer to this residue would not be favorable. Nonetheless, as described in the biochemical experiments to characterize reaction intermediates in the subsequent sections, phosphorylation of the C99S Srx variant is possible to a minor extent (27). This finding resulted in an alternative proposal in which Srx accepts the phosphate moiety first and then transfers this group to the Prx sulfinic acid. The analysis of additional mutants and the determination of the Srx•ATP•Mg^{2+}•PrxI complex, however, support a direct in-line attack by the Prx Cys-$S_PO_2^-$ moiety (27, 29, 31).

The Srx-Prx embrace:
active-site and backside interfaces

One of the conundrums of Srx-mediated repair is exemplified by the crystal structure of hPrxII in the hyperoxidized state (30, 58). In this structure, the Cys-SO_2^- moiety is not accessible to Srx because of its stable interaction with a conserved Arg residue and the presence of the overlying GGLG and YF motifs. Therefore, the helix containing Cys-SO_2^- must partially unfold, an attribute already known to occur during normal catalysis, to allow an attack on the ATP molecule within the Srx active site (21). Moreover, the YF motif must change conformation (*i.e.*, the entire C-terminus of the adjacent Prx molecule must move out of the way). A variety of complexes of human PrxI with Srx have been successfully determined with the implementation of protein engineering. In these efforts, strategic site-directed mutants have been generated within the active sites of both molecules, the
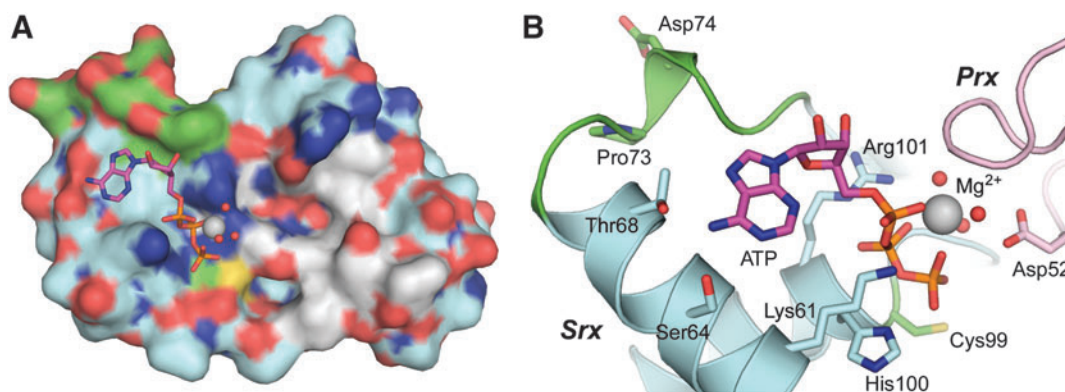
FIG. 4. Surface features and nucleotide-binding motif of sulfiredoxin. (A) Surface representation of the ATP•Mg^{2+} complex (PDB code 3CYI) (31). Residues lining the hydrophobic pockets near the γ-phosphate (orange) and Mg^{2+} ion (gray) are highlighted in white. Blue and red surface features indicate the nitrogen and oxygen atoms of the surface side chains. The location of the Cys-containing loop insertion in yeast Srx and Cys^{99} of human Srx are highlighted in green. **(B)** Closeup of the human Srx active site. The novel ATP-binding motif of Srx consists of Lys^{61}, Ser^{64}, Thr^{68}, His^{100}, and Arg^{101}. Cys^{99} is located at the bottom of the active site ~ 5 Å away from the γ-phosphate of ATP. In this image from an engineered Srx(C99A)• PrxI(C52D)•ATP•Mg^{2+} complex (PDB code 3HY2), Asp52 mimics the incoming sulfinic acid moiety (see text and Fig. 5 for additional details) (29). The Mg^{2+} ion and its associated water molecules are shown as gray and red spheres, respectively. The position of Cys^{99} (green) was modeled from the crystal structure of wild-type, human Srx in complex with ATP•Mg^{2+} in panel **A**. Pro73 and Asp74 have been labeled and colored green to indicate the location of the 17 residue, Cys-containing insert found in *S. cerevisiae* Srx (Fig. 2). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

C-terminus of the Prx molecule, and the Prx dimer-dimer interface. It was also necessary to screen different N-terminal truncation variants of Srx, a common technique used in x-ray crystallography. The remarkable structural rearrangements observed in the Prx molecule support the inability to predict computationally this unique interaction between these two proteins (38).

