

STRUCTURAL AND CATALYTIC FEATURES AFFECTING INACTIVATION OF
TYPICAL 2-CYS HUMAN PEROXIREDOXINS 2 AND 3

BY

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LIST OF ABBREVIATIONS

amu = atomic mass units

ATP = adenosine triphosphate

ADP = adenosine diphosphate

CBD = chitin binding domain

Cys-S_PH = peroxidatic cysteine

Cys-S_PO₂⁻ (Cys-SO₂H) = cysteine sulfinic acid

Cys-S_PO₂PO₃²⁻ = cysteine sulfinic phosphoryl ester

Cys-S_PO₃²⁻ = cysteine sulfonic acid

Cys-S_POH (Cys-SOH) = cysteine sulfenic acid

Cys-SN (Cys-S_PN) = cysteine sulfenamide

Cys-S_RH = resolving cysteine

DTT = dithiothreitol

DNA = deoxyribonucleic acid

EDTA = ethylene diamine triacetic acid

ESI-TOF = electrospray ionization – time of flight

FF = fully folded

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

GGLG = glycine glycine leucine glycine

GrxI = glutaredoxin I

GSH = glutathione

IRP = iron-regulatory protein

IRE = iron-response element

HEPES = (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer

H₂O₂ = hydrogen peroxide

KDA = lysine, aspartate and alanine

LU = locally unfolded

MS = mass spectrometry

MsrA = methionine sulfoxide reductase A

MW= molecular weight

NADPH = nicotinamide adenine dinucleotide phosphate reduced

NRA = asparagine, arginine and alanine

OhrR = organic hydroperoxide resistance enzyme

PARP 1 = poly (ADP-ribose) polymerase 1

PMSF = phenylmethanesulfonyl fluoride

Prx = peroxiredoxin

Prx-S_pO-S-Srx = thiosulfinate intermediate

PTP1B = protein tyrosine phosphatase B

RA = reducing agent

R.M.S = root mean square

ROS = reactive oxygen species

ROOH = lipid peroxides

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

S_P-S_R = intermolecular disulfide between peroxidatic and resolving cysteine

std. dev. = standard deviation

Srx = sulfiredoxin

Tris = Tris (Hydroxymethyl)aminomethane buffer

Trx = thioredoxin

YF = tyrosine and phenylalanine

ABSTRACT

Reactive oxygen species are key mediators of intracellular signaling and significantly influence the progression of several pathophysiologies. Oxidative stress damages macromolecules (lipids, nucleic acids and proteins). Antioxidant enzymes play a critical homeostatic role in modulating the survival and death signaling pathways in the cells of the complex interconnected systems within the human body. One such class of enzymes, human typical 2-cys peroxiredoxins, is involved in redox regulation through the cyclic oxidation and reduction of cysteine residues during its normal catalytic cycle. This redox cycling facilitates hydrogen peroxide (H_2O_2) based cell signaling, protects cells from oxidative stress and inhibits apoptosis. There are four members of this class with disparate subcellular distributions: Prx1, Prx2 (both in the cytoplasm), Prx3 (mitochondria) and Prx4 (endoplasmic reticulum). They possess an evolutionary adaptation within the C-terminus that allows these 'sensitive' Prxs to be susceptible to hyperoxidation. Under normal conditions this allows the propagation of localized H_2O_2 signaling ('Floodgate Hypothesis') and resumption of peroxidase activity when the hyperoxidized Prx is repaired by sulfiredoxin (Srx). However, during extreme oxidative stress, antioxidant defense mechanisms fail, resulting in damage to several organs, such as the myocardium, liver, ovaries, pancreas and brain, leading to cardiomyopathy, cancer, metabolic disorders and neurodegenerative diseases.

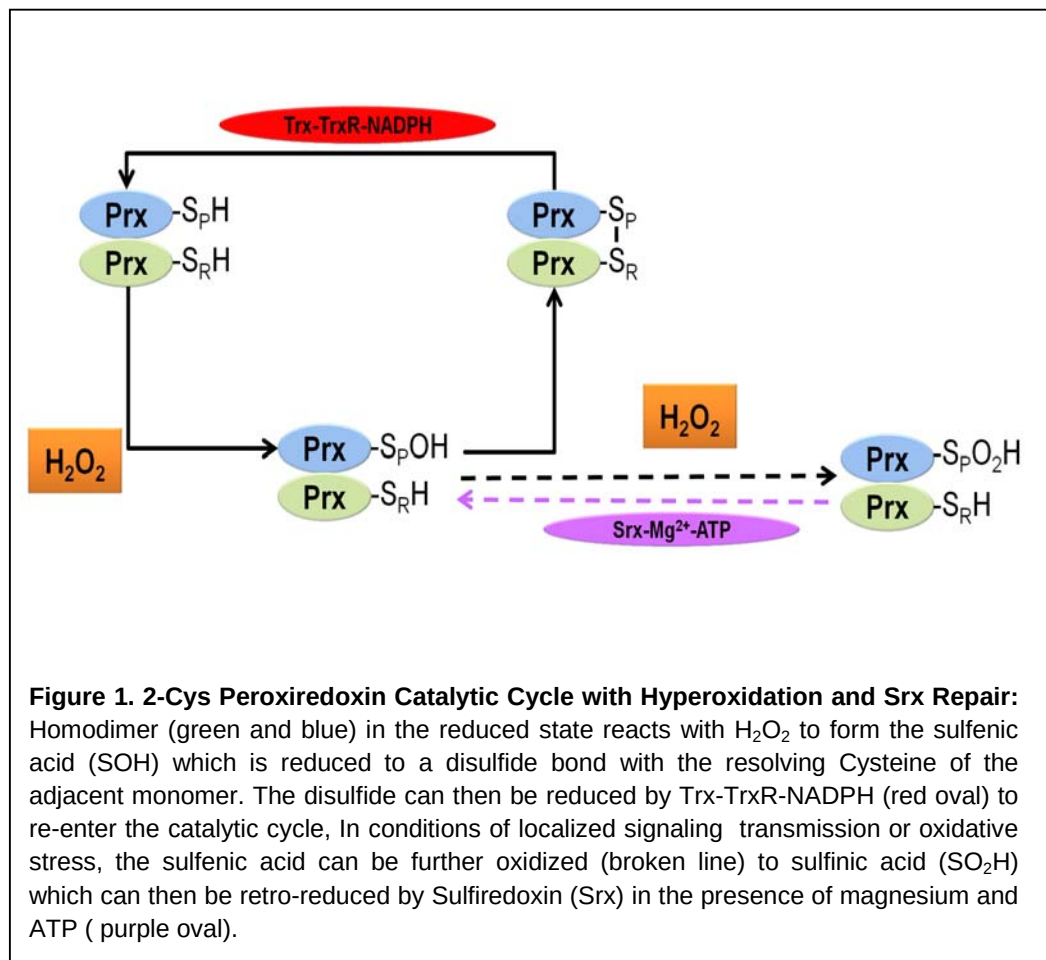
Research within this thesis is focused on a comparative study between cytoplasmic Prx2 and mitochondrial Prx3 using X-ray crystallography, homology modeling and mass spectrometry. Previous studies conducted in cells indicate that Prx3 is less susceptible than Prx2 to hyperoxidation. A sequence alignment of Prx2 and Prx3 reveals differences within the C-terminus that are surmised to be the reason for this observation. Mass spectrometry studies using Prx2/3 mutants involving unique residues within this region along with mutants that contain only the

peroxidatic cysteine confirmed that this region is crucial to regulation of hyperoxidation susceptibility. These studies also revealed a novel sulfenamide intermediate that adds another layer to the regulation of the Prx catalytic cycle and hyperoxidation susceptibility. Structural studies further support the notion that this C-terminal region influences the ease of hyperoxidation and also identify other potential structural features responsible for this phenomenon.

CHAPTER ONE

INTRODUCTION

Human typical 2-Cys peroxiredoxins (Prxs) are highly-expressed antioxidant enzymes that can convert hydrogen peroxide to water, maintaining redox homeostasis within cells. In addition to being protective in nature, this peroxidase function also plays a key role in modulating H_2O_2 -mediated cell signaling in normal and pathophysiological contexts, such as: cell growth, differentiation, aging, diabetes, neurodegeneration, myocardial infarction and cancer [1,2]. The basic catalytic subunit of the typical 2-Cys subclass (Prx1-Prx4) consists of two catalytic Cys residues (peroxidatic and resolving) on each monomer of an obligate homodimer [3,4]. A simple schematic of the 2-Cys Prx catalytic cycle is presented in Figure 1.

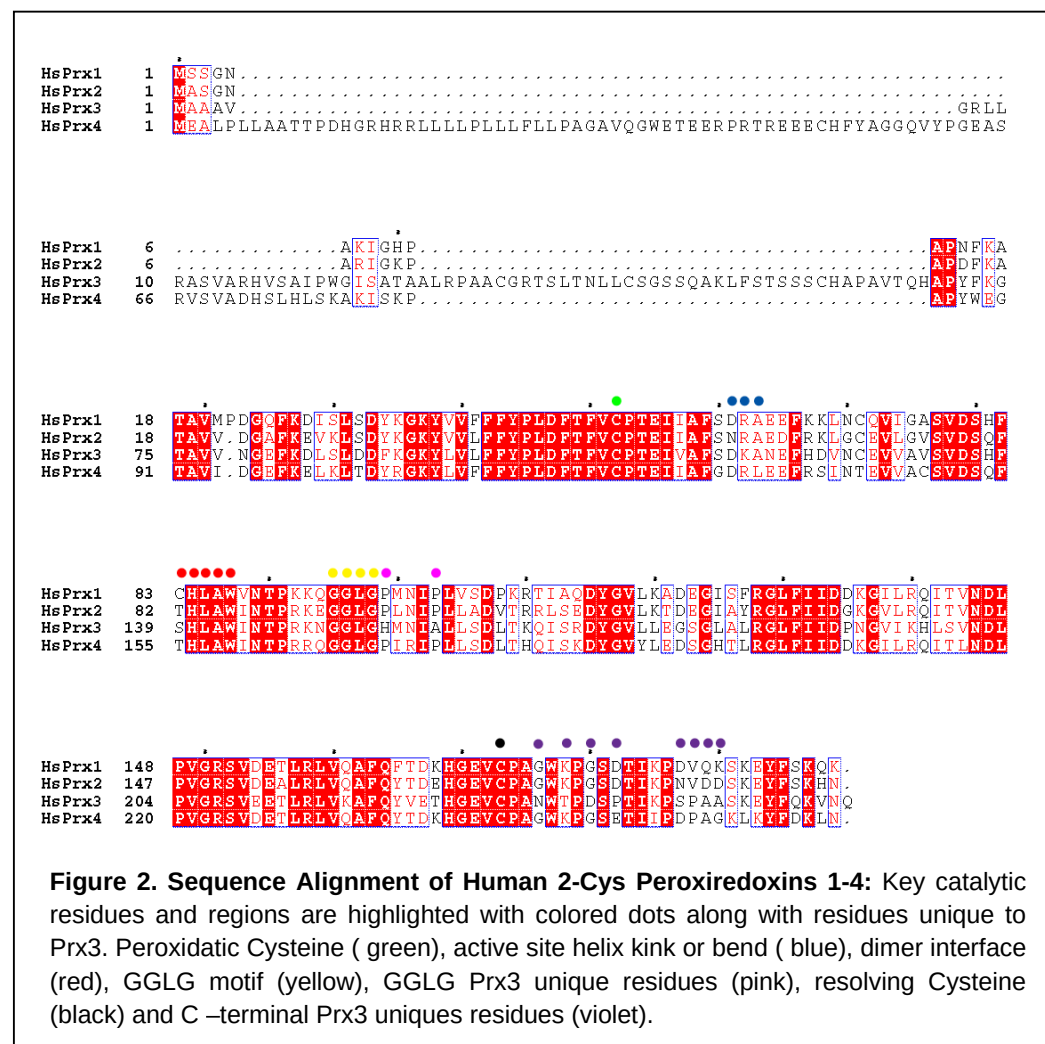


The “peroxidatic” Cys residue ($-S_P H$) attacks a H_2O_2 molecule to form a sulfenic acid intermediate ($-S_P OH$). This is followed by the resolution step involving structural rearrangements within active site near the peroxidatic Cys and the “resolving” Cys residue ($-S_R H$), near the C-terminus of the adjacent monomer, creating an intermolecular disulfide with the release of water. In the subsequent reduction step, this disulfide (S_P-S_R) is reduced by the thioredoxin-thioredoxin reductase-NADPH system (Trx-TrxR-NADPH) [5].

During propagation of hydrogen peroxide signaling or high oxidative stress, a second H_2O_2 molecule can react with the $-S_P OH$ hyperoxidizing it to sulfinic acid ($-S_P O_2 H$) which results in inactivation. Repair of the Prx molecules by sulfiredoxin (Srx) restores the peroxidase activity, lowers peroxide levels, and switches off H_2O_2 based cell signaling events [6-8]. It has been reported that the susceptibility to hyperoxidation phenomenon varies among this subclass of Prxs. Prx1, Prx2 (both cytosolic) and Prx4 (endoplasmic reticulum) are more easily hyperoxidized than Prx3 (mitochondria) [9]. A sequence alignment of the 2-Cys Prxs reveals that Prx3 has a unique primary sequence in the C-terminus near the resolving Cysteine as well as two residues following the GGLG motif within the active site region (Figure 2). This led to the hypothesis governing this thesis that the observed C-terminal sequence variation affected the flexibility of the overall Prx structure thus affecting the stability of the sulfenic acid and the subsequent rate of disulfide or sulfinic acid formation.

There is a structural basis for the susceptibility of typical 2-Cys Prxs to hyperoxidation [4]. It is believed to be an evolutionary adaptation to enable participation in H_2O_2 cell signaling events. Based on the currently published literature, all bacterial and other eukaryotic Prxs have the same basic structural features and utilize a similar catalytic mechanism that involves major structural rearrangements from a fully-folded state to a locally unfolded state [10]. For typical 2-Cys Prxs, the reduced and hyperoxidized forms are in the fully-folded conformation with the active site helix containing the peroxidatic Cysteine folded and approximately 12-14 Å away

from the resolving cysteine, whose side chain is buried in the folded C-terminus in the nearby monomer (refer to Chapter 3 for more information) [11]. When Prx is fully-folded, the loop containing the GGLG motif and C-terminal helix containing the YF motif are positioned adjacent to each other, allowing the peroxidatic cysteine to be buried.



After peroxidation, the disulfide is formed due to a transition from the fully-folded to the locally-unfolded state, where the C-terminus and the active site unfold to enable the resolution of the sulfenic acid with the thiol of the resolving cysteine. This can be considered the slow step in the catalytic cycle, as the YF motif within the C-terminus is positioned above the active site restricting the mobility of this region and prolonging exposure of the sulfenic acid to excess H_2O_2 . This allows the sulfenic acid

to be overoxidized to the inactive sulfinic acid. This facile hyperoxidation of the typical 2-Cys Prxs has categorized them as 'sensitive' when compared to the resistant bacterial Prxs that are referred to as 'robust' and lack this C-terminal region [4]. In addition to lacking this C-terminus region, the 'robust' bacterial Prxs also lack the GGLG motif [11]. Studies have demonstrated that truncation of the C-terminus of eukaryotic Prxs enables them to be resistant to hyperoxidation like 'robust' bacterial Prxs [12]. Therefore, it is surmised that sequence variations within the C-terminus that disrupt its orientation and/or interaction with the rest of the Prx molecule may lead to a favorable rate of disulfide formation and thus decrease the susceptibility to hyperoxidation [9,13].

While conformational changes within the homodimer play an essential role in regulation of catalysis and subsequent hyperoxidation, other quaternary changes are also observed to have an effect on catalysis. The shift between the dimer and higher MW oligomers adds an additional layer of structural complexity to the regulation of peroxidation and hyperoxidation. Prxs can exist as octamers, decamers, dodecamers and hexa-decamers with the decameric state being the most common [10]. In the specific case of typical human 2-Cys Prxs, the decamer appears to be the most common form [14,15]. There is the possibility that human Prx3 can exist as a dodecamer and even as a two ring catenane, a species first observed in bovine Prx3 that has an 89% homology with human Prx3 [16]. The doughnut or toroidal ring form is favored in all steps of catalysis with the exception of the disulfide state, as the unfolding action required appears to disrupt the dimer-dimer interface that stabilizes the higher MW oligomer [17]. However, recent X-ray crystal structures solved for oxidized human 2-Cys Prx4 and Prx2 reveal that the disulfide form can also be stable as a decamer (refer to chapter 3 for Prx2 SS structure) [15,17,18]. The decameric state is observed to be more catalytically efficient than the dimeric state and less prone to thioredoxin mediated reduction [19,20].

Human typical 2-Cys Prxs function as peroxidases and their susceptibility to hyperoxidation enables them to play a central role in cellular signaling networks and in several pathologies. This dichotomy can be better understood with an appreciation for the role that reactive oxygen species, such as H_2O_2 , play in cellular processes. Low concentrations of H_2O_2 , derived from metabolism within the mitochondria (which are the primary generators of endogenous ROS), are essential in several normal processes such as signaling, apoptosis and regulation of gene expression [21,22]. High concentrations of H_2O_2 that perturb the ROS/antioxidant balance can lead to deleterious effects on the cell including damage to nucleic acids, lipids and proteins [2]. Damage to DNA is particularly crucial as it results in mutations leading to aging and cancer.

Prx3 is very important in several pathologies due to its mitochondrial localization and comparative resistance to inactivation through hyperoxidation. Therefore, Prx3 is the first line of defense against mitochondrial ROS and calculations have shown that it is targeted by 90% of H_2O_2 [23]. A key concept in the aging process is that mitochondrial dysfunction increases leading to increased ROS production. Increased ROS, especially H_2O_2 , can lead to the production of far more damaging hydroxyl radicals through the Fenton-Harding reaction in the presence of iron. The regulation of hydrogen peroxide is thus absolutely essential to prevent apoptosis and regulate iron homeostasis. Apoptosis has been shown to be controlled by H_2O_2 levels in the mitochondria through the action of p66Shc, a proapoptotic protein, which is activated by cysteine oxidation to form disulfide bonds and subsequently must be reduced by glutathione or thioredoxin [21]. This fascinating protein is regulated by the combination of H_2O_2 and thioredoxin, which implicates the involvement of 2-Cys Prx3 whose catalytic cycle utilizes both (Fig.1). Even more fascinating is H_2O_2 regulation of iron homeostasis through the mediation of the iron-response element and iron-regulatory protein (IRE- IRP) coupled processes [24,25]. This coupled system controls the amount of iron within the cells by regulating the

expression of ferritin (iron storage) and transferrin receptor-1 (iron transport). A specific example is cysteine oxidation of IRP2 which leads to lower association with the IRE and lowers the expression of transferrin receptor-1 [26].

Prx3 has also been implicated in several cardiovascular diseases. In an animal model of transient cerebral ischemia, delayed neuronal death was observed in the CA1 region of the hippocampus coincident with the subcellular localization of Prx3 and its thiol reductant, Thioredoxin 2 (Trx2) [27]. Also, overexpression of Prx3 protected the murine heart from post myocardial infarction remodeling and heart failure, as measured by the reduction in: left ventricular cavity dilation, myocyte hypertrophy, interstitial fibrosis and apoptosis [28]. Recently, Prx2 has also been implicated in cardiovascular disease by reducing post ischemic injury, and significantly reducing apoptosis by inhibiting the cell death pathways associated with: poly (ADP-ribose) polymerase 1 (PARP1) and p53 [29]. Also, cancer cells have been discovered to contain high levels of Prx3 within their mitochondria protecting them from apoptosis-inducing drugs [30-32]. Prx2 localized to the nucleus has also been observed to protect cancer cells from death caused by DNA damage, and knockdown of Prx2 has sensitized resistant head and neck cancer cell lines to radiation [33]. A more profound understanding of the molecular basis for hyperoxidation of Prx2 and Prx3 and the relative resistance of Prx3 to inactivation will provide a more detailed insight into catalysis and feasibility as a drug target in cardiovascular, drug-resistant cancer and neurodegenerative diseases [34-36].

This thesis used a combination of time-resolved and chemical quench mass spectrometry, X-ray crystallography in addition to homology modeling to elucidate the molecular basis of hyperoxidation susceptibility and the observed difference in this regard between Prx2 and Prx3. Previous studies have used low resolution techniques such as non-reducing SDS-PAGE coupled with Western blotting to demonstrate this difference in hyperoxidation among the 2-Cys Prxs. The time-resolved technique, along with the mutagenesis of cysteines to serines, enabled the

trapping of intermediates involved in the 2-Cys Prx catalytic cycle. Several insights were gained including the direct observation of the sulfenic acid intermediate. Of even greater interest, a sulfenamide intermediate was observed during catalysis which has never before been observed for a human 2-Cys Prxs. The protein sulfenamide intermediate was initially identified in GAPDH when its sulfenic form reacted with a small organic amine molecule, benzylamine [37]. Additionally, it has been previously observed in other redox active proteins such as protein tyrosine phosphatase 1B (PTP1B), *Bacillus subtilis* organic peroxide sensor OhrR and more recently the mouse Methionine sulfoxide Reductase A (MsrA) [38,39]. Also, an intrinsic difference in the reactivity of the peroxidatic cysteine was observed between Prx2 and Prx3, with Prx2 forming the sulfenic acid much faster. In studies done with synthetic peptides, it was discovered that the cysteine sulfenic reacted with amino or guanidino groups from lysine and arginine to form inter- and intra-molecular sulfenamide cross-links [40]. For 2-Cys Prxs, the peroxidatic cysteine is adjacent to a valine on its N-terminus and a proline on its C-terminus, neither of which can form a sulfonamide. Within the active site, however, is a arginine (Arg127 for Prx2), which does contain a guanidino group capable of nucleophilic attack, and thus can form a sulfenamide [14]. To further understand the role of the C-terminus and active site GGLG motif region in hyperoxidation, the unique Prx3 residues (four in the C-terminus and two in the active site) were mutated to those in Prx2 and vice versa, both singly and in combination. The C-terminal mutations had the largest impact on resistance to hyperoxidation with the residues near the GGLG motif playing a minimal role. Neither the C-terminal mutations alone or in combination with the GGLG mutations fully converted Prx3 to becoming Prx2-like sensitive indicating that other residues, regions and perhaps the oligomeric state of the Prx molecule may be involved in regulating the ease of hyperoxidation.

Structural studies in Chapter 3 have provided further insight into how the observed sequence differences between Prx2 and Prx3 influence the conformational changes that control susceptibility to hyperoxidation. Additional residues within the C-terminus and the active site have been identified that play a role in catalysis and hyperoxidation. Within this thesis the Prx2 disulfide structure was solved to 2.1 Å as a decamer. Homology modeling was used to generate models of Prx2 and Prx3 in the reduced state as a monomer, dimer and decamer. This survey revealed an interesting difference between Prx2 and Prx3 at the active site helix kink (blue dots in Fig. 2) where there is a non-conserved change in amino acid residues from NRA (N60, R61 and A62) in Prx2 to DKA (D117, K118 and A119) in Prx3. This appears to introduce flexibility into this region of the active site helix containing the peroxidatic cysteine lending further credence to the theory that Prx2 can adopt multiple conformations, thus slowing the formation of the protective disulfide and leaving the sulfenic acid exposed to further oxidation. Prx3, in contrast, has limited flexibility in this region and in the C-terminus allowing less conformational transitions, and thus a faster disulfide bond formation, protecting it from hyperoxidation. Additionally Prx3 disulfide and the Prx3 C108D hyperoxidation mimic were modeled as decamers using a similar technique. Prx3 was also modeled as dodecamer and concatenated dodecamers to gain further insight into potential effects of this oligomeric state on the dimer interface and active site. It is of interest to note that Prx3 decamer and dodecamer showed no differences in the dimer interface and active site. However, with the concatenated dodecamers, the points of contact between the two dodecamers revealed a hydrogen bonding network directly interacting with the active site helix which could potentially have an impact on catalysis and hyperoxidation.

This dissertation consists of five chapters. Chapter one introduces the hypothesis governing the thesis research and summarizes the key experimental results. Chapter 2 is an analysis of the molecular basis of hyperoxidation using mass

spectrometry and the identification of important catalytic intermediates including the novel sulfenamide. This chapter also addresses the impact of the C-terminus and GGLG on catalysis. Chapter 3 compares the structures of Prx2 and Prx3 in different oligomeric states and in all three redox states: reduced, oxidized and hyperoxidized. Chapter 4 is a review article that outlines the Srx repair of the hyperoxidized 2-Cys Prxs which is the logical future direction of this project. Chapter 5 is a summary of the main conclusions of this thesis project and identifies potential future directions for typical 2-Cys Prxs field.

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CHAPTER TWO
MOLECULAR BASIS FOR THE RESISTANCE OF HUMAN MITOCHONDRIAL 2-CYS
PEROXIREDOXIN 3 TO HYPEROXIDATION

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Running Title: Molecular Basis for Resistance of hPrx3 to Hyperoxidation

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Background: Human 2-Cys peroxiredoxins (Prxs) defend against oxidative stress but are susceptible to inactivation by oxidation.

Results: Prx2 and Prx3 variants demonstrate that C-terminal residues modulate the propensity toward oxidative inactivation.

Conclusion: Rapid disulfide bond formation protects Prx3 from inactivation, consistent with its cellular localization.

Significance: Prx3 is an attractive therapeutic target for mitochondrial dysfunction in heart disease and cancer.

SUMMARY

Peroxiredoxins (Prxs) detoxify peroxides and modulate H₂O₂-mediated cell signaling in normal and numerous pathophysiological contexts. The typical 2-Cys subclass of Prxs (human Prx1-4) utilizes a Cys sulfenic acid (Cys-SOH) intermediate and disulfide bond formation across two subunits during catalysis. During oxidative stress, however, the Cys-SOH moiety can react with H₂O₂ to form Cys sulfinic acid (Cys-SO₂H), resulting in inactivation. The propensity to hyperoxidize varies greatly among human Prxs. Mitochondrial Prx3 is the most resistant to inactivation, but the molecular basis for this property is unknown. A panel of chimeras and Cys variants of Prx2 and Prx3 were treated with H₂O₂ and analyzed by rapid chemical quench and time-resolved ESI-TOF mass spectrometry. The latter utilized an online, rapid-mixing setup to collect data on the low seconds' timescale. These approaches enabled the first direct observation of the Cys-SOH intermediate and a novel Cys sulfenamide (Cys-SN) for Prx2 and Prx3 during catalysis. The substitution of C-terminal residues in Prx3, residues adjacent to the resolving Cys residue, resulted in a Prx2-like protein with increased sensitivity to hyperoxidation and decreased ability to form the

intermolecular disulfide bond between subunits. The corresponding Prx2 chimera became more resistant to hyperoxidation. Taken altogether, the results of this study support that the kinetics of the Cys-SOH intermediate is key to determine the probability of hyperoxidation or stabilization into Cys-SN and disulfide states. Given the oxidizing environment of the mitochondrion, it makes sense that Prx3 would favor disulfide bond formation as a protection mechanism against hyperoxidation and inactivation.

Peroxiredoxins (Prxs) are ubiquitous, highly expressed antioxidant enzymes that can convert hydrogen peroxide (H_2O_2), peroxynitrite (ONOO^-), and lipid peroxides (ROOH) to water. While this function was originally thought to be primarily protective in nature, Prxs also play a key role in modulating H_2O_2 -mediated cell signaling in normal and pathophysiological contexts including cell growth, differentiation, adrenal steroidogenesis, neurodegeneration, and cancer (2-6). Human cells contain six Prx isoforms with differences in subcellular localization and content of Cys residues (7). The typical 2-Cys or Prx1 subclass (human Prx1-4) contains two catalytic Cys residues on each monomer of an obligate homodimer (Fig. 1A). Under normal conditions, the “peroxidatic” Cys residue ($\text{Cys-S}_\text{P}\text{H}$) attacks a H_2O_2 molecule to form a sulfenic acid intermediate ($\text{Cys-S}_\text{P}\text{OH}$). Subsequent structural rearrangements within the active site near the $\text{Cys-S}_\text{P}\text{H}$ residue and the “resolving” Cys residue ($\text{Cys-S}_\text{R}\text{H}$), located near the C-terminus of the adjacent subunit, enable an intermolecular disulfide to be formed. This disulfide ($\text{S}_\text{P}\text{-S}_\text{R}$) is ultimately reduced by the thioredoxin-thioredoxin reductase-NADPH (Trx-TrxR-NADPH) system. Additionally, during the catalytic cycle, an interchange between dimeric and higher-order oligomeric states occurs, with the reduced decamer typically being the most active form (8,9).

Under conditions of high oxidative stress, a second H_2O_2 molecule can react with the $\text{Cys-S}_\text{P}\text{OH}$ moiety to form a Cys sulfinic acid ($\text{Cys-S}_\text{P}\text{O}_2\text{H}$) moiety within some Prx

isoforms (10). This hyperoxidation of the Prx molecule results in inactivation and is thought to enable H_2O_2 to modulate the activity of a variety of other proteins including phosphatases and the master redox transcription factor Nrf2 (11-13). Repair of the Prx molecules by sulfiredoxin (Srx) restores the peroxidase activity, lowers peroxide levels, and terminates subsequent downstream signaling events (10,14-16). However, the susceptibility of human 2-Cys Prxs to hyperoxidation varies greatly, with the cytoplasmic Prx1 and Prx2 being more susceptible than the mitochondrial Prx3 (17). The resistance of Prx3 to hyperoxidation is consistent with its localization, but the molecular basis for this characteristic is not known. Moreover, a detailed analysis of Prx3 is needed to understand its ability to protect the murine heart from the damage caused by myocardial infarction and cancer cells from apoptosis-inducing drugs (3,18,19).

An alignment of human Prx1-4 reveals that Prx3 has a unique primary sequence near the GGLG motif within the active site region (Fig. 1B) and near the C-terminus. A close-up of the hyperoxidized Prx2 structure (Fig. 1C) illustrates the proximity of these regions to the Cys-S_PH residue (1). In particular, the GGLG motif interacts with the C-terminal helix of the adjacent Prx subunit, which contains the conserved Tyr and Phe residues of the YF motif. This specific interaction is postulated to slow the rate of formation of the intermolecular disulfide intermediate (S_P-S_R) during catalysis, enabling hyperoxidation to occur (7,11). The changes in the Prx3 sequence in the proximity of the Cys-S_RH residue and the YF motif have been postulated to alter the interaction with the rest of the Prx molecule, resulting in a decreased susceptibility to hyperoxidation (10,17). Therefore, changes in both regions in Prx3 may result in its unique biochemical and physiological properties.

In this study, a panel of Prx2 and Prx3 variants and chimeras was analyzed to investigate the contribution of the observed sequence changes near the GGLG motif and the C-terminus to hyperoxidation. Previous reports have used long timescales, non-

reducing SDS-PAGE, 2D-PAGE, and Western blotting to monitor the hyperoxidation of Prx molecules (17, 20, 21). In contrast, the data presented herein was collected using a combination of rapid chemical quench and time-resolved ESI-TOF mass spectrometry methods to facilitate analysis under both denaturing and non-denaturing conditions (22, 23). These improvements and the strategic use of Cys variants have enabled the direct observation of the Cys-S_POH intermediate during catalysis. Moreover, the stability of this intermediate in Prx2 is supported by the time-dependent formation of an intramolecular Cys-sulfenamide (-SN) product. Changing the C-terminal residues of Prx2 and Prx3 had the largest impact on resistance to hyperoxidation. The residues near the GGLG motif appeared to play a minimal role. While Prx3 could be converted into a Prx2-like molecule and vice versa, the transformations were incomplete suggesting that additional residues, regions of the protein, and perhaps the equilibrium of the oligomeric states may also be involved in regulating the ease of hyperoxidation.

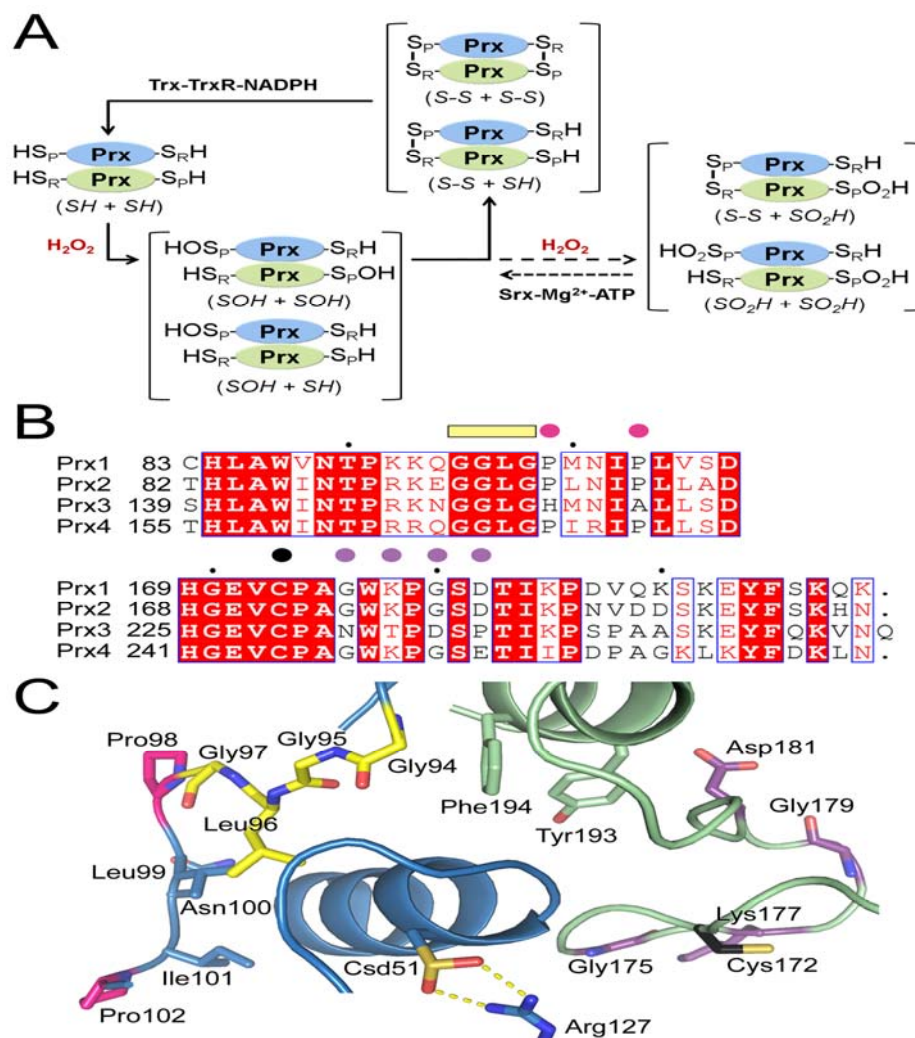


FIGURE 1. Key residues involved in 2-Cys Prx catalysis and hyperoxidation. (A) 2-Cys Prx catalytic cycle showing oxidation, hyperoxidation and repair by sulfiredoxin. The monomers of the obligate Prx homodimer are shown in blue and green. Depending on the concentration of peroxide present, one or both of the peroxidatic Cys residues (Cys-S_pH) may be oxidized to the Cys sulfenic acid (Cys-S_pOH) or hyperoxidized to the Cys sulfinic acid (Cys-S_pO₂H). The resolving Cys residue, Cys-S_rH, is located near the C-terminus and forms an intermolecular disulfide bond with the Cys-S_pH residue during normal catalysis. Reduction of this disulfide and the Cys-S_pO₂H moiety is performed by the thioredoxin-thioredoxin reductase-NAPDH (Trx-Trx-NADPH) system and sulfiredoxin (Srx), respectively. The abbreviation used within the main text for each species is indicated in *italics*. (B) Sequence alignment of key residues within the active site. The following motifs and residues are highlighted: GGLG motif, yellow bar; residue differences between the Prxs, pink and purple circles; Cys-S_rH residue, black circle. (C) Active site of hyperoxidized, human Prx2. The same coloring scheme from panel B is used. The peroxidatic Cys is hyperoxidized and labeled as Csd51. The Cys-S_rH residue for Prx2 is Cys172. PDB code 1QMV (1).

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The human Prx2 and Prx3 genes were subcloned into the pET17 (Novagen) and pTYB21 (New England Biolabs), respectively, in a manner that ultimately resulted in the mature form of each protein without any additional N-terminal or C-terminal residues. This was necessary as additional residues at either location could negatively impact catalytic activity. All Prx variants were created using the QuikChange site-directed mutagenesis method (Stratagene) with the appropriate primers. All proteins were expressed in BL21-Gold (DE3) *Escherichia coli* cells (New England Biolabs).

For the Prx2 variants [WT, PP→HA (P98H and P102A), C2S (C70S and C172S), CT (G175N, K177T, G179D and D181P), PP→HA+CT], the *E. coli* cells were grown at 37° C until an OD₆₀₀ of 0.8 and induced with 0.5 mM IPTG at 25° C for 4-5 hr. Given the absence of an affinity tag, the purification required four chromatographic steps. The cells were lysed in 100 mL of 20 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA containing protease inhibitors (PMSF and benzamidine; both at 0.1 mM) using an Emulsiflex C5 homogenizer (Avestin, Inc.). This mixture was then centrifuged and the supernatant treated with 2.5% streptomycin sulfate following by centrifugation. Ammonium sulfate was added to a final concentration of 20% to the supernatant and the solution filtered. This solution was loaded onto a Phenyl Sepharose High Performance (Low Sub) column (GE Healthcare) and eluted with 600 mL linear gradient to buffer without ammonium sulfate. The fractions corresponding to the Prx molecule, as determined by SDS-PAGE, were dialyzed into 20 mM Tris pH 7.9 and subsequently loaded onto a Q-Sepharose FF column (GE Healthcare) and eluted with a 600 mL linear gradient to 500 mM NaCl. The

Prx fractions were pooled and dialyzed into 7 mM potassium phosphate pH 7.0. The dialysate was subsequently loaded onto a CHT ceramic hydroxyapatite column (Bio-Rad) and eluted with a 600 mL linear gradient to 400 mM potassium phosphate pH 7.0. The Prx2-containing fractions were concentrated to 5 mL and loaded onto a Superdex 200 column equilibrated with 20 mM HEPES pH 7.5, 100 mM NaCl. The Prx fractions were pooled, concentrated, flash frozen with liquid nitrogen, and stored at -80° C until use. All Prx2 proteins were stored in a buffer without DTT with the exception of Prx2C2S, which was stored in 20 mM HEPES pH 7.5, 100 mM NaCl and 10 mM dithiothreitol (DTT).