The first crystal structure of the human Srx•PrxI complex (PDB code 2RII) was made possible by mimicking the proposed thiosulfinate intermediate (Fig. 3) with a disulfide bond between the two active-site Cys residues (28). Importantly, disulfide-bonded Srx-Prx complexes have also been observed *in vivo* and *in vitro* (6, 27, 55). To form the disulfide between Cys^{99} of Srx and Cys^{52} of PrxI, the remaining Cys residues of PrxI were mutated to Ser to stabilize the complex and to prevent disulfide shuffling. No mutations were required in Srx, as it only has one Cys residue. A step-wise process involving the formation of a thio-2-nitrobenzoic acid adduct of PrxI and the subsequent addition of Srx generated the Srx•PrxI complex (*i.e.,* each Prx molecule of the decamer is in complex with one Srx molecule). To increase the diffraction quality of the crystals, a mutation was also made at the dimer–dimer interface. The mutation of Cys^{83} to Glu results in the juxtaposition of two negative charges and the disruption of the decamer into dimeric units (22, 51). Crystals of the latter complex diffracted to 2.6-Å resolution and revealed the interaction between the two molecules. Moreover, the superposition of this dimeric structure onto the hPrxII-SO$_2^-$ structure enabled a model of the full, toroidal complex (Fig. 5A) to be made. Two interfaces between the molecules were observed: between the active-site regions of both proteins and the "backside" of Srx with the C-terminus of the adjacent Prx molecule (Fig. 5B).

The active-site interface showed that the helix containing the Cys-S$_P$H residue did unfold to establish the disulfide bond

with Cys^{99} of Srx (28). This change placed Phe^{50} of PrxI within the primarily hydrophobic surface pocket (Figs. 4A and 5C) generated by Leu^{53}, Asp^{80}, Leu^{82}, Phe^{96}, Val^{118}, Val^{127}, and Tyr^{128} of Srx. Analysis of the toroid model (Fig. 5A) also indicates that Phe^{26}, Phe^{82}, and Leu^{85} of PrxI may contribute to this pocket. To determine the structure of the quaternary complex between Srx, PrxI, ATP, and Mg^{2+}, the engineered disulfide bond was moved to the backside interface, described in more detail later, between residue 43 of Srx and residue 185 of PrxI (29). In an effort to approximate the Cys-SO$_2^-$ moiety, Cys^{52} was mutated to Asp (*i.e.,* substitution of the sulfur atom for a carbon atom; R-SO$_2^-$ *vs.* R-CO$_2^-$). These modifications enabled crystals to be soaked with ATP and Mg^{2+}. The resulting complex (Fig. 5C, PDB code 3CYI) recapitulated the docking of Phe^{50} within the Srx pocket and the unwinding of the active-site helix. Moreover, the sulfinic acid mimic was within ~ 4 Å of the γ-phosphate atom of ATP and positioned correctly for an inline attack. The quaternary complex also revealed the role of the Mg^{2+} ion to orient the γ-phosphate of ATP and the possibility that the GGLG motif and backbone atoms of the preceding three residues, Gln^{92}, Arg^{93}, and Arg^{94}, may play a role in the Srx–Prx interaction.