For the Prx3 variants [WT, HA→PP (H155P and A159P), C2S (C127S and C229S), CT (N232G, T234K, D236G and P238D), HA→PP+CT], the *E. coli* cells were grown at 37° C until an OD₆₀₀ of 0.8 and induced with 0.5 mM IPTG at 18° C for 16 hr. Expression from the pTYB21 vector results in the addition of an N-terminal chitin binding domain (CBD) contained within an intein sequence, enabling the self-processing and removal of the CBD-intein tag after incubation with DTT. The cells were lysed in 150 mL of 20 mM Tris pH 8.5, 500 mM NaCl and 1 mM EDTA containing protease inhibitors (PMSF and benzamidine; both at 0.1 mM). The supernatant was loaded onto a chitin column (New England Biolabs) and extensively washed. Intein-mediated cleavage was initiated by the equilibrating the column with 20 mM Tris pH 8.5, 500 mM NaCl, 1mM EDTA and 50 mM DTT followed 40 hrs incubation at room temperature. The mature form of Prx3, residues 62-255, was eluted from the column, dialyzed against 20 mM Mes pH 6.5, 1 mM DTT and subsequently loaded onto a Q-Sepharose FF column (GE Healthcare) and eluted with a 600 mL (0-50%) linear gradient to 1M NaCl . The Prx3-containing fractions were concentrated and purified further using the Superdex 200 column, as described for the Prx2 variants. All Prx3 variants were stored in 20 mM HEPES pH 7.5, 100 mM NaCl with the exception of Prx3 WT which had 10 mM DTT.

Preparation of Samples for Mass Spectrometry Analysis—Immediately prior to analysis, the Prx variants were thawed and reduced with 10 mM DTT at room temperature for 30 min. DTT was removed by passing the protein solution through a Bio-Gel P6 spin column (Bio-Rad) pre-equilibrated with either 50 mM Tris buffer pH 7.5 or 50 mM ammonium acetate pH 6.9. Protein concentrations were determined, in duplicate at a minimum, using the absorbance at 280 nm and the theoretical extinction coefficients for each protein (Prx2 WT, 20,460 M⁻¹cm⁻¹; Prx2-C2S, 21,430 M⁻¹cm⁻¹; Prx2-CT, 21,555 M⁻¹cm⁻¹; Prx2 PP→HA, 21,555 M⁻¹cm⁻¹; Prx2 PP→HA+CT, 21,555 M⁻¹cm⁻¹; Prx3 WT, 20,065 M⁻¹cm⁻¹; Prx3-C2S, 19,940 M⁻¹cm⁻¹; Prx3 CT, 20,065 M⁻¹cm⁻¹; Prx3 HA→PP, 20,065 M⁻¹cm⁻¹; Prx3 HA→PP+CT, 20,065 M⁻¹cm⁻¹). (<http://web.expasy.org/protparam/>). The protein samples were immediately diluted and analyzed using the chemical quench and time-resolved methods described below.

Mass Spectrometry Data Collection and Analysis—For the chemical quench experiments, each DTT-free Prx protein was diluted further in 50 mM Tris pH 7.5 to a final concentration of 50 μM. Oxidation was initiated by the addition of 0.8 equivalents of standardized H₂O₂ ($\epsilon_{240} = 43.6 \text{ M}^{-1}\text{cm}^{-1}$) to the protein solution. The solution was incubated at 25°C in a Thermomixer (Eppendorf) with gentle mixing. In control experiments, all conditions were the same as above except the same volume of H₂O instead of H₂O₂ was used. At 30 s incubation time, the sample was applied to a Bio-Gel P6 spin column pre-equilibrated with 0.03% formic acid in H₂O to quench the oxidation reaction. The flow through was then used directly for ESI-TOF MS analysis.

In the comparative time-resolved experiments using the Prx2 and Prx3 variants, protein oxidation was performed using an online rapid-mixing setup. The experimental setup contained two Hamilton syringes: one containing 100 μM DTT-free Prx variant and

the other 100 μM H_2O_2 , both in 50 mM ammonium acetate pH 6.9. The syringes were individually connected to separate fused silica capillaries and simultaneously advanced using a syringe pump (KD Scientific). The solutions were combined through a zero dead volume-mixing tee (Upchurch Scientific) into a connecting fused silica capillary (volume: 0.362 μL). The mixture was then continuously flowed into an ESI needle (volume: 1.269 μL) inserted in a stainless steel electrospray probe for ESI-TOF MS analysis. Varying flow rates were applied to achieve reaction time points lower than 30 s.

All ESI-TOF MS data were recorded in a positive ion mode on an Agilent MSD TOF system with the following settings: capillary voltage (V_{Cap}) 3500 V, nebulizer gas (N_2) 30 psig, drying gas (N_2) 5.0 L min^{-1} ; fragmentor 140 V; gas temperature 325°C. The chemical quench samples were injected for analysis by ESI-TOF MS at a flow rate of 25 $\mu\text{L min}^{-1}$ from a 250 μL syringe via a syringe pump. For the time-resolved experiments, the samples were injected as described above. The averaged MS spectra were deconvoluted using the Agilent MassHunter workstation software v. B.01.03. Data for the Prx2-C2S variant were fitted using SigmaPlot v. 11.0 (Systat Software Inc) and KinTek Explorer (KinTek Corporation) based on a simple kinetic model $\text{E} + \text{S} \leftrightarrow \text{EI}$; $\text{EI} + \text{S} \leftrightarrow \text{EP}$, where E is Prx-C2S, S is H_2O_2 , EI is the Prx-C2S- $\text{S}_\text{P}\text{OH}$, and EP is Prx-C2S- $\text{S}_\text{P}\text{O}_2\text{H}$.

RESULTS AND DISCUSSION

Hyperoxidation of wild-type Prx2 and Prx3—While human Prx2 and Prx3 exhibit second order rate constants of $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ with H_2O_2 , these enzymes represent divergent 2-Cys Prx molecules with respect to their susceptibility to hyperoxidation of the catalytic Cys- $\text{S}_\text{P}\text{H}$ residue to Cys sulfinic acid (Cys- $\text{S}_\text{P}\text{O}_2\text{H}$) (24). In order to evaluate this difference, a panel of Prx2 and Prx3 variants was expressed and purified from *Escherichia coli*. Importantly, the expression construct for each protein was designed

with the requirement that no affinity tags or additional N- and C-terminal residues remain at the final step of purification, as these can greatly influence the oligomeric state and peroxidase activity (9,25). While Prx2 was readily expressed and purified without affinity tags, Prx3 was more problematic requiring the screening of a variety of expression tags and an evaluation of their ease of removal by proteases. In the end, only an N-terminal chitin binding domain-intein fusion led to sufficient expression levels for all variants analyzed, resulting in a mature N-terminus at residue 62 following DTT treatment.

Previous *in vitro* and cellular studies have used gel-based and Western blotting methods to monitor the hyperoxidation of Prx2 and Prx3 (17,20,21). While these low resolution techniques do illustrate the differences in reactivity with H_2O_2 , they have missed critical reaction intermediates that may shed light into the molecular mechanism of resistance to hyperoxidation in Prx3. Quantitative ESI-TOF mass spectrometry approaches were used in this study to dissect the appearance and disappearance of reaction intermediates (Fig. 1) associated with oxidation (Cys-S_POH, M+16) and hyperoxidation (Cys-S_PO₂H, M+32). A key feature of this approach has been to pre-reduce the samples with DTT and to desalt immediately prior to analysis. Moreover, all data presented herein were collected without the presence of DTT or other external reductant like thioredoxin in the reaction mixture. This simplification prevents the Prx molecule from cycling and enables partial-turnover analysis of Prx oxidation.

Reduced Prx2 or Prx3 (50 μM) was mixed with 0.8 equivalents of H_2O_2 at pH 7.5 and incubated for 30 s. The reaction was chemically quenched by passing the sample through a desalting column equilibrated with 0.03% formic acid in H_2O and immediately analyzed by ESI-TOF mass spectrometry (Fig. 2). The addition of H_2O_2 to Prx2 results in the conversion of the reduced monomer (SH, M) to the hyperoxidized monomer (SO₂H, M+32) and two intermolecular, disulfide-linked species, the oxidized (SS+SH, M+M-2) and hyperoxidized (SS+SO₂H, M+M+30) dimers (Fig. 1; all theoretical and experimental

mass values are given in Table 1). In contrast, the addition of H_2O_2 to Prx3 results in the same species, but with more of the reduced (SH) monomer remaining. These results are consistent with cell-based and *in vitro* studies showing that Prx3 is more resistant to hyperoxidation (17, 20, 21). Moreover, the concentrations of Prx and H_2O_2 used were directly comparable to those found within cells (26, 27). These experiments demonstrate for the first time that hyperoxidation of Prx2 and Prx3 can occur on a physiologically relevant time scale without catalytic cycling when the concentration of H_2O_2 is similar to the amount of Prx protein. These observations also support the notion that the lifetime of the Cys- $\text{S}_\text{P}\text{OH}$ intermediate (Fig. 1) is crucial to enable subsequent hyperoxidation, but this species has not been directly observed during Prx turnover before (11, 28).

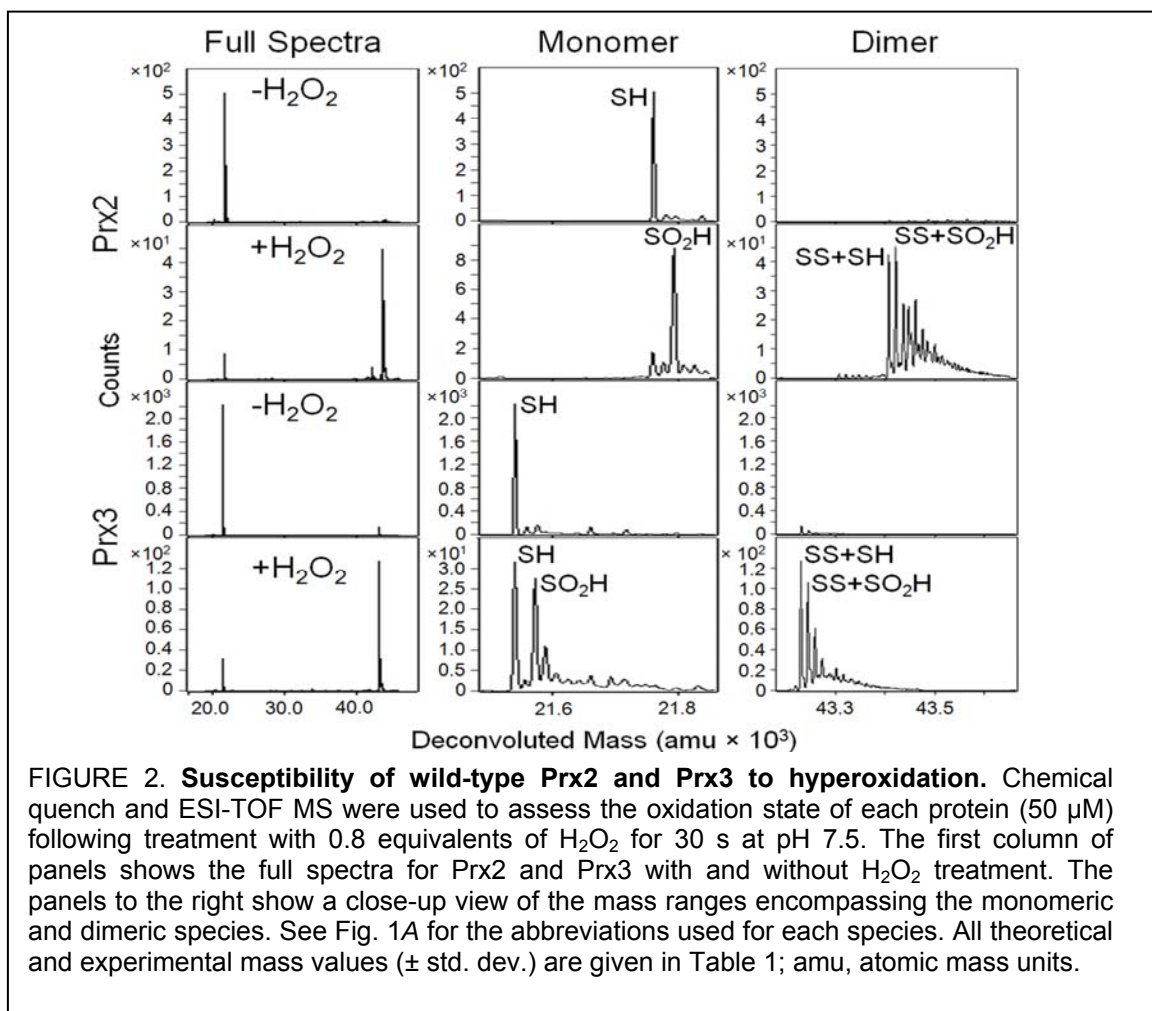


Table 1. Theoretical and experimental mass values for the different oxidation states of Prx2 and Prx3 variants.

Theoretical Mass Values $[M+H]^{+1}$ (amu)					
Oxidation State	Prx2 WT	Prx2-C2S	Prx2 PP→HA	Prx2 CT	Prx2 PP→HA+CT
SH	21761.7	21729.6	21775.7	21831.7	21845.7
SOH	21777.7	21745.6	21791.7	21847.7	21861.7
SN	21759.7	21727.6	21773.7	21829.7	21843.7
SS+SH	43520.4	- ^a	43548.4	43660.4	43688.4
SS+SO ₂ H	43552.4	- ^a	43580.4	43692.4	43720.4
SO ₂ H	21793.7	21761.6	21807.7	21863.7	21877.7
	Prx3 WT	Prx3-C2S	Prx3 HA→PP	Prx3 CT	Prx3 HA→PP+CT
SH	21540.5	21508.4	21526.5	21470.4	21456.4
SOH	21556.5	21524.4	21542.5	21486.4	21472.4
SN	21538.5	21506.4	21524.5	21468.4	21454.4
SS+SH	43078.0	- ^a	43050.0	42937.8	42909.8
SS+SO ₂ H	43110.0	- ^a	43082.0	42969.8	42941.8
SO ₂ H	21572.5	21540.4	21558.5	21502.4	21488.4
Experimental Mass Values $[M+H]^{+1}$ (amu)^{b,c}					
Oxidation State	Prx2 WT	Prx2-C2S	Prx2 PP→HA	Prx2 CT	Prx2 PP→HA+CT
SH	21762.2 ± 0.6	21729.2 ± 0.4	21775.2 ± 0.5	21831.8 ± 0.1	21845.6 ± 0.1
SOH	21776.9 ± 0.5	21745.1 ± 0.4	- ^b	- ^b	- ^b
SN	21760.3 ± 0.3	21727.2 ± 0.3	- ^b	- ^b	- ^b
SS+SH	43520.2 ± 0.3	- ^a	43546.9 ± 0.2	43659.6 ± 0.4	43687.0 ± 0.2
SS+SO ₂ H	43554.2 ± 0.7	- ^a	43582.2 ± 0.1	43693.1 ± 0.5	43723.4 ± 0.3
SO ₂ H	21793.8 ± 0.9	21760.8 ± 0.1	21808.1 ± 0.1	21863.7 ± 0.2	- ^b
	Prx3 WT	Prx3-C2S	Prx3 HA→PP	Prx3 CT	Prx3 HA→PP+CT^c
SH	21540.4 ± 0.2	21508.3 ± 0.2	21526.1 ± 0.4	21470.3 ± 0.3	21455.9 ± 0.2
SOH	- ^b	21524.2 ± 0.2	- ^b	- ^b	- ^b
SN	- ^b	21506.7 ± 0.1	- ^b	- ^b	- ^b
SS+SH	43077.3 ± 0.1	- ^a	43049.1 ± 0.1	- ^b	42908.3 ± 0.1
SS+SO ₂ H	43112.0 ± 0.9	- ^a	43083.8 ± 0.5	- ^b	42942.8 ± 0.1
SO ₂ H	21572.7 ± 0.3	21540.4 ± 0.1	21558.5 ± 0.2	21502.4 ± 0.3	21488.4 ± 0.3

^aThe removal of the Cys-S_RH residue by mutagenesis prevents the possibility of this species forming. See main text for details.

^bSpecies not observed. Please see main text for experimental details, as some species can only be captured with the time-resolved approach. Not all Prx2 and Prx3 variants were analyzed with the latter approach.

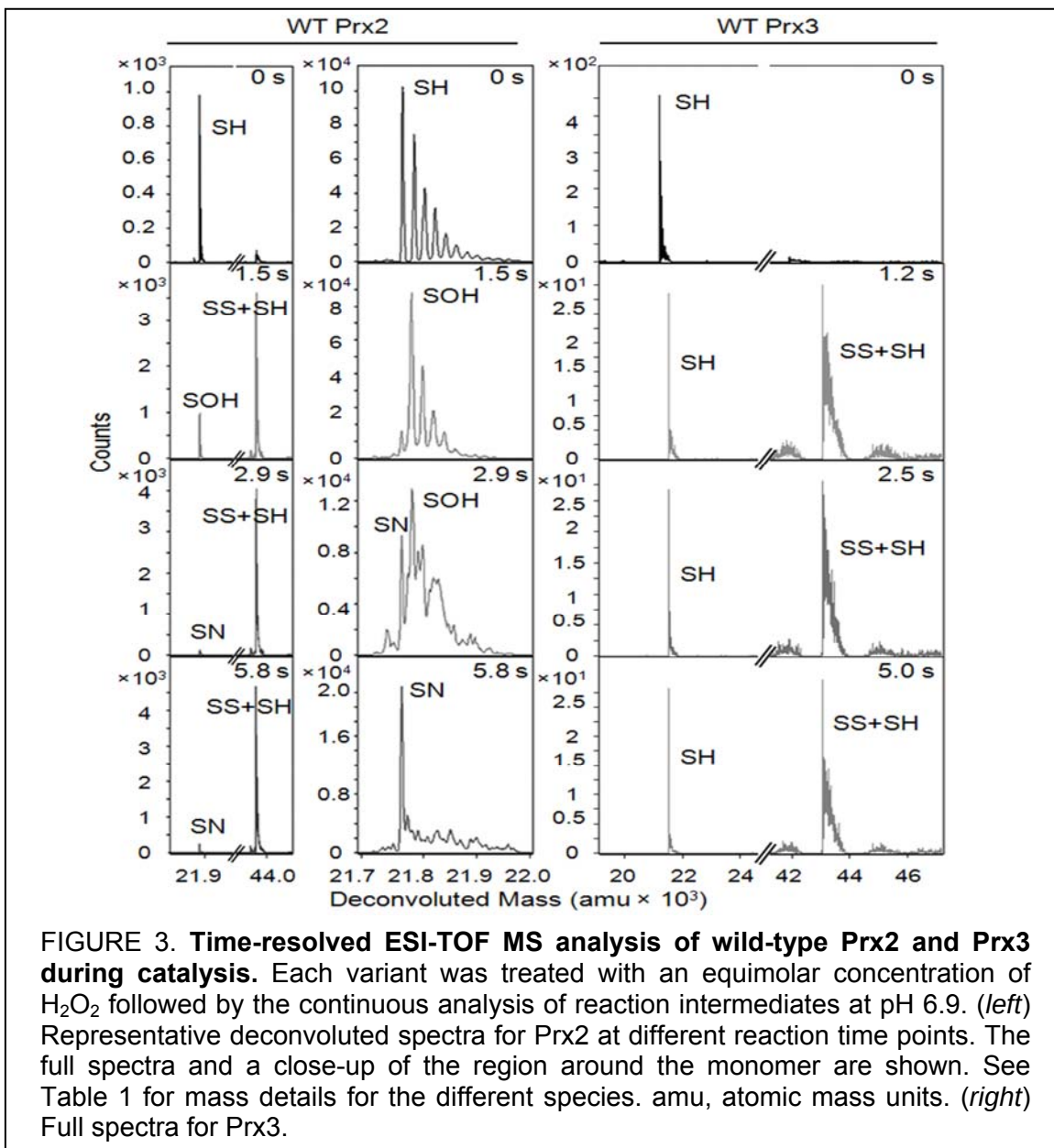
^cAll mass values were determined in triplicate except for Prx3 HA→PP+CT, which was performed in duplicate, due to the paucity of material available.