On closer inspection of dimeric Srx-PrxI complex structure (Fig. 5B), it was a surprise to find that the C-termini of the Prx molecules, containing the YF motif, completely unfolded to "embrace" the adjacent Srx molecules (28). Fluorescence anisotropy studies and activity analyses of site-directed mutants showed that this backside interface (Fig. 5D) was conserved and essential for Srx binding and repair. The necessity for the C-terminus of 2-Cys Prxs to bind Srx highlights its varied cellular roles. For example, the interaction of the hPrxI C-terminus with the PDZ domain of Omi/HtrA2 is necessary to promote protease activity (23). The interactions with the Abl and Myc proteins, MIF, phospholipase D1, and the PDGF receptor also raise the possibility that the binding of the
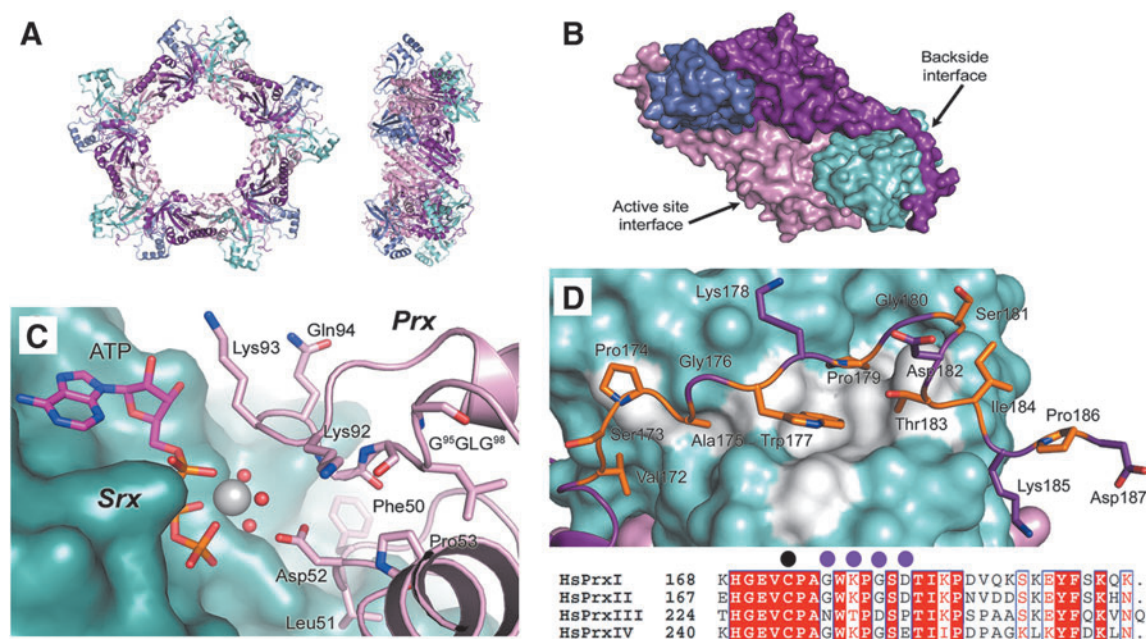
FIG. 5.   The human Srx•PrxI complex. (A) Front and side views of the toroidal Srx-PrxI complex model containing 10 Prx (pink/purple) and 10 Srx molecules (blue/cyan) (28). **(B)** Surface representation of one Prx dimer and its active-site and backside interactions with two Srx molecules. **(C)** Close-up of the active-site interface in the Srx(C99A)•PrxI(C52D)• ATP•Mg^{2+} complex. Same coloring scheme used as in Fig. 4B. **(D)** Close-up of the backside interface highlighting the local secondary structure of the PrxI C-terminus. In this complex, the resolving Cys residue, Cys^{173}, was mutated to Ser, indicated by the black dot in the sequence alignment. The white surface on the Srx molecule highlights conserved residues. Orange highlighting on PrxI indicates conserved residues that interact with Srx. The purple dots on the alignment denote those residues that are different for PrxIII. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

Pro-rich C-terminus of Prx to Srx represents a general mechanism for 2-Cys Prxs to associate with key regulatory or signaling proteins (12, 35, 36, 76). It is also important to note that hPrxIII has four key substitutions in this region (alignment in Fig. 5D) and is considerably more resistant to hyperoxidation than are hPrxI and hPrxII (13). Thus, it is intriguing to speculate that these substitutions in some way affect the hyperoxidation process and may also influence repair by Srx.

Cys-sulfinic phosphoryl ester formation

In an effort to stabilize and trap the phosphorylated intermediate in the first step of the Srx reaction (Fig. 3), Jeong *et al.* (27) mutated the catalytic Cys^{99} of hSrx to Ser and Ala, known to inactivate the protein and to still allow ATP binding (27). Analysis of the reactions, through the use of [γ-^{32}P]-ATP, SDS-PAGE, and autoradiography, revealed that less than 1% of Ser^{99} had been phosphorylated when incubated for 4 h with wild-type, hyperoxidized hPrxI, but not reduced hPrxI. These data were taken as evidence for the phosphorylation of Cys^{99} of Srx before the phosphorylation of the sulfininc acid group of Prx, contrary to the original mechanistic proposal (6). Another group compared these same Srx variants with wild-type hPrxI and hPrxI-C52D, the Cys sulfinic acid mimic (31). In this setup, the addition of wild-type Srx led to the rapid phosphorylation of Asp52 (<1 min) followed by the phosphorylation of the C99S and C99A Srx mutants to some degree. The latter observation suggests that another residue in the active site of Srx can be phosphorylated, if given enough time; perhaps this residue is His^{100}. A different study using ^{18}O-labeled

PrxI-S^{18}O$_2^-$ also showed that the phosphorylation of the Prx molecule is readily reversible ($k = 0.35 \, min^{-1}$) (33). Further support for this notion comes from studies in which the exogenous reductant, such as GSH, was omitted from the reaction (24, 27, 55). In reactions monitoring P_i release from ATP, more P_i was liberated than predicted, based on the amount of Prx added to the reaction. The phenomenon also was dependent on the amount of ATP and Srx in the reaction. Thus, a futile cycle has been proposed to occur from the collapse of either or both the sulfinic phosphoryl ester and thiosulfinate intermediates (24, 27). Altogether, these data and the positioning of Asp^{52} relative to the γ-phosphate of ATP within the ATP•Mg^{2+} complex (Fig. 5) support the direct phosphorylation of the Prx molecule as the first step of the reaction.

Protein–protein thiosulfinate formation and resolution

The second step of the reaction (Fig. 3) was originally proposed to involve the formation of a thiosulfinate between the Prx and Srx molecules (6). The observation of DTT-sensitive linkages between Srx and Prx molecules from *in vitro* reactions with recombinant proteins and cell studies support this view. Alternatively, based on the futile cycle in the absence of GSH, GSH could also be involved in the formation of a thiosulfinate intermediate (27). It is important to note, however, that GSH and Trx are not required for the repair of the Prx molecule. As long as enough active Srx, ATP, and Mg^{2+} are present in the reaction, the Prx molecule will be repaired. Therefore, in an effort to simplify the reaction

conditions and to stabilize reaction intermediates, site-directed mutants of the Prx Cys-S_RH residue (*i.e.,* Cys[172] in hPrxII; Cys[171] in *S. cerevisiae* Tsa1) and other Cys residues not required for catalysis (*i.e.,* Cys[70] in hPrxII; Cys[48] and Cys[106] in *S. cerevisiae* Srx) were generated (33, 55). Moreover, GSH and Trx were not added to the reaction, as their addition could readily lead to the collapse of sensitive intermediates and enable disulfide-bond shuffling. Another critical experimental aspect of the studies was the use of low-pH conditions (0.08 % trifluoro-acetic acid or $50\,mM$ ammonium acetate, pH 3) to stabilize the labile thiosulfinate intermediate.

With all of the experimental precautions described in place, both studies readily observed the formation of a thiosulfinate intermediate between the Srx and Prx molecules (Prx-S_PO-S-Srx; $k = 1.2–1.4\,min^{-1}$). This rate is similar to the overall rate of the reaction $0.1–1.8\,min^{-1}$ (11, 24, 28, 55, 56), establishing the chemical competence of the Srx-Prx–based thiosulfinate intermediate. Interestingly, the thiosulfinate intermediate from both organisms readily collapsed with the formation of the disulfide-bonded complex between Srx and Prx ($k = 0.14\,min^{-1}$ for hSrx).