Time-resolved ESI-TOF MS analysis of early reaction intermediates—The tracking of the formation of the Cys-S_POH species in Prxs was first studied using molecular probes specific for this functional group including dimedone (29). Advances in the development of chemical probes have revolutionized the isolation and identification of other proteins that form a Cys sulfenic acid within cells exposed to a variety of stress conditions (7,30,31). The reactivity of these probes is, however, not high enough to capture the transient Cys-S_POH intermediate during the Prx reaction cycle (32). The

reported reaction rates vary from 0.003 min^{-1} to 1.65 min^{-1} at a saturating concentration of dimedone (33). Even when using chemical quench methods and ESI-TOF MS at a 30 s time point (Fig. 2), the Cys-S_POH species is not captured for wild-type Prx2 and Prx3. Therefore, a more rapid analysis of the reaction intermediates is necessary.

Time-resolved ESI-TOF MS experiments, employing an online, rapid-mixing setup, were used to monitor the formation of the Cys-S_POH species for Prx2 and Prx3 during catalysis. In this approach the Prx proteins were pre-reduced with DTT, desalted into a volatile buffer, and loaded into a Hamilton syringe. The samples were then mixed on-line in an equimolar ratio with H₂O₂ at varying flow rates (10–80 $\mu\text{L}/\text{min}$) to achieve the acquisition of mass spectra at short reaction time points (1–15 s). With this 30-fold reduction of the reaction time-scale, the detection of the Cys-S_POH intermediate at pH 7.5 was still not possible. However, decreasing the reaction pH to 6.9 enabled the detection of the Cys-S_POH species for wild-type (WT) Prx2 at 1.5 s (Fig. 3), even when the majority of the protein was present as the oxidized dimer (SS). By 5.8 s the Cys-S_POH intermediate was consumed and a new peak emerged with a mass consistent with the formation of a Cys sulfenamide (Cys-SN, M-2) (Table 1), a novel finding to our knowledge for human Prx proteins. While at this point we cannot determine whether the Cys-SN exists in solution or it was formed in gas-phase during MS analysis, its presence suggests that the Cys-SOH microenvironment supports its formation in Prx2. In contrast, only the reduced monomer (SH) and the oxidized dimer (SS+SH) were observed in the mass spectra for Prx3 in the 1.0–5.0 s reaction time range. The direct observation of the Cys-S_POH species followed by its conversion to Cys-SN support the relative stability of this intermediate in Prx2. The absence of these species for Prx3 suggests that the lifetime of the Cys-S_POH intermediate is considerably shorter than that for Prx2, as a consequence of intermolecular disulfide bond formation (S_P-S_R) being favored. Thus, the

presence of the Cys-S_RH residue and the ability to form the intermolecular SS species appears to greatly impact the lifetime of the Cys-S_POH intermediate.



Hyperoxidation of Prx2 and Prx3 Cys variants—In order to dissect the contribution of the Cys-S_RH residue and SS intermediate formation to the hyperoxidation of Prx2 and Prx3, the Cys-S_RH residue and one other non-catalytic Cys residue were mutated to Ser (Prx2-C2S, C70S and C172S; Prx3-C2S, C127S and C229S; numbering scheme based

on full-length gene sequence). These mutations leave only the Cys-S_PH residue for each protein, and therefore the dimeric SS species cannot form and the potential for unwanted thiol-disulfide exchange reactions is removed. An analysis of these variants at pH 7.5, with the addition of two equivalents of H₂O₂ for 30 s (Fig. 4), was performed using the chemical quench method coupled with ESI-TOF mass spectrometry. The Prx3-C2S variant remained in the reduced state while the Prx2-C2S variant was fully hyperoxidized, further highlighting the intrinsic differences between Prx2 and Prx3.

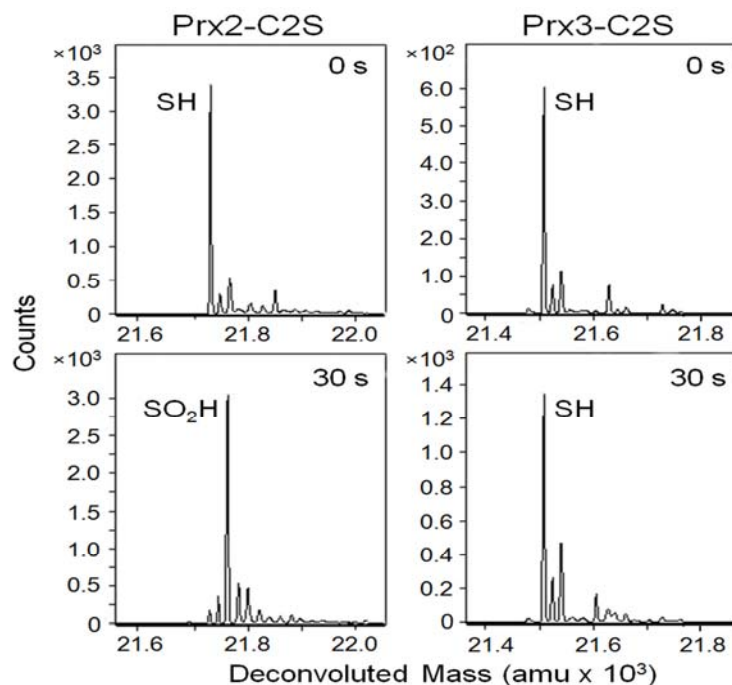


FIGURE 4. Susceptibility of Prx2-C2S and Prx3-C2S to hyperoxidation. Chemical quench and ESI-TOF MS were used to assess the oxidation state of each protein (50 μ M) following treatment with 2 equivalents of H₂O₂ for 30 s at pH 7.5. These Prx2 and Prx3 variants contain only the Cys-S_PH residue and cannot form the intermolecular disulfide reaction intermediate. Therefore, the deconvoluted spectra focus on the monomeric species, as indicated.

These Prx2 and Prx3 Cys variants were also analyzed by time-resolved ESI-TOF MS at pH 6.9 in order to evaluate the formation of reaction intermediates. For Prx2-C2S, the addition of 1 equivalent of H₂O₂ resulted in the formation of primarily Cys-S_POH species by 1.2 s (Fig. 5A). By 15 s, three species were present; Cys-S_POH, Cys-S_PN

and Cys-S_PO₂H. At the 600 s time point, the hyperoxidized species predominated. Additional time points were collected and the relative intensities converted to concentration to generate a plot (Fig. 5B) of the reduced, oxidized and hyperoxidized species versus time. The intensities for the Cys-S_POH and Cys-S_PN intermediates were combined, as the Cys-S_PN intermediate can only form from the Cys-S_POH. A global fit of the data using KinTek Explorer was used to determine the following rate constants: $k_{\text{SH} \rightarrow \text{SOH}}$, $2.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$; $k_{\text{SOH} \rightarrow \text{SO}_2\text{H}}$, $1.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$. An exponential fit to the formation of the Cys-S_PO₂H species yielded the $k_{\text{SH} \rightarrow \text{SO}_2\text{H}}$ rate constant of $9.2 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$, consistent with the conversion of the Cys-S_POH intermediate to the Cys-S_PO₂H species being the rate-limiting step in Prx2-C2S hyperoxidation.

One caveat to the Prx2-C2S studies was the unanticipated observation of more oxidization than expected, considering the equimolar proportion of H₂O₂ added. It is unclear why this occurred. Nonetheless, the data for Prx2-C2S is consistent with the increased lifetime of the Cys-S_POH intermediate and the inability to form the normal Cys-S_P-S_R-Cys intermolecular disulfide. In marked contrast, the Cys-S_POH, Cys-S_PN, and Cys-S_PO₂H species were observed at similar levels at 600 s for Prx3-C2S (Fig. 5C). These data parallel the wild-type MS data (Fig. 3), supporting further the resistance of Prx3 to hyperoxidation. Importantly, the $k_{\text{SH} \rightarrow \text{SOH}}$ rate of $\sim 10^4$ Prx2-C2S is consistent with the rate reported for turnover for the WT enzyme ($\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$), especially given the mutation of the resolving Cys residue used in the time-resolved MS studies (24,34). These observations are also consistent with the decrease in hyperoxidation observed when mutating the Cys-S_RH residue to Ser or Ala in other eukaryotic Prxs (35-37). Thus, the C-terminus of the adjacent Prx monomer can dramatically influence the reactivity and hyperoxidation of the neighboring Cys-S_PH residue.

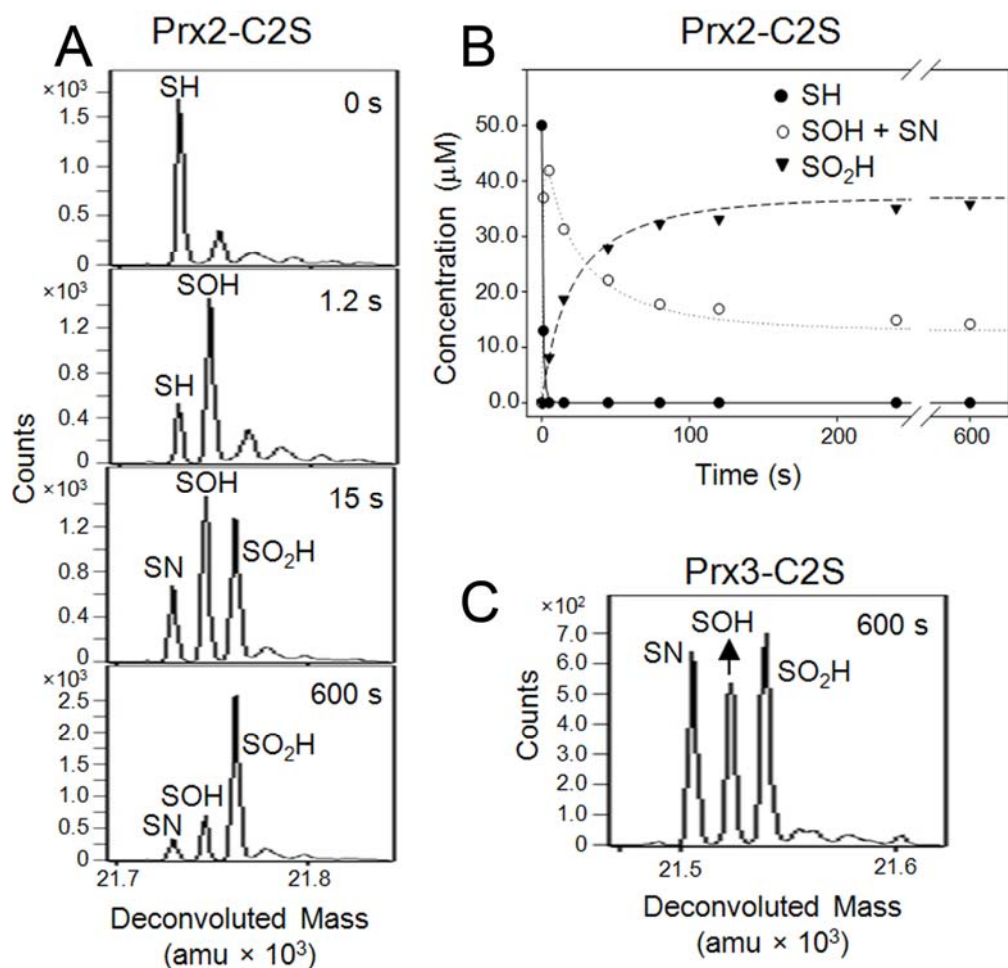


FIGURE 5. Time-resolved ESI-TOF MS analysis of the Prx2-C2S and Prx3-C2S variants during catalysis. (A) Representative deconvoluted spectra for Prx2-C2S at the indicated reaction time points. The protein was treated with an equimolar concentration of H₂O₂ (50 μ M of each final) at pH 6.9 followed by the analysis of reaction mixture, by ESI-TOF mass spectrometry. The spectra are focused on the following species: Cys-S_PH, Cys-S_POH, Cys-SO₂H, and a novel Cys-sulfenamide (Cys-S_PN) intermediate (Table 1). (B) Global kinetic modeling of the Prx2-C2S kinetic data. The plot shows the determined kinetic profiles for the -S_PH and -S_PO₂H and the combined -S_POH/-S_PN species, as the -S_PN intermediate logically originates from the -S_POH species. (C) Deconvoluted spectra for Prx3-C2S treated with H₂O₂ for 600 s.

Hyperoxidation of C-terminal and GGLG motif chimeras of Prx2 and Prx3–As

briefly described earlier, the packing of the C-terminal, YF-containing helix against the GGLG motif (Fig. 1C) is a prominent feature of eukaryotic Prxs. This interaction and the

resultant stabilization of the active site are thought to slow the rate of formation of the intermolecular SS intermediate during catalysis, enabling hyperoxidation (7,11). In fact, the mutation and truncation of the C-terminus results in an increased resistance to hyperoxidation in other Prxs (35,38-40). The appendage of a C-terminus from a Prx molecule sensitive to hyperoxidation to one that is normally resistant can also result in an increase in sensitivity to hyperoxidation (39). Similar studies have not been performed with human Prx2 and Prx3 in an effort to address their differences in hyperoxidation.

A sequence alignment (Fig. 1B) of human Prx1-4 reveals that two Pro residues, Pro98 and Pro102 of Prx2, are substituted to His and Ala in Prx3, respectively. Their position next to the GGLG motif suggests that these Pro residues may be important for the positioning of this motif to interact with the C-terminus of the adjacent Prx subunit. Four additional differences between Prx2 and Prx3 were identified adjacent to the Cys-S_RH residue (17). In this region, Gly175, Lys177, Gly179, and Asp181 of Prx2 are substituted with Asn, Thr, Asp and Pro in Prx3, respectively. A panel of Prx2 and Prx3 variants was generated where these sequence differences were swapped as a group to generate chimeras. The panel was evaluated using the same experimental conditions as for the wild-type proteins and using the chemical quench method. Importantly, these variants all contain the Cys-S_RH residue and can therefore undergo normal catalytic cycling.

Following the addition of 0.8 equivalents H₂O₂ for 30 s, the Prx2 GGLG region chimera (Prx2 PP→HA) (Fig. 6A) had a similar profile to WT Prx2, with prominent monomeric and dimeric species containing the Cys-S_PO₂H moiety. In contrast, the Prx2 C-terminal chimera (Prx2 CT) was more resistant to hyperoxidation, as indicated by the lack of formation of the Cys-S_PO₂H monomeric species. In addition, the proportion of the SS species increased, similar to that found for WT Prx3, suggesting that the rate of SS

bond formation had increased. The combination of the variants, Prx2 PP→HA+CT, did not result in a further increase in protection from hyperoxidation. These observations support that the sequence changes near the GGLG motif of Prx2 do not influence hyperoxidation. The changing of the C-terminal residues of Prx2 to those of Prx3, however, resulted in a Prx3-like protein with an increased resistance to hyperoxidation.

The analysis of the Prx3 HA→PP chimera revealed an increase in hyperoxidation; i.e., the monomeric and dimeric species containing the Cys-S_PO₂H moiety increased (Fig. 6B) over the WT protein. The Prx3 CT chimera was even more sensitive to hyperoxidation, as only the monomeric Cys-SO₂H species was observed in addition to a complete loss of SS and SS+SO₂H species. The combination of the GGLG and CT variants, Prx3 HA→PP+CT, yielded a similar increase in the monomeric SO₂H species, but also exhibited an increase in the SS+SO₂H species. It is unclear at this time how the combination of the two sets of mutations could lead to a compensatory effect. Altogether, these data support that the C-terminus of Prx3 is a key determinant to the resistance of the wild-type enzyme to hyperoxidation, and that the residues near the GGLG motif can modulate this resistance. It is interesting to note that none of the Prx2 and Prx3 chimeras exhibited a full transformation in their sensitivity or resistance to hyperoxidation. This finding suggests that other regions of the proteins and their dynamic oligomeric states may also influence the ease of hyperoxidation.

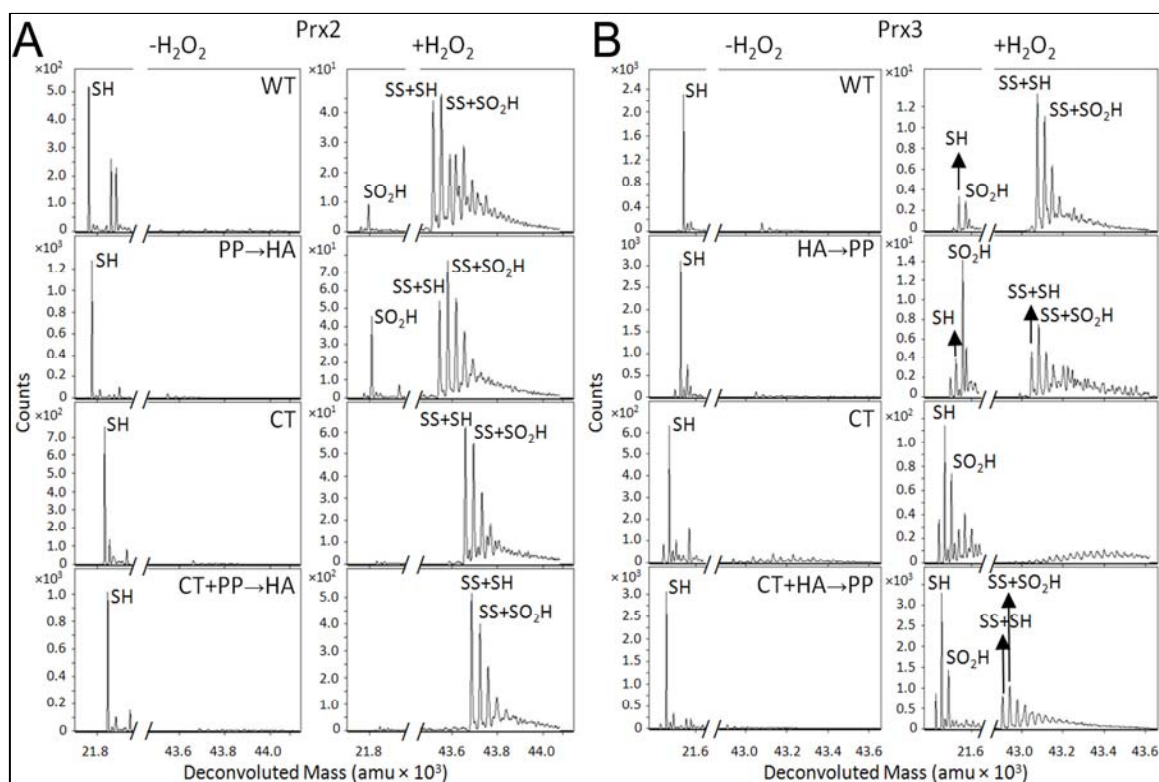


FIGURE 6. Susceptibility of Prx2 and Prx3 GGLG and C-terminal chimeras to hyperoxidation. A, Prx2 variants: WT; PP→HA (P98H and P102A); CT (G175N, K177T, G179D and D181P); PP→HA+CT. B, Prx3 variants: WT, HA→PP (H155P and A159P); CT (N232G, T234K, D236G and P238D); HA→PP+CT]. Chemical quench ESI-TOF analyses were performed with 0.8 equivalents of H_2O_2 for 30 s at pH 7.5. A close-up of the mass range for the monomeric (left) and dimeric (right) species is presented within each panel

Implications of Cys-sulfenamide formation—The observation of a Cys-S_PN intermediate for WT Prx2 and Prx2-C2S (Figs. 3 and 5) supports a Cys-S_POH–Cys-S_PN equilibrium that prolongs the lifetime of this intermediate and increases its susceptibility to hyperoxidation. On the other hand, the inability to observe the Cys-S_POH and Cys-S_PN intermediates for WT Prx3 also supports the resistance of this protein to hyperoxidation. The latter observation, however, is contrary to what one would think when examining the literature of other proteins that utilize the Cys-SN intermediate, including protein phosphatase PTP1B and OhrR (41-43). For example, the five-membered Cys-SN intermediate in PTP1B occurs between Cys215 and the backbone

amide group of Ser216. This intermediate is thought to protect Cys215 from hyperoxidation and irreversible inactivation, but facilitates glutathionylation (41,42). Thus, we were surprised to observe the Cys-S_PN species for Prx2 since it is more sensitive to hyperoxidation than Prx3. It is certainly possible that the Cys-S_PN intermediate of Prx2 is readily collapsed back to the Cys-S_POH species by the addition of a water molecule. This scenario would extend the lifetime of the Cys-S_POH species enabling hyperoxidation.