This disulfide-bonded complex could arise from the following scenarios (Fig. 3). First, if another reduced Srx molecule attacked the thiosulfinate at the Srx sulfur atom, Prx-S_POH would be released, with the concomitant formation of the Srx-S-S-Srx dimer, a species observed in the yeast Srx study (55). Because the Cys-S_RH residue has been mutated, and an intermolecular disulfide bond cannot be formed, the Prx-S_POH species could readily react with any free Srx molecule to generate the disulfide, Srx-S-S_P-Prx. Second, if another Srx molecule attacked the latter complex, a fully reduced Prx molecule could be released, along with another equivalent of the Srx-S-S-Srx dimer. The former does occur with wild-type yeast Srx, but in this case, Cys[48], uniquely present within a surface loop (Fig. 4), attacks the Prx-S_PO-S-Srx species to form an intramolecular disulfide (*i.e.,* Cys[48]-Cys[84]) (56). Reduction of this disulfide is facilitated by Trx, suggesting that the reduction of the thiosulfinate intermediate and the recycling of Srx are different for the human enzyme system.

The preceding discussion most likely means that either GSH or Trx directly reduces the human Srx-Prx thiosulfinate (Fig. 3, indicated by RSH). The observation that yeast Trx was not as efficient at reducing the Srx-Tsa1(C48S) thiosulfinate supports that GSH may play a key role in the resolution of the thiosulfinate in humans (56). Importantly, the formation of the Srx-Prx thiosulfinate intermediate is consistent with the proximity of Cys[99] of hSrx to the ATP molecule and the formation of a Prx sulfinic phosphoryl intermediate (Figs. 4B and 5C). Nonetheless, in all the presented mass-spectrometry experiments, GSH was omitted from the reaction. The addition of GSH to the reaction has the potential to establish a Prx-GSH–based thiosulfinate that could be reduced by another GSH molecule (Fig. 3) (27). It is clear that additional time- and concentration-dependent mass-spectrometry experiments will be required to deconvolute the GSH contribution to the kinetics of thiosulfinate formation and resolution.

Sulfiredoxin as a Deglutathionylating Agent

Dissecting the role of GSH in the Srx reaction could be complicated by observations in the literature that indicate that Srx has a second function. The initial experiments suggested that Srx can modulate the glutathionylation status of a number of key proteins, including actin and PTP1B (19). By reactivating phosphatases and influencing the activity of regulatory kinases, Srx may be a regulator of cell proliferation and influence the response of cancer cells to drugs (39). A recent study, however, found that the deglutathionylating activity of Srx is specific for typical 2-Cys Prxs, when compared with glutaredoxin 1 (GrxI) (49). Srx was able to remove GSH from Cys[83] and Cys[173] of hPrxI *in vitro* to a greater extent than the peroxidatic Cys[52], which was readily removed by GrxI. The reaction resulted in the glutathionylation of Srx on Cys[99]. Srx was unable to remove GSH from glutathionylated Cys, BSA, and PrxV. Moreover, the siRNA-mediated knockdown of Srx resulted in an increase in PrxI glutathionylation in A549 and HeLa cells after H_2O_2 exposure. Overexpression of Srx had the opposite effect. Based on the Srx-PrxI complex structure and the ability of the proteins to form a disulfide linkage readily (Fig. 5), it is difficult at this time to rationalize why Srx would not preferentially deglutathionylate the peroxidatic Cys residue. This problem is particularly evident, as the mutation of Pro[174] and Pro[179] of PrxI and Tyr[92] of Srx at the backside interface decreased the deglutathionylating activity. Why the mutation of these residues would affect the release of GSH from the other Cys residues is also not clear at this time. Therefore, the design and interpretation of future experiments to determine how GSH affects the sulfinic acid reductase activity of Srx will need to be conducted with caution.

Conclusions and Additional Open Questions

Hyperoxidation of typical 2-Cys Prxs to the Cys sulfinic acid (Fig. 6A) and their reactivation by Srx represents a compelling cellular strategy to modulate peroxide-based cell signaling. Under some conditions, this hyperoxidation can switch the activity of the peroxidase to a molecular chaperone. Srx is able to restore peroxidase activity by relying on novel interactions with the Prx molecule to juxtapose the sulfininc acid moiety properly for nucleophilic attack on the ATP molecule. Current studies support the direct phosphorylation of the sulfinic acid moiety followed by the formation a Srx-Prx thiosulfinate intermediate. To simplify these studies, GSH was omitted from the reaction. Thus, cellular GSH could ultimately play a key role in the Srx reaction. Future experiments are clearly needed in this area.

It is important to note, however, that the activity of Prxs can be modulated by a variety of other covalent modifications, including acetylation, further oxidation to the Cys sulfonic acid (Fig. 1), *S*-nitrosylation, and phosphorylation. A complex relationship appears to exist between these modifications and the modulation of peroxidase activity, hyperoxidation, and chaperone activity (1, 2, 4, 15, 37). For example, N-terminal acetylation of PrxII and not PrxI (Fig. 6B) prevents the Prx molecule from being oxidized to the sulfonic acid derivative, an irreversible modification (59). Acetylation of Lys197/196 of PrxI/II near the YF motif (Fig. 6C) increases peroxidase activity and confers resistance to oxidation and high-molecular-weight chaperone formation (50). The histone deacetylase HDAC6 has been implicated in controlling this modification. *S*-nitrosylation of both the peroxidatic and resolving Cys residues of PrxII appears to promote oxidative-stressed induced
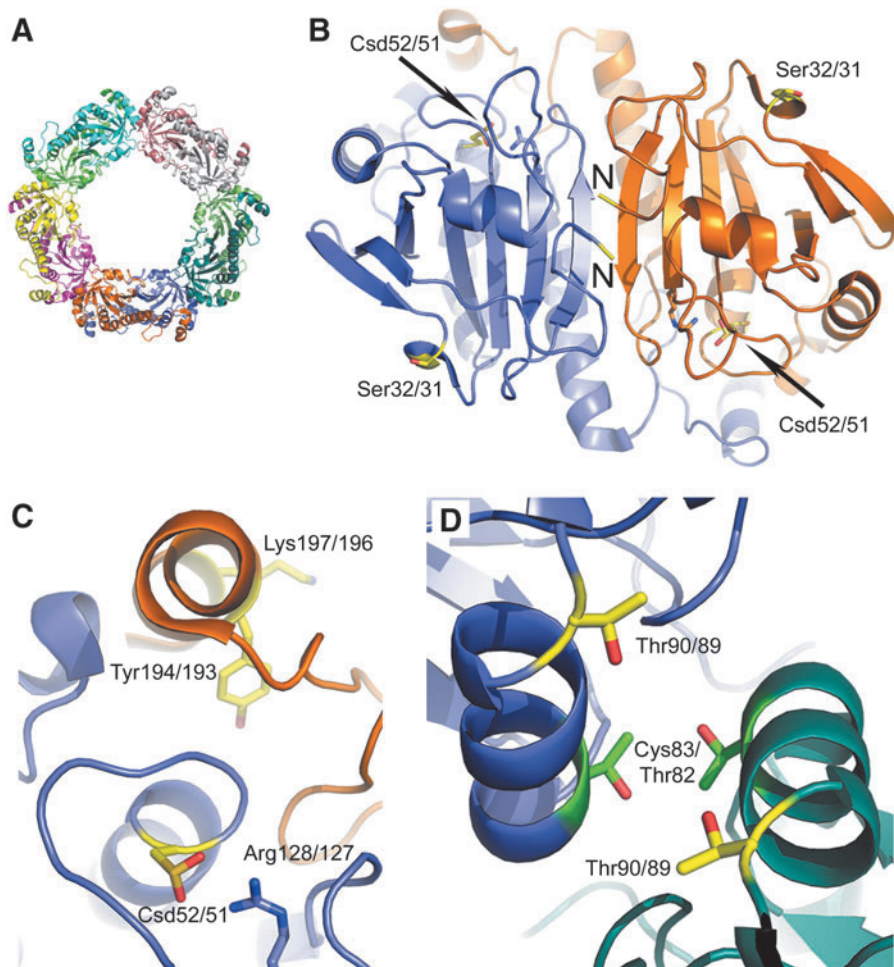
FIG. 6. **Sites of covalent modification for human PrxI and PrxII.** **(A)** The hyperoxidized PrxII decamer with each monomer represented in a different color (PDB code 1QMV) (58). **(B)** Close-up of one Prx dimer highlighting the monomer–monomer interface near the N-termini, labeled N. Sites of covalent modification in all panels are colored yellow. The Cys-S_PH residue is present in the sulfinic acid form (Csd). Numbering scheme used: PrxI residue number/PrxII residue number. Ser[32/31] and the N-termini are located on the back of the Prx dimer away from the Prx active sites. **(C)** Close-up of the active site. Tyr[194/193], part of the YF motif, and Lys[197/196] are proximal to the peroxidatic Cys residue. **(D)** The dimer–dimer interface. Thr[90/89] can be phosphorylated. For reference, the mutation of Thr[82]/Cys[83] to Glu (green) results in the disruption of the decamer into its dimeric constituents (28). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