Inspection of the Prx2 active site (Fig. 1C) and the residues surrounding the Cys51-S_PH residue reveals that Prx2 would not be able to form a backbone-mediated Cys-S_PN intermediate similar to PTP1B. Cys51 is adjacent to the conserved amino acid Pro52, which lacks an amide proton and cannot attack the sulfenic acid moiety. Based on studies with synthetic peptides, it is possible that the Cys-S_PN formation in Prx2 is mediated through the amine groups of a Lys or Arg side chain (44). Arg127, a conserved residue, is the only residue adjacent to Cys51 that could be involved in Cys-SN formation. It is unclear whether the Cys-S_PN intermediate of Prx2 has biological relevance other than providing evidence for an extended lifetime of the Cys-S_POH intermediate. While the stabilization of the latter is essential to enable hyperoxidation and inactivation, as proposed in the floodgate hypothesis for H₂O₂-mediated cell signaling, it may be that a stable Cys-S_POH intermediate is necessary to facilitate the formation of disulfide bonds with other proteins (7, 11, 45). Similarly, the Cys-S_PN intermediate in Prx2 would be amenable to attack by the resolving Cys172 residue, a target protein thiol, or glutathione. In each of these scenarios, the Prx2 molecule could ultimately be returned to the reduced state capable of further rounds of catalysis. WT Prx3 appears to bypass the formation of the Cys-SN intermediate since the formation of intermolecular SS bond between the Cys-S_POH intermediate and Cys-S_RH residue is facile. Given the highly oxidizing environment of the mitochondria, it makes sense that

the Prx3 molecule would favor rapid SS formation in order to protect the Cys-S_PH residue. Nonetheless, hyperoxidation of Prx3 and its subsequent repair by Srx does occur within the mitochondrion and plays a crucial role in adrenal steroidogenesis (6).

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CHAPTER THREE

**COMPARATIVE ANALYSIS OF STRUCTURAL FEATURES INFLUENCING
CATALYSIS AND INACTIVATION OF HUMAN TYPICAL 2-CYS
PEROXIREDOXINS 2 AND 3**

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ABSTRACT

Mitochondria are major generators of reactive oxidative species (ROS) and free radicals produced as dangerous byproducts of the electron transport chain. Oxidative stress results from the imbalance between ROS production and removal by antioxidants; it damages cellular macromolecules, including carbohydrates, lipids, nucleic acids and proteins. From a clinical perspective, mitochondrially-generated ROS have a major role in injury to the myocardium. Mitochondrial antioxidant enzymes, including peroxiredoxin 3 (Prx3), are the first line of defense against oxidative heart damage. The transgenic overexpression of Prx3 has a protective effect on the heart in a murine model of myocardial infarction. Therefore, therapeutic approaches designed to mitigate mitochondrial oxidative stress utilizing Prx3 have the potential to prevent heart failure. Prx3 is a unique member of the typical 2-Cys sub-family of peroxiredoxins (Prxs). Prxs catalyzes the reduction of hydrogen peroxide via a catalytic, 'peroxidatic' cysteine. In the presence of excess hydrogen peroxide, however, the sulfur atom of this cysteine residue is covalently modified through hyperoxidation to Cys sulfinic acid (Cys-SO₂H). The inactivated Prx is repaired by an enzyme called sulfiredoxin (Srx). The rates of hyperoxidation of Prx3 differ from those of other human, 2-Cys Prxs, such as Prx1, Prx2, and Prx4. Interestingly, the C-terminus of Prx3 contains an unusual primary sequence in the region that interacts with Srx and contributes to these differences. A combination of X-ray crystallography and homology modeling was used to identify structural features that explain differences observed between the more susceptible to hyperoxidation Prx2 and the less susceptible Prx3. Regions within the Prx C-terminus (CT) of the adjacent monomer (including the Srx interface), the last C-terminal Helix (α 5 or CT-Helix2), and within the active site helix (α 2), residues near the GGLG motif as well as key conserved residues within the active site reveal variations that favor flexibility in Prx2, slowing formation of the disulfide bond and favoring hyperoxidation.

INTRODUCTION

Significance of ROS and Oxidative Stress to Cardiovascular Diseases:

Reactive oxygen species (ROS) are key mediators of cardiovascular disease (1). The term ROS refers to a diverse set of chemical species derived from molecular oxygen metabolism which includes superoxide anions, hydroxyl radicals, hydrogen peroxide and peroxynitrite. Up to 1-2% of oxygen molecules are converted to ROS intracellularly by the mitochondrial electron transport chain (2). The balance between ROS production and removal by antioxidants determines the extent of oxidative stress. Oxidative stress causes the deleterious modification of several macromolecules including lipids, nucleic acids and proteins (3). Antioxidant enzymes, particularly those situated within the mitochondria, play a critical homeostatic role in modulating the survival and death signaling pathways in the cells of the myocardium (1,4). One such enzyme, Prx3, is involved in redox regulation through the cyclic oxidation and reduction of cysteine (Cys) residues during its normal catalytic cycle. This redox cycling enables Prx3 to protect myocardial cells from oxidative stress and inhibits apoptosis (5).

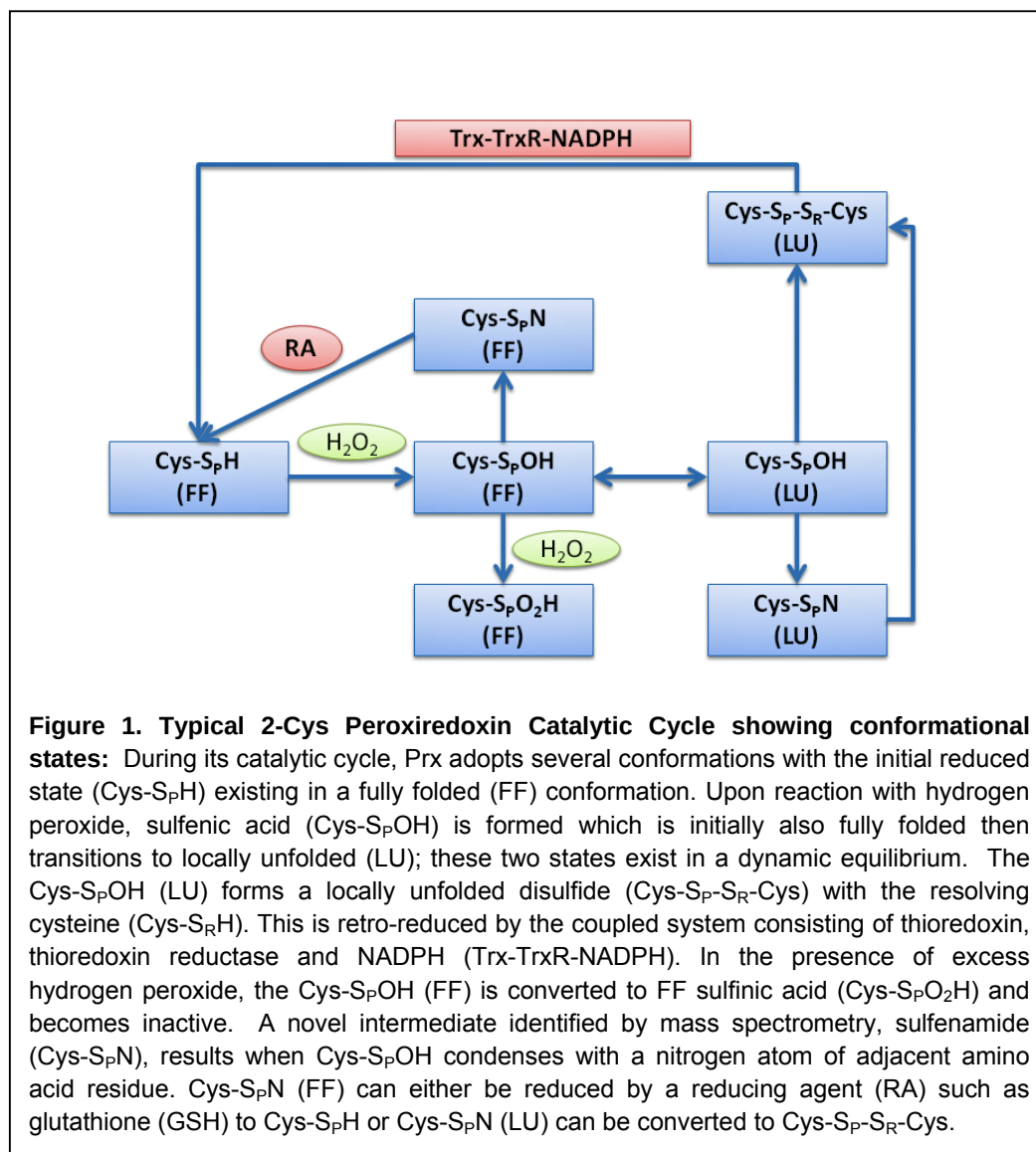
During extreme oxidative stress, however, the antioxidant defense mechanisms often fail resulting in damage to the myocardium, leading to contractile and structural failure, myocyte hypertrophy, apoptosis and interstitial fibrosis (5). The aberrant ROS production affects many cell signaling pathways (6). For example, increased levels of ROS generated in the mitochondria modulate hypoxia induced factor 1 alpha (HIF-1 α) in response to oxygen tension (7). The myocardium has the highest oxygen absorbance rate in the human body, with a basal rate of 0.1 ml g⁻¹ min⁻¹ (8). Thus, by physiological necessity, cardiomyocytes have the highest concentration of mitochondria per unit volume in order to sufficiently generate ATP via oxidative metabolism. Heart ROS are generated by several cellular sources including cardiomyocytes, vascular endothelial cells and neutrophils (9). Importantly,

mitochondria from failing hearts produce more oxygen radicals (10). Therefore, there is a link between mitochondrial dysfunction and oxidative stress (11).

Impact of Oxidative Stress on the Mitochondria of Cardiomyocytes: Normal mitochondrial functions are regulated by both mitochondrial DNA (mtDNA) and factors that control mtDNA replication and/or transcription (12-14). A decrease in mitochondrial function and mtDNA copy number is associated with heart failure post myocardial infarction (10,15). Mitochondria lack histone-mediated DNA protection; have minor DNA repair activity, and must withstand constant bombardment with oxygen radicals (16). It is hypothesized that mtDNA oxidative damage, as well as dysregulation of replication and transcription, play a role in myocardial failure (17-21). Fortunately, there are several mitochondrial antioxidant enzymes that scavenge for ROS. These include peroxiredoxin 3 (Prx3), peroxiredoxin 5 (Prx5), glutathione peroxidase (GPx), manganese superoxide dismutase (MnSOD) (4,5). Prx3 is ~30 times more abundant than GPx and MnSOD in mitochondrial extracts, and 90% of mitochondrial H₂O₂ preferentially reacts with Prx3 (22,23).

Peroxiredoxin 3, a Unique 2-Cys Peroxiredoxin: Prx3 belongs to a ubiquitous family of thiol-dependent peroxidases involved in redox regulation of cell signaling and antioxidant defense (1). The six mammalian isoforms (Prx1-6) are divided into three categories (2-Cys, atypical 2-Cys and 1-Cys) according to the number and location of their cysteine residue(s) (24). In the 2-Cys Prx (Prx1-4) catalytic cycle (Figure 1), the reduced Prx (Cys-S_PH) attacks a H₂O₂ molecule to form a sulfenic acid intermediate. An intermolecular disulfide bond is then formed between the Prx molecules within the dimeric unit (Cys-S_P-S_R-Cys) and ultimately reduced by thioredoxin (Trx). Kinetic analysis using time-resolved ESI-TOF mass spectrometry (Chapter 2), revealed an interesting difference between Prx2 and Prx3, in that the sulfenic acid is more stable and thus longer lasting for Prx2 than Prx3 allowing it to be converted to a sulfenamide (Fig. 1). The sulfenic acid intermediate was not observed for WT Prx3 indicating that it was not long-lived and thus the subsequent

conversion to the sulfenamide is not seen. In high oxidative stress conditions, a second H_2O_2 molecule reacts with the sulfenic acid intermediate to form a sulfinic acid (Cys-SO₂H). This hyperoxidized, catalytically inactive species is reduced by the unique repair enzyme sulfiredoxin (Srx) (25).



Prx3 is a protective factor in the myocardium: Overexpression of Prx3 protects the murine heart from post myocardial infarction remodeling and heart failure, as measured by the reduction in left ventricular cavity dilation, myocyte hypertrophy, interstitial fibrosis and apoptosis (18). In the same study, Prx3 overexpression was shown to decrease oxidative stress, mtDNA copy number

decline and dysfunction. Therefore, Prx3 has a protective function and can influence the progression of cardiomyopathy. Importantly, Prx3 undergoes oxidation to its locally unfolded disulfide-bonded dimer (Fig. 1) during ischemia, and this is reversed during reperfusion (26). Moreover, during apoptosis Prx3 is rapidly oxidized to its disulfide-bonded form which precedes major apoptotic events and caspase activation (27). Oxidative stress can also result in human Prx3 (Prx3) hyperoxidation and higher order oligomerization, but Prx3 is more resistant to hyperoxidation than human Prx1 and Prx2 present in the cytoplasm (28). Data presented in Chapter 2 confirmed this and identified residues involved in this regulation.

A detailed investigation of the three-dimensional structures of the critical redox states of Prx3 and Prx2 *i.e.* reduced (SH), oxidized (S-S) and hyperoxidized (SO_2^-) forms is necessary to understand why Prx3 is more resistant to oxidation. There is no structural data for human Prx3, but there is a low resolution structure of bovine Prx3 (3.3 Å resolution, 88.7% sequence identity to human Prx3). This structure is, however, controversial in the field as it is a concatenated dodecamer (*i.e.* 2 interlocked rings of 6 dimers each) and not a single decamer like other 2-Cys Prxs (5 dimers) (29). Detailed analysis of Prx2 and Prx3 structures should provide insight into how they rearrange during oxidation/hyperoxidation, and reveal what the Prx3 substrate of Srx looks like before repair.

Prx3 has a unique C-terminal sequence. The C-terminal region of 2-Cys Prx molecules is thought to facilitate hyperoxidation by slowing down the formation rate of the intermolecular disulfide bond during catalysis by hindering the transition from the fully folded to locally unfolded state (Fig. 1) (30). The protection from hyperoxidation observed in C-terminal truncation variants of other eukaryotic 2-Cys Prxs supports this notion (31,32). Importantly, the C-terminal region also undergoes major structural rearrangements required for Srx-mediated repair (25,33). As described in more detail below, a sequence alignment has revealed significant differences in the C-terminal region of Prx3 when compared to Prx1, 2 and 4. It is

hypothesized that these differences result in the greater resistance of Prx3 to hyperoxidation. The results of an analysis of chimeras of Prx2 and Prx3 in this region (Chapter 2) support this notion. However, these chimeras did not fully “switch” in their phenotype, suggesting that other regions of the proteins contribute to activity and hyperoxidation. The crystal structures and homology models presented here identify additional regions that may play a role in the observed differences in peroxidase activity and hyperoxidation of Prx3 when compared to the readily hyperoxidized Prx2.

Structural features that facilitate catalytic conformational changes: Based on the literature available and previous comparisons of bacterial and other eukaryotic Prxs, the reduced and hyperoxidized forms exist in a fully folded (FF) conformation, *i.e.* the active site helix containing the peroxidatic cysteine is folded and approximately 13 Å away from the resolving cysteine located near the C-terminus (30). In this conformation the GGLG motif and the C-terminal region which contains the YF motif are positioned next to each other causing the peroxidatic cysteine to be buried. In order for the disulfide bond to form there is a conformational change from the FF to the locally unfolded (LU) state. The unfolding of the C-terminal region and the active site helix facilitate the reaction of the sulfenic acid of the peroxidatic cysteine with the thiol of the resolving cysteine to generate the disulfide (Fig. 1). Any changes in the C-terminus that affect its orientation and/or interactions with the rest of the Prx molecule may lead to a favorable rate of disulfide bond formation and thus decrease the rate of inactivation via hyperoxidation.

In the series of homology modeling and X-ray crystallography experiments presented here, specific attention was paid to any changes in the active site and C-terminal conformations when comparing Prx2 and Prx3 and other bacterial Prxs, also known to be more resistant to hyperoxidation (34). These comparisons identified additional key residues for site directed mutagenesis studies and kinetic evaluations. Moreover, Prx3 was modelled as decamer, dodecamer and concatenated

dodecamers and the dimer-dimer interface was carefully examined to identify additional residues unique to Prx3 that may facilitate this unusual arrangement and whether or not these interactions contribute to the resistance of Prx3 to hyperoxidation. This yielded a surprising result in that the dimer-dimer interface was structurally conserved between the decamer and dodecamer. However, at the points of contact between the two dodecamers an interesting hydrogen bonding network was observed.

EXPERIMENTAL PROCEDURES

Purification of Human 2-Cys Peroxiredoxin 2 in Disulfide (Oxidized State):

The human Prx2 gene was subcloned into the pET17 (Novagen) without any additional N-terminal or C-terminal residues. It was expressed in BL21-Gold (DE3) *Escherichia coli* cells (New England Biolabs). The *E. coli* cells were grown at 37° C until an OD₆₀₀ of 0.8 and induced with 0.5 mM IPTG at 25° C for 4-5 hr. The purification required four chromatographic steps. The cells were lysed in 100 mL of 20 mM HEPES pH 7.9, 100 mM NaCl, 1 mM EDTA containing protease inhibitors (PMSF and benzamidine; both at 0.1 mM) using an Emulsiflex C5 (Avestin, Inc.). This mixture was then centrifuged and the supernatant treated with 2.5% streptomycin sulfate following by centrifugation. Ammonium sulfate was added to a final concentration of 20% to the supernatant and the solution filtered. This solution was loaded onto a Phenyl Sepharose High Performance (Low Sub) column (GE Healthcare) and eluted with 600 mL linear gradient to buffer without ammonium sulfate. The fractions corresponding to the oxidized Prx molecule, as determined by reducing and non-reducing SDS-PAGE, were dialyzed into 20 mM Tris pH 7.9 and subsequently loaded onto a Q-Sepharose FF column (GE Healthcare) and eluted with a 600 mL linear gradient to 500 mM NaCl. The Prx fractions were pooled and dialyzed into 7 mM potassium phosphate pH 7.0. The dialysate was subsequently

loaded onto a CHT ceramic hydroxyapatite column (Bio-Rad) and eluted with a 600 mL linear gradient to 400 mM potassium phosphate pH 7.0. The Prx2-containing fractions were concentrated to 5 mL and loaded onto a Superdex 200 column equilibrated with 20 mM HEPES pH 7.5, 100 mM NaCl. The Prx2 fractions were pooled, concentrated, and flash frozen with liquid nitrogen. This was stored at -80° C until ready to be used.

Crystallographic analysis of Prx2 (SS): Prx2 disulfide (SS) crystals initially appeared in a condition which consisted of 0.2 M zinc acetate, 0.1 M sodium acetate pH 4.5 and 10% PEG 3000 at room temperature using the sitting drop vapor diffusion technique. Several rounds of optimization were carried out varying parameters that included protein concentration, drop size, temperature, buffer concentration and pH, and the precipitant concentration (35). The condition that yielded the best diffracting crystals were: a protein concentration of 10mg/ml, a drop size of 14µl (7µl protein and 7µl well solution), 6.1% PEG 3350, 0.1 M sodium acetate pH 4.1, 0.2 M zinc acetate at a temperature of 25° C. Cryo-cooling conditions were determined by testing the following reagents: glycerol, MPD, ethylene glycol, PEG400 and paratone (36). The best cryo cooling reagent was found to be 25% glycerol. The best method to cryo protect the crystals involved using a mother liquor containing 10% PEG3350, 0.2 M zinc acetate, 0.1 M sodium acetate pH 4.1, 5% glycerol and then slowly increasing the glycerol content to 25% glycerol. The best approach was to slow soak and transfer the crystal to mother liquor solutions with increasing concentrations of glycerol. Data was collected at the Brookhaven National Synchrotron Light Source (NSLS) on the X25 beamline. Data collection statistics are displayed in Table 1. Phasing was done using molecular replacement with the search model as a dimer of Prx2-SO₂H (1QMV) (37). This model was modified by the removal of the GGLG motif region, the C-terminus and active site helix regions of the Prx molecule to prevent model bias during structure solution and rebuilding. The PHASER program was used to solve the phases by searching for five dimers (38). Model building,

refinement and validation of model quality were completed using COOT, CNS/REFMAC5/PHENIX and MOLPROBITY, respectively (39-43).

Homology Modelling:

(A) Overall Homology Modeling Rationale

The ultimate goal of homology modeling is to predict the structure of a protein from its sequence with accuracy comparable to experimentally obtained structures (44). Homology modeling protocols consist of seven steps: template recognition and alignment; correction of the initial alignment; generation of the backbone; modeling of loops; modeling of side-chains; optimization of the model and finally validation of the model. Recent advances have introduced high resolution methods for model refinement that incorporate information using full atomic details that can take into account secondary structure and core packing rearrangements to generate physically realistic models (45). Based on these recent advances in field, YASARA Energy minimization server was selected to further refine models generated by the following homology servers, PHYRE2 and SWISSPDB homology modeling.

(B) Peroxiredoxin 2

Prx2 reduced (SH) monomers were modeled using PHYRE2 in intensive mode using the Prx2SO₂H Chain A as the template (1QMV) (37,46,47). Prx2 SH dimers were generated using the monomers generated by PHYRE2 and the protein-protein docking server ClusPro 2.0 (48-51). YASARA Energy Minimization Server was used to refine the model and the quality of the model was assessed using MOLPROBITY web server (43,45). Prx2 SH decamer model was generated using Prime in Maestro (Schrödinger Suite 2012) based on 1QMV then further refined using YASARA Energy Minimization Server and analyzed using MOLPROBITY (43,52,53).

(C) Peroxiredoxin 3

Prx3 reduced (SH) monomers and dimers were generated and refined using the same approach mentioned above for Prx2, using the template *Saccharomyces cerevisiae* thiol specific antioxidant protein 1 (Tsa1 C47S) PDB code 3SBC Chain G

(54). Prx3 SH and Prx3 C108D (hyperoxidation mimic) decamer models were generated using the same approach mentioned above for the Prx2 decamer with the template being Prx4 SH (PDB code, 2PN8). The same procedure was also used to produce the Prx3 SH dodecamer model using as the template a single dodecamer of bovine Prx3 SH, a single dodecamer (PDB code, 1ZYE) (29). The SWISSPDB homology modeling server in the automatic modeling mode was used to generate Prx3 SH concatenated dodecamers based on the 1ZYE template and then refined using YASARA (55-57). The same approach was used to generate Prx3 disulfide (SS) decamer using Prx4 SS (PDB code, 3TJB) as a template (58).

RESULTS AND DISCUSSION

Peroxiredoxin 2 reduced monomer, dimer and decamer homology models:

The model of Peroxiredoxin 2 in the monomeric form revealed some intriguing differences not seen in other monomeric Prxs such as BCP (59). The overall monomeric structure is comparable to previous Prx structures, but there were differences observed in the final helix of the C-terminus also called CT Helix 2 or $\alpha 5$ in purple (Fig. 2A&B) containing the YF motif and also in the bend of the active site helix ($\alpha 2$) also referred to as the NRA (N60, R61, A62) region in blue. These regions were partially unfolded from canonical helices into loops. Of greater interest is that the peroxidatic cysteine (Cys-SP) is also not on a canonical helical turn, as previously observed. This indicates a certain degree of flexibility within these regions in the monomeric form. This can be attributed to the absence of an adjacent monomer which restrains the active site region allowing a specific range of motion and provides interacting partners for CT Helix 2.

By contrast, the dimer model reveals that the NRA region is not partially unwound, but within a canonical helical turn and Cys-SP now also becomes part of a canonical helical turn situated on the very edge of the helix (Fig. 2C&D). This is

indicative of the fact that having a partner monomer is needed to introduce restraint into the active site region. The YF motif region remains a loop, i.e., not part of a canonical helical turn (purple residues). The distance between Cys-SP and the resolving cysteine Cys-SR is 17.1 Å, which is larger than the distance observed for other obligate Prx homodimers (Fig. 2D) (60). Three key catalytic residues showed changes in their positions when compared to those in the literature, R127 is 7.6 Å from Cys- SP, 2.9 Å from E54, and E54 is 9.5 Å from R150, and these distances are suboptimal for catalysis (Fig. 2D) (54,61).

In the decamer model, the NRA region is similar to that observed for the dimer model. Cys-SP is now located within the first helical turn. The YF motif in CT Helix 2 is now part of the helical turn (Fig. 2E&F). The peroxidatic cysteine and the resolving cysteine are now situated 13.6 Å apart. This suggests an increasing restriction of the active site region. An overlay of Prx2 dimer (pink and grey) and the Prx2 decamer (green and cyan) reveal that the differences (orange regions) significantly affect the active site (Fig. 2G). There is a major shift observed within the N-terminus, the active site helix and GGLG motif, which causes the peroxidatic cysteines to be displaced by 6.3 Å. A smaller shift occurs within the C-terminus especially CT Helix 2 resulting in the resolving cysteines being only 2.6 Å shifted with respect to one another. Key conserved residues also show shifts when compared with the dimer, with Arg 127 now 3.8 Å and 3.3 Å from Cys-SP and Arg 150 rotated in and 2.9 Å away from E54 (Fig. 2H). No change in distance was observed between R127 and E54 where the distance remained at 2.9 Å. These distances and positions of these key residues are now optimally positioned for catalysis.

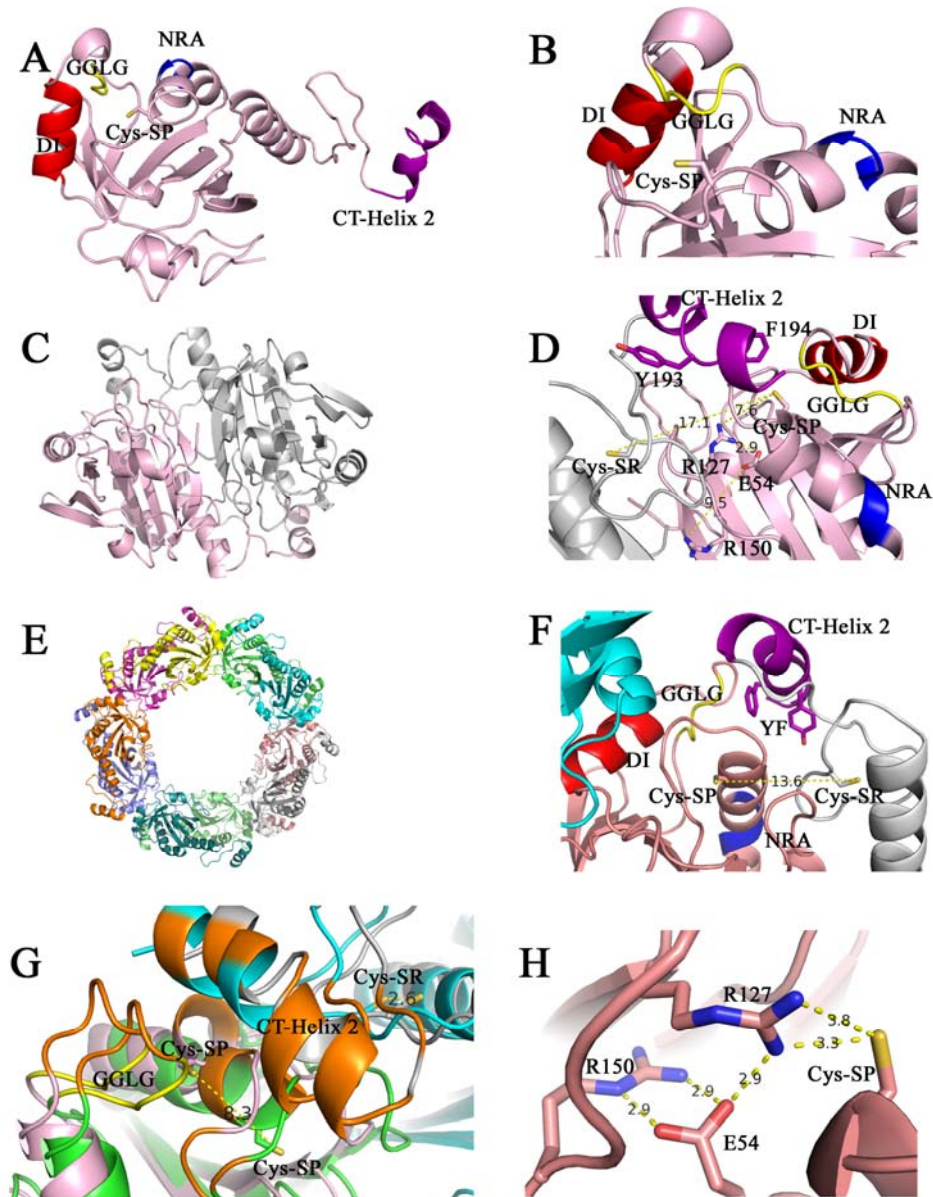


Figure 2. Differences between monomer, dimer and decamer of Peroxiredoxin 2 in the reduced form: (A) Prx2 SH monomer (B) Prx2SH monomer active site showing the peroxidatic cysteine, the GGLG motif (yellow), the extra C-terminus (purple) with the YF motif, the dimer interface -DI (red) and the NRA region (blue). (C) Prx2SH dimer (D) Prx2SH dimer active site showing the peroxidatic cysteine (pink), the resolving cysteine (gray), along with other sites mentioned in (B). (E) Prx2SH decamer. (F) Prx2SH decamer active site (G) Overlay of dimer (pink and grey) and decamer (green and cyan) structures with regions showing differences highlighted in orange. (H) Decamer active site highlighting two catalytically important arginines (127 and 150) and a conserved glutamate (54).

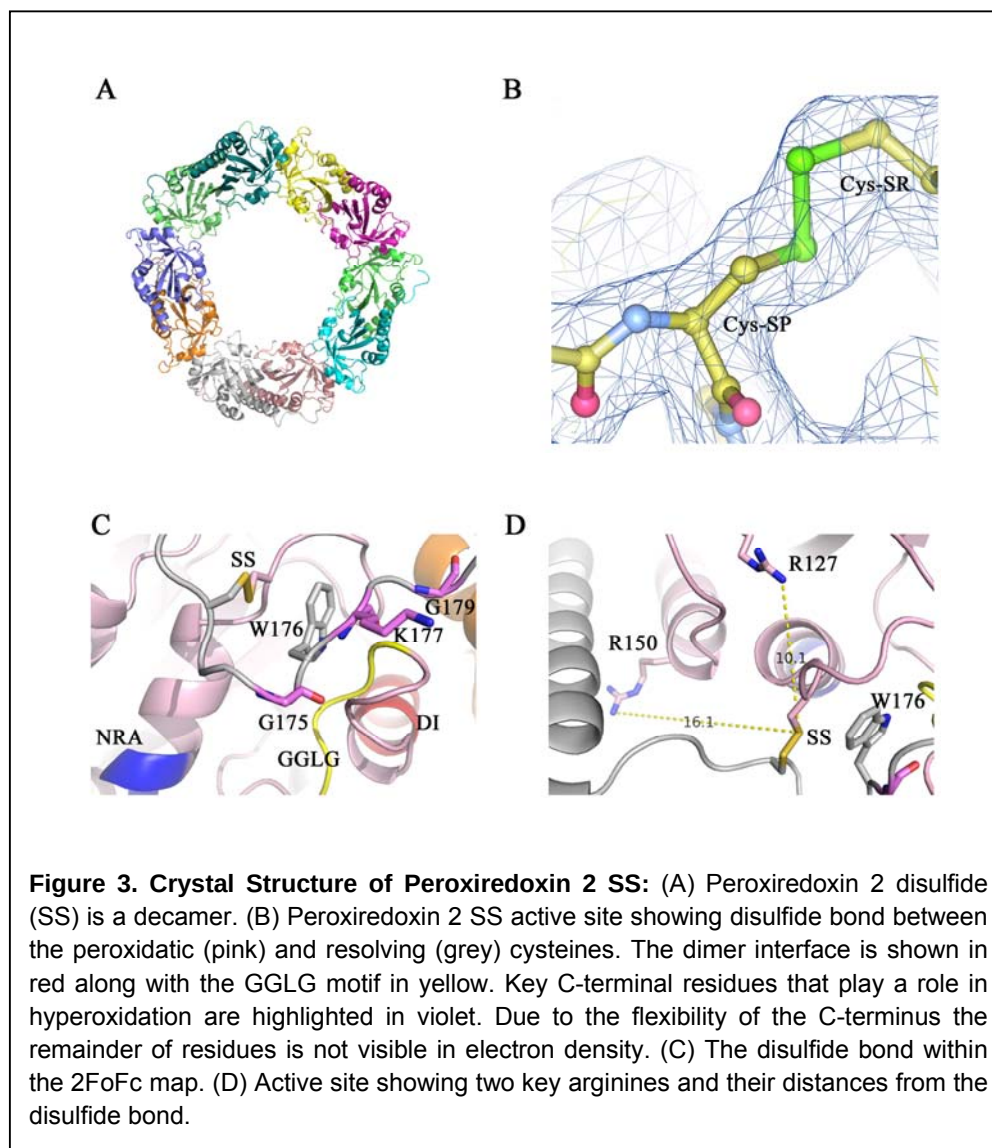
These models provide great insight into the observations that the decamer is more active than the dimer and why typical 2-Cys Prxs function catalytically as an obligate homodimer (62). It appears that the partner monomer of the homodimer and then their subsequent assembly into decamers restricts the active site and the positioning of key catalytic residues. This restriction of the active site therefore provides the optimal orientation for catalysis. The residues are now positioned to bind a H₂O₂ molecule. This impact of the oligomeric state on activity of a 2-Cys Prx, specifically tryparedoxin peroxidase from *Trypanosoma cruzi*, was studied using molecular dynamics simulations (63). These simulations indicated that the oligomeric state affected the pK_a of the peroxidatic cysteine with the decameric state possessing the ideal pK_a value for optimum reactivity. Altogether the evidence within the literature and results of the homology modeling presented herein support the idea that the decameric state is the most active.

Peroxiredoxin 2 oxidized decamer (SS) crystal structure: The human 2-Cys Prx2 disulfide-bonded crystal structure (data collection and refinement statistics shown in Table 1) was determined as a stable decamer, similar to that seen for Prx4 SS (Fig. 3A) (58). There is good electron density observed for the disulfide bond as shown by the 2Fo-Fc map (Fig. 3B). The active site shows major rearrangement with part of the active site helix containing Cys-SP unwinding to form the locally unfolded disulfide bond (Fig. 3C). No changes were observed in the NRA region further confirming that only part of the helix unwound. The conserved tryptophan within the C-terminus (W176) is now packed adjacent to the disulfide. Conserved Arg residues, R127 and R150, are now located 10.1 Å and 16.1 Å away from the Cys-SP, providing further evidence of the disruption and unfolding of active site from fully folded to locally unfolded state. In this LU state, the enzyme is inactive and cannot react with any incoming H₂O₂ molecule until the disulfide is reduced by Trx, allowing the Prx molecule to return to its active fully folded state (Fig.1 and Fig. 2D).