neuronal cell death in Parkinson disease (18). Phosphorylation of PrxI/II leads to differential effects. Phosphorylation of Thr[90/89] (Fig. 6D) by cyclin-dependent kinases dramatically reduces peroxidase activity, promotes oxidative stress, and can lead to chaperone formation (10, 26, 53, 63). Interestingly, phosphorylation of Tyr[194] (Fig. 6C) PrxI can also lead to inactivation, whereas PrxII was not affected by modification at this site (72). These observations dramatically contrast with the activation of Prx activity by Lys[196/197] acetylation, described earlier. Stimulation of peroxidase activity has also been observed when Ser[32] (Fig. 6B) of PrxI is phosphorylated by TOPK (79).

From each of the brief examples and the biochemical and structural data described throughout this review, it is clear that disruption of the dimer–dimer interface should and typically does lead to decreased peroxidase activity. Therefore, it is unclear how the phosphorylation of Thr[90] of PrxI should induce chaperone activity, as the Prx molecule must be able to initiate the catalytic cycle for hyperoxidation to occur. Moreover, any mutation or covalent modification that stimulates peroxidase activity (*e.g.,* Lys[197/196] acetylation) could have been the result of an increased intermolecular disulfide bond-formation rate for the Prx molecules. By analogy, one would expect that the phosphorylation of the Tyr residue within the YF motif would lead to an increase in Prx activity, when exactly the op-

posite was observed. It also unclear how phosphorylation at Ser[32], located far from the active site, could stimulate Prx activity. Therefore, much is still to be learned about the molecular basis for the regulation of Prx activity and its repair by Srx.

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Abbreviations Used

Cys-S_PH = peroxidatic cysteine
Cys-$S_PO_2^-$ = cysteine sulfinic acid
Cys-$S_PO_2PO_3^{2-}$ = cysteine sulfinic phosphoryl ester
Cys-$S_PO_3^{2-}$ = cysteine sulfonic acid
Cys-S_POH = cysteine sulfenic acid
Cys-S_RH = resolving cysteine
GrxI = glutaredoxin I
GSH = glutathione
H_2O_2 = hydrogen peroxide
Prx = peroxiredoxin
Prx-S_PO-S-Srx = thiosulfinate intermediate
PTP1B = protein tyrosine phosphatase B
Srx = sulfiredoxin
Trx = thioredoxin