Table 1 Data collection and refinement statistics (molecular replacement)

Human Typical 2-Cys Peroxiredoxin 2 SS Decamer	
Data collection	
Wavelength (Å)	1.1
Space group	P 1 21 1
Cell dimensions	
<i>a, b, c</i> (Å)	50.04, 198.75, 116.63
α, β, γ (°)	90, 96.17, 90
Resolution (Å)	44.49 - 2.15 (2.23 - 2.15)*
<i>R</i> _{merge}	0.066(0.737)
<i>I</i> / σ	19.05 (2.83)
Completeness (%)	99.77 (97.86)
Redundancy	6.61(6.57)
Wilson B-factor	49.64
Refinement	
Resolution (Å)	44.49 - 2.15 (2.23 - 2.15)
No. reflections	122212 (11959)
<i>R</i> _{work} / <i>R</i> _{free}	0.2077/ .2436
No. atoms	26670
Protein	13199
Ligand/ion	-
Water	233
B-factors	
Protein	59.00
Ligand/ion	-
Water	43.80
R.m.s. deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.65
Clash score	11.77
Ramachandran	
Favored (%)	96
Outliers (%)	0.96

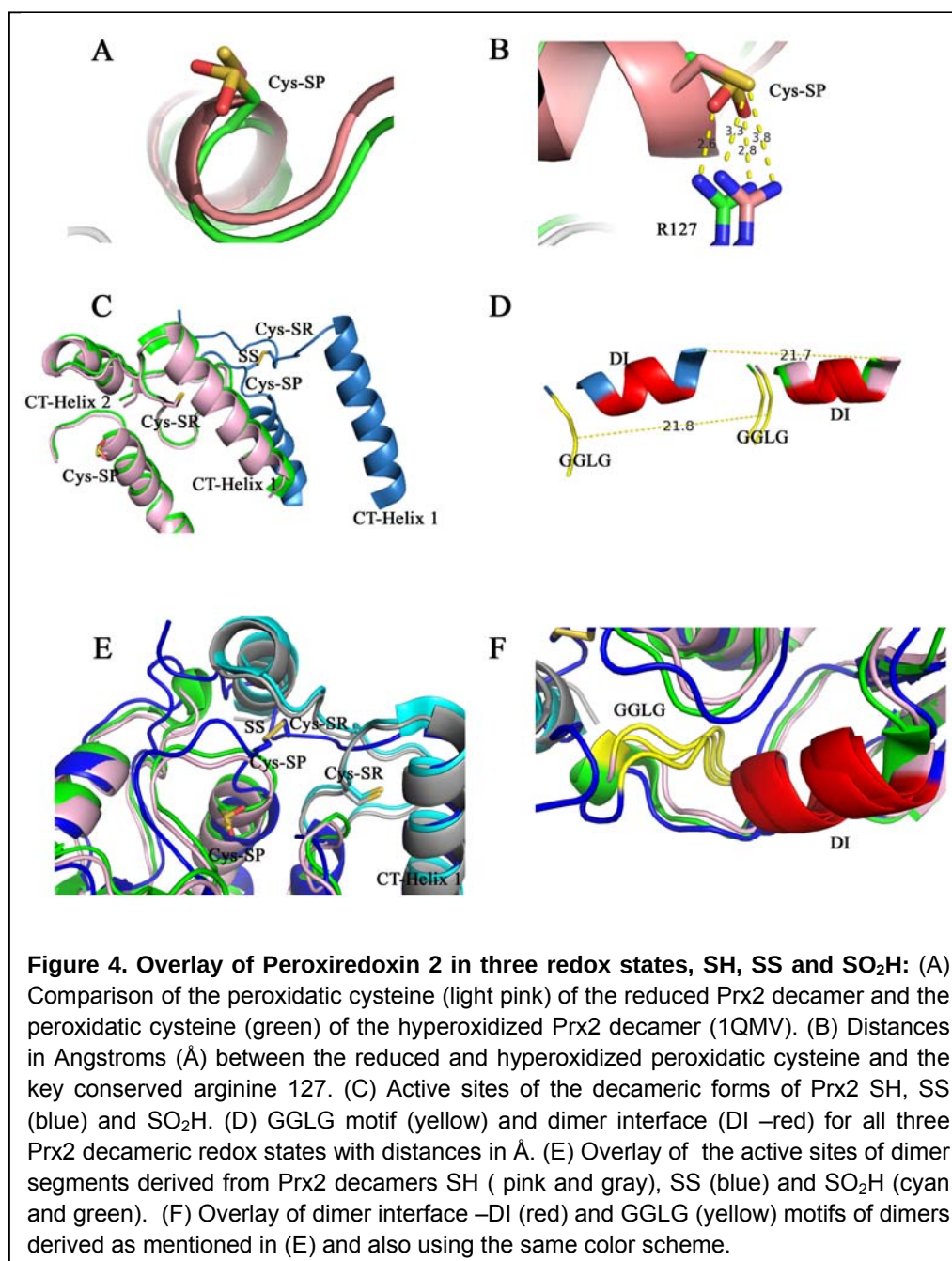
*Data collected from a single crystal. *Values in parentheses are for highest-resolution shell.



Comparison of Peroxiredoxin 2 SH, SS and SO₂H decamers: An overlay of Prx2 SH (pink) and Prx2 SO₂H (green) reveals a very similar overall structure, but there are still subtle differences (Fig. 4A). Cys-SP of the hyperoxidized form is not part of a canonical helical turn while that of the reduced form is, thus indicating the hyperoxidized form has begun to partially unfold. This unfolding may make it easier for the further unwinding needed for favorable orientation towards repair enzyme sulfiredoxin (Srx). Arg127 is located at distances of 2.6 Å and 2.8 Å from Cys-SP in the hyperoxidized structure, when contrasted to distances of 3.3 Å and 3.8 Å in the reduced structure (Fig. 4B). This hydrogen bonding to the Cys-SP in the

hyperoxidized form could compensate for the loss of restriction created by the partial unwinding of the active site. This orientation can be likened to a locked door, with the lock being the H-bonded Cys-SP and Arg127 and hinge being the partially unwound helix loop. The key in this scenario would be the properly oriented Srx molecule in complex with its cofactors magnesium and ATP. In other words, the hyperoxidized Cys-SP is perfectly positioned for an attack by the Srx repair enzyme and the active site region will not unwind until Srx attacks.

An overlay of all three redox states Prx2 SH (light pink), Prx2 SS (blue) and Prx2 SO₂H (green) reveals that, as expected, the fully folded states SH and SO₂H are quite similar whereas the locally unfolded SS shows major structural rearrangements of the active site region (Fig. 4C). The active site helix and CT-Helix 1 shows a dramatic shift in the disulfide form away from their positions in the fully folded form. The C-terminus unfolds in such a manner that the CT-Helix 2 is not visible in the electron density. Another orientation shows the major shift in positions between the dimer interface also referred to as DI (red) of the fully folded state and locally unfolded state; they are shifted by 21.7 Å. Likewise, a large shift of GGLG motif (yellow) of 21.8 Å is observed between the locally unfolded state and the fully folded states (Fig. 4D). These movements concur with previous observations in the field; that the movement from the fully folded to the locally unfolded state requires major rearrangements that could eventually lead to destabilization of the decamer. These necessary rearrangements also slow the unfolding of the active site allowing the Cys-SP to remain in the fully folded state longer and in the correct orientation for an attack by a second H₂O₂ molecule. This facilitates hyperoxidation and introduces susceptibility to it. The larger the rearrangements needed, the longer the Cys-SP will remain fully folded and exposed. This leads to a greater susceptibility to hyperoxidation and could point to a key difference between more sensitive Prx2 and less sensitive Prx3.

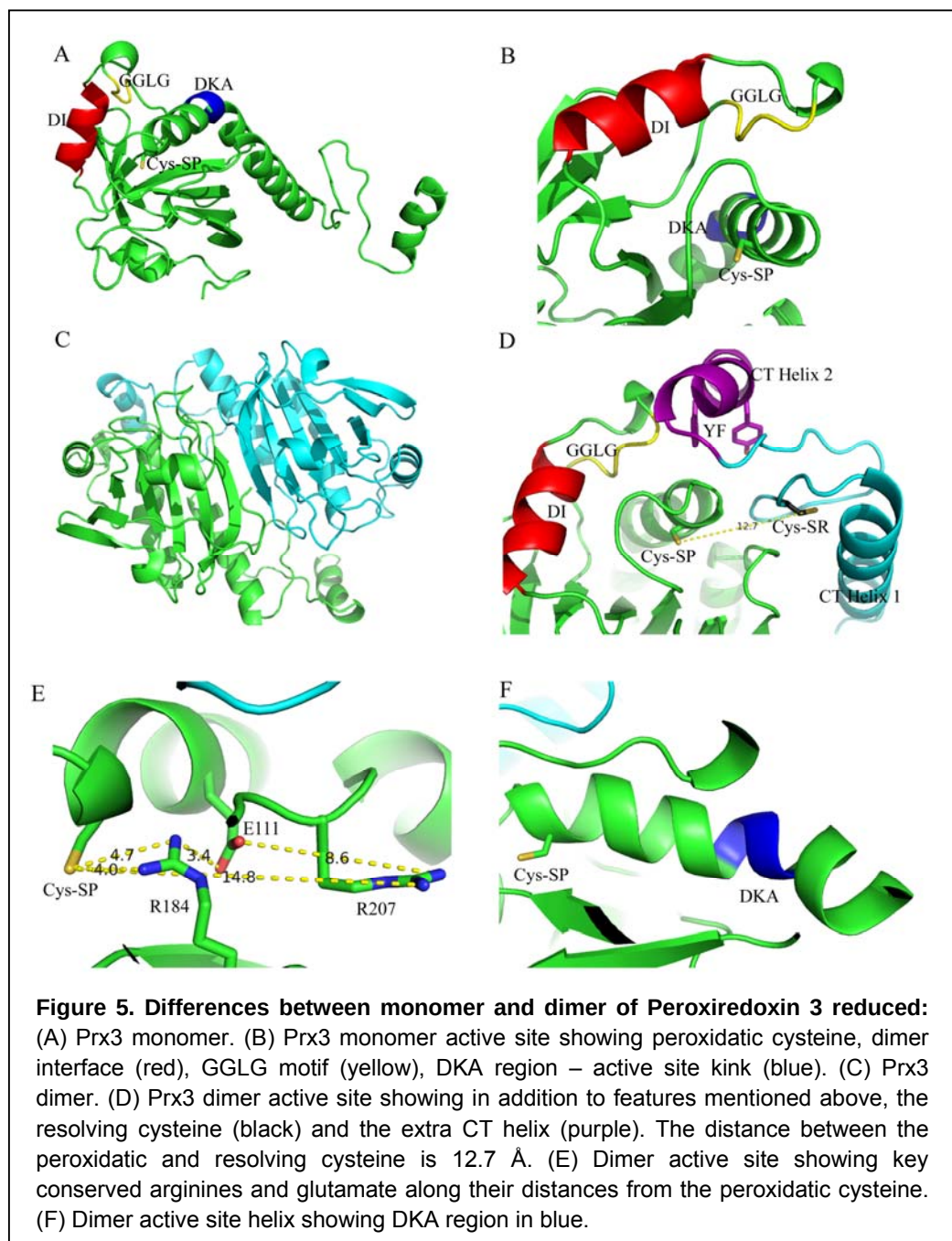


An interesting difference was observed when dimer segments from each decamer (Prx2 SH, SS and SO₂H) were structurally aligned to each other. The secondary structural elements of the SS dimer and the SH dimer display a perfect superimposition which is in sharp contrast to that observed in the decamer overlays (Fig. 4 C&D). A partial unfolding of the active site helix adjacent to Cys-SP along with movements of Cys-SR to form the disulfide is seen as previously described within the

literature (Fig. 4E) (60). The GGLG and dimer interface regions perfectly superimpose upon each other (Fig. 4F). This interesting anomaly observed between the alignment of decamer-derived dimer segments and decamers demonstrate the major impact the decamer arrangement has on its individual dimer segments. Independent of the restraints imposed by the decamer, the dimer segments can rotate and translate into perfect alignment. Within the decamer environment, the disulfide bonded LU dimers have a different orientation to that of the reduced and hyperoxidized FF dimers. This lends support to the hypothesis, presented within the literature, that the disulfide bond introduces changes within the decamer that could lead to its eventual destabilization (64).

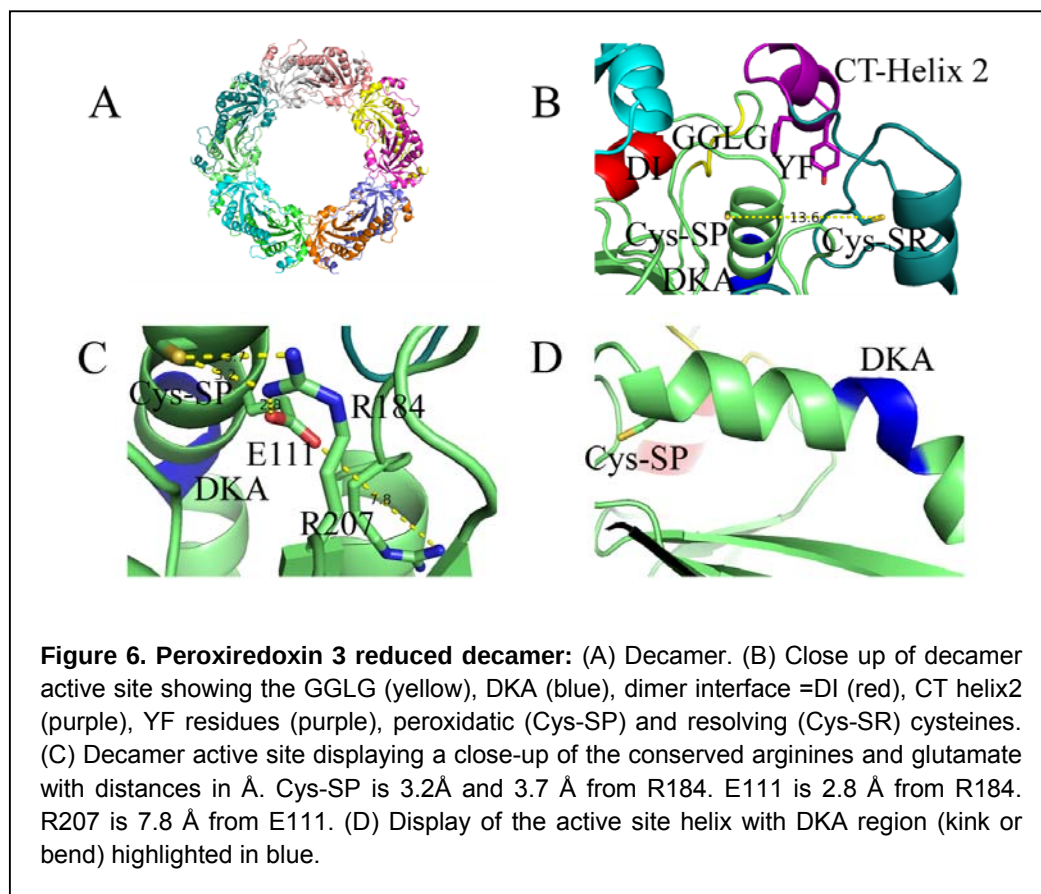
Peroxiredoxin 3 reduced monomer, dimer and decamer homology models:

The homology model of Prx3 SH monomer appears to match descriptions of other 2-Cys Prxs within the literature (Fig. 5A&B) (60). The active site helix ($\alpha 2$) kink or bend, DKA (D117, K118, A119) region, in blue is a part of a helical turn and not a loop. Likewise, the Cys-SP is part of the first helical turn and not on a loop. Both of these changes contrast to the Prx 2 monomer (Fig. 2A&B). The homology model of the Prx3 SH dimer is also quite different, and the active site is completed with the YF motif on CT Helix-2, which is fully helical with no loop regions which contrasts with that observed for Prx2 (Fig. 5C&D). Also, the Cys-SP and Cys-SR residues are 12.7 Å apart, well within the range reported in the literature. Within the active site, Arg184 is 4.0 Å away from Cys-SP and R207 is 14.8 Å (Fig. 5E). Additionally, E111 is 3.4 Å apart from Cys-SP and 8.6 Å away from R207. This suggests that these key residues in the dimer form are not in the perfect position for catalysis. A closer view of the active site helix reveals that the DKA region is within the fourth helical turn and Cys-SP is part of the first helical turn, further emphasizing the lack of unfolding around the active site helix (Fig. 5F).



In the decamer model, the active site region is quite similar to the dimer, especially the GGLG motif, DI and active site helix (Fig. 6A, 6B&5D). An overlay of the dimer (light pink) and the decamer (green), confirms that there are differences (orange) in the C-terminus, both helix 1 and 2, as well as the loop containing the Cys-SR (Fig. 9A). These differences cause a 4.0 Å shift and a change in the orientation of the resolving cysteine residue (Fig. 9B). A closer look at the conserved arginines

within the active site reveals that Arg184 is 3.2 Å and 3.7 Å apart from Cys-SP (Fig. 6C). Arg184 is 2.8 Å away from the conserved E111 which is located 7.8 Å from R207. The active site helix is overall structurally similar to that of the dimer, in that it is well folded in the KDA region and Cys-SP (Fig. 6D).

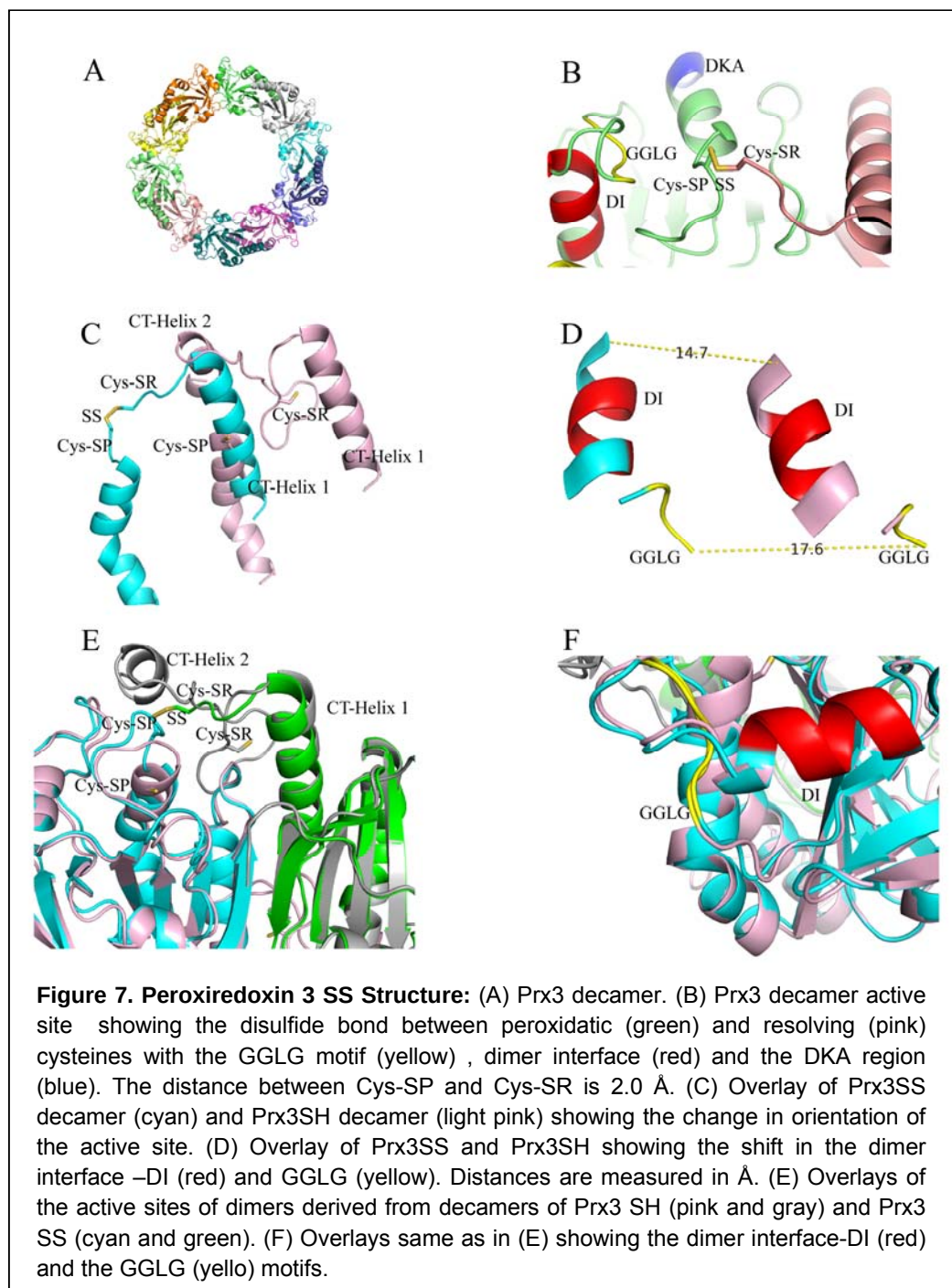


Analysis of these three states from the monomer to decamer reveal that Prx3 may have evolved to have better folded helices irrespective of its oligomeric state and favors a restricted range of movement within its active site. The active site of the Prx3 decamer is better positioned for catalysis than the dimer due to the closer positioning of Arg184 and Glu111. The conserved Arg207 is rotated away from the active site of both the decamer and dimer when compared to Arg150 in Prx2, revealing potential reasons for the observed catalytic differences between Prx2 and Prx3, seen in the literature and in Chapter 2 (61). Mutagenesis of these arginines and subsequent catalytic studies revealed that these arginines are important for the

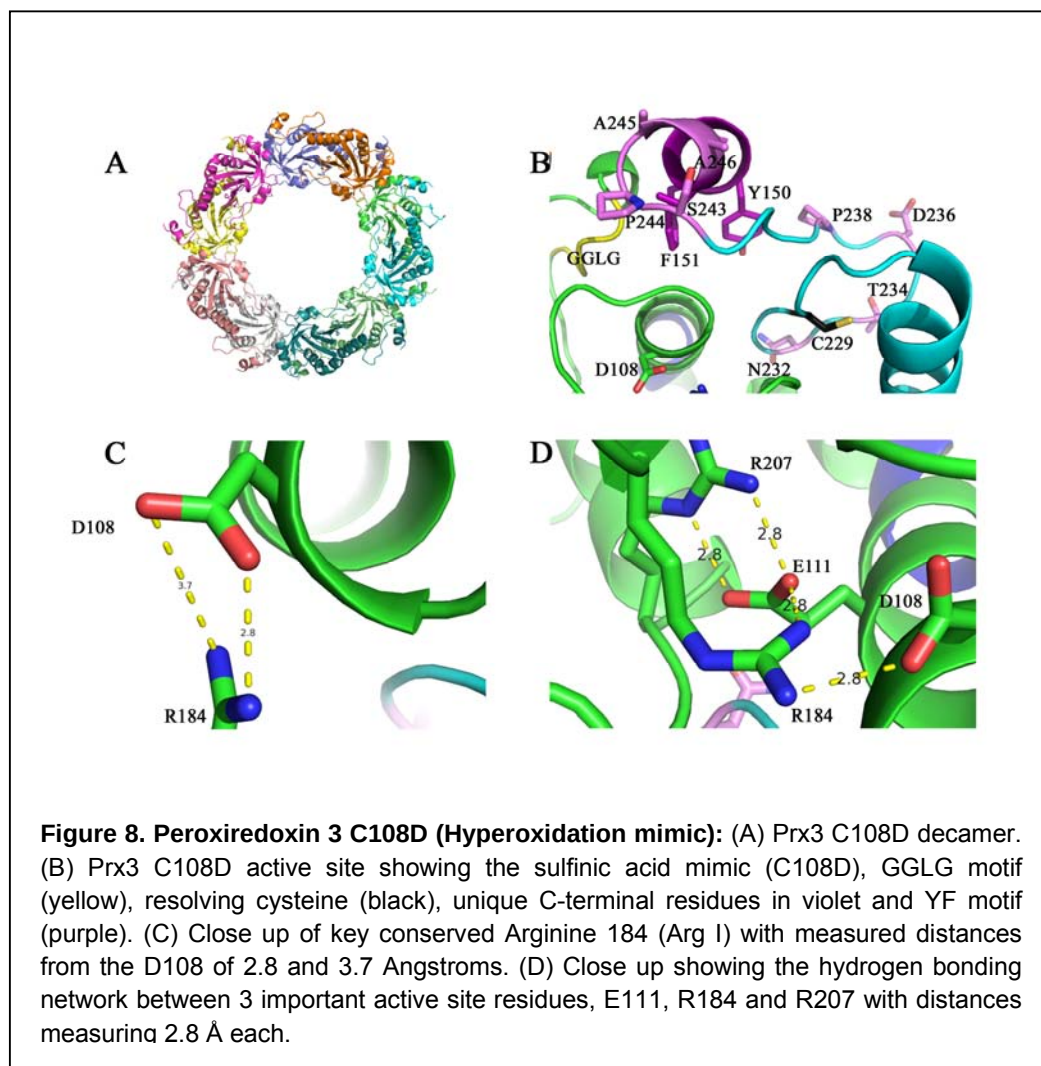
reaction with H_2O_2 (61). Additionally, the Arg207, either in dimer or decamer, is further away from the peroxidatic cysteine than is observed for bovine Prx3 (29,61).

Peroxiredoxin 3 decamer disulfide (SS) and Prx3 C108D (hyperoxidation mimic) homology models: Prx3 SS was modeled as a decamer (Fig. 7A). As expected the GGLG and DI shifted with respect to the SH form to allow the active site helix around the Cys-SP to unwind and form the disulfide with Cys-SR (Fig. 7B). There was a major shift that occurred within the active site between the fully folded reduced state and locally unfolded oxidized state (Fig. 7C). This confirms that like other Prxs, Prx3 also requires major rearrangements to transitions from the fully folded to the locally unfolded state. The shift between the dimer interface (red) of the fully-folded conformation and the locally unfolded conformation is 14.7 Å (Fig. 7D). The GGLG motif (yellow) had a shift of 17.6 Å between the fully folded and locally unfolded states. The shift that occurred in the dimer interface is much smaller than that of Prx2 and likewise for the GGLG motif shift observed (Fig. 4D). This difference between Prx2 and Prx3 shows that there is a limited range of movement for Prx3 in the active site. This allows Prx3 to undergo less conformational transition states on its way to forming a disulfide. As a result, Prx3 can form the disulfide much faster than Prx2, providing an important evasive mechanism to hyperoxidation. This notion is supported by the data presented in Chapter 2.

It is interesting to note that a similar anomaly to that observed in Prx2 was seen in the alignment of Prx3 SS to Prx3 SH decamers and decamer-derived dimer segments. Independent of the decamer, there was perfect alignment of these dimers (Fig. 7E&F). However, placed within context of the decamer, there was a clear difference in orientation between the decamer-bound disulfide bonded dimers and those of the fully folded reduced and hyperoxidized states. Further investigation is needed to elucidate the structural or procedural reasons behind these observed differences in the alignment of dimers and decamers.



Prx3 C108D, the hyperoxidation mimic, was modeled as a decamer (Fig. 8A). The strategy of using an aspartate residue as a mimic for sulfinic acid was previously employed to solve the Prx1-Srx complex crystal structure (25,33). A closer look at the active site helix reveals a very similar structure to the fully folded reduced active site (Fig. 8B).



The residues highlighted in violet near the Cys-SR (black) are the unique C-terminal residues (Chapter 1, Fig. 2) that had the major impact on hyperoxidation, imparting a limited range of motion to this region of Prx3 contrasted with Prx2. The substitution in this region of Prx3 for two glycine residues in favor of an asparagine and an aspartate indicates a change to reduce the overall flexibility in this region. Lower flexibility means less conformational transition states possible. This allows a shorter structural and kinetic route to disulfide bond formation in Prx3 when compared to Prx2. Arg184 of Prx3 is 3.7 Å and 2.8 Å away from Cys-SP; this indicates a move to more closely associate with Cys-SP similar to that observed for Prx2 SO₂H (Fig. 8C&4B). The difference observed with the 3.7 Å distance is due to the fact that

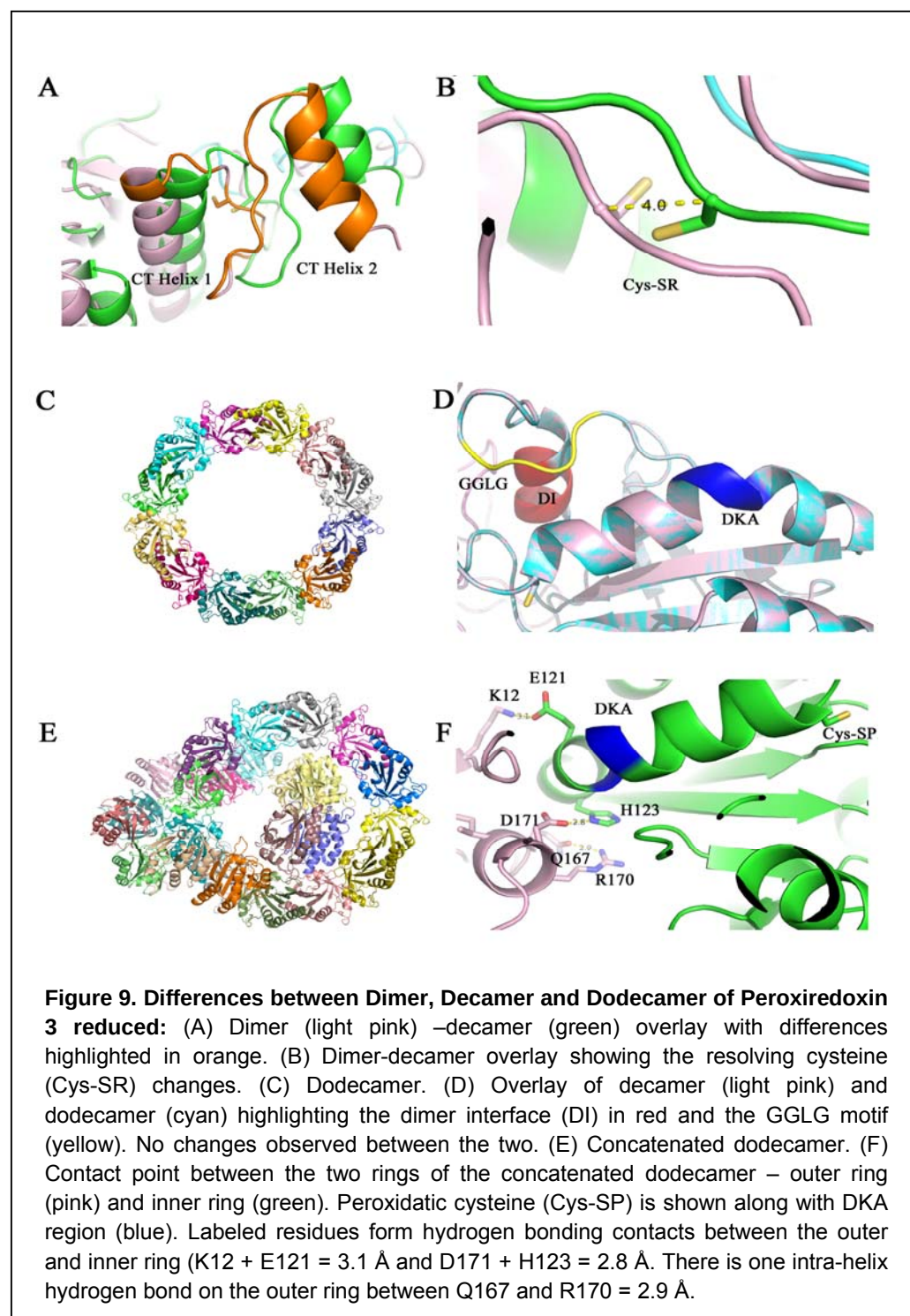
aspartate, though an excellent mimic for the sulfinic acid, has a planar side chain unlike the sulfinic acid. Thus, only one of its side chain oxygens is properly positioned for the optimal hydrogen bonding distance. Interestingly, Arg207 is now oriented towards the active site and 2.8 Å away from E111; these distances indicate a change in its orientation when compared to the reduced structure (Fig. 8D&6C).

Comparison of Peroxiredoxin 3 reduced dimer, decamer, dodecamer and concatenated dodecamer homology models: There were differences with the C-terminus observed for the dimer and decamer, with the Cys-SR residues having a different orientation and a shift of 4.0 Å (Fig. 9A&B). The C-terminus affects the rate of disulfide bond formation. It should be noted that Prx2 reduced dimer and decamer models displayed differences throughout the active site region across both monomers in the obligate homodimer, indicating a propensity for a greater range of movement within these regions of its structure (Fig. 2G). Additionally for Prx2, the differences were major within the N-terminal active site with a shift of 6.3 Å between the Cys-SP residues and only a 2.6 Å shift between the Cys-SR residues.

A far more fascinating comparison was done between the decamer and dodecamer using portions of each truncated to 5 monomers (Chains A-E). This comparison revealed a quite shocking result that there are no major differences between the two oligomeric states and that the dimer interface (DI) is remarkably similar (Fig. 9C&D). The rationale behind this truncation was based on the geometry of circles. If the oligomers are imagined to be circles with differing circumferences, the align algorithm aligns them at a single arbitrary point by tilting one circle into the other at an angle of x degrees. This angle introduced by the algorithm will distort any true angular difference between the two oligomers. To avoid this effect, the decamer and dodecamer were subdivided into equal segments so a single part of the circle of fixed distance can be aligned independent of the need to introduce this tilt.

Analysis of points of the contact between the dodecamers in the two-ring catenane revealed three interesting hydrogen bonding contacts between the active

site helix ($\alpha 2$) of the inner dodecamer and outer dodecamer helix (Fig. 9E&F).



The inter-ring contacts are between: (i) $\alpha 2$ E121 and inner ring K12 (3.1 Å); (ii) $\alpha 2$ H123 and inner ring D171 (2.8 Å) and (iii) inner ring inter-helix contacts Q167 and R170 (2.9 Å). These contacts, of which there are three, stabilize the catenane. A

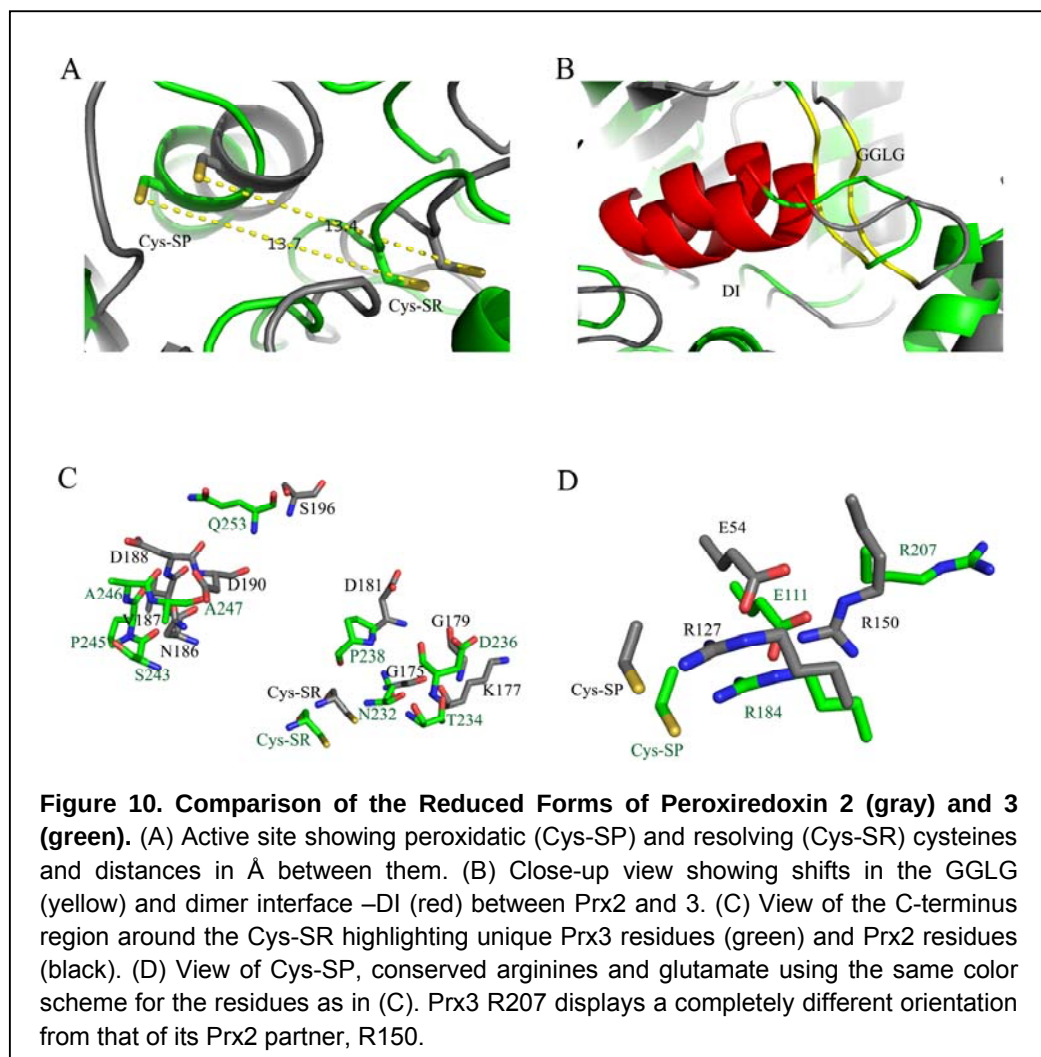
further fascinating observation is that these contacts are below the DKA region away from the Cys-SP residue, and thus do not block the peroxidatic cysteine from reacting with H_2O_2 . It also will not affect the disulfide formation as only the region of the helix immediately adjacent to the Cys-SP unwinds to form the disulfide.

Analysis of structural differences that govern susceptibility to hyperoxidation:

When 2-Cys Prxs are exposed to high levels of peroxide during oxidative stress, the peroxidatic cysteine is hyperoxidized to cysteine sulfinic acid. 2-Cys Prxs have different subcellular localizations: Prx1 and Prx2 (cytosol), Prx3 (mitochondria) and Prx4 (endoplasmic reticulum). It can be inferred that these Prxs have unique properties based on their location within the cell and the differences in the redox environment. Mass spectrometry data (Chapter 2) and previous studies have further supported this hypothesis revealing differences in hyperoxidation between Prx2 and Prx3. It was observed that Prx3 is more resistant to hyperoxidation than Prx2. This is not a surprising result as mitochondria are exposed to the most reactive oxygen species from the electron transport chain. This essential mitochondrial antioxidant would be non-functional as a peroxidase in the harsh mitochondrial environment containing a high concentration of endogenous hydrogen peroxide. Intriguingly, this leads to the possibility that if Prx3 is hyperoxidized at a toxic level of hydrogen peroxide, this could be an initiating signal for apoptosis. Increased mitochondrial ROS production has been implicated in the initiation of apoptosis. Therefore, resistance of Prx3 to hyperoxidation is critical for cell survival and must be efficiently repaired by Srx to protect cells from death.

Structural differences must exist to enable this variation in susceptibility to hyperoxidation. A comparison of the reduced forms of Prx2 (gray) and 3 (green) reveals subtle global structural differences but a strikingly similar distance between the Cys-SR of each Prx (Fig. 10A). There are also subtle differences observed between the dimer interface (red) and GGLG (yellow) motifs (Fig. 10B). With the exception of Prx3, the C-terminal region is highly conserved across the 2-Cys Prx

family (Chapter 1, Fig. 2); they all have the C-terminal motif: **GWKPGSD**. The C-terminal region of Prx3 contains the unique motif: **NWTPDSP**.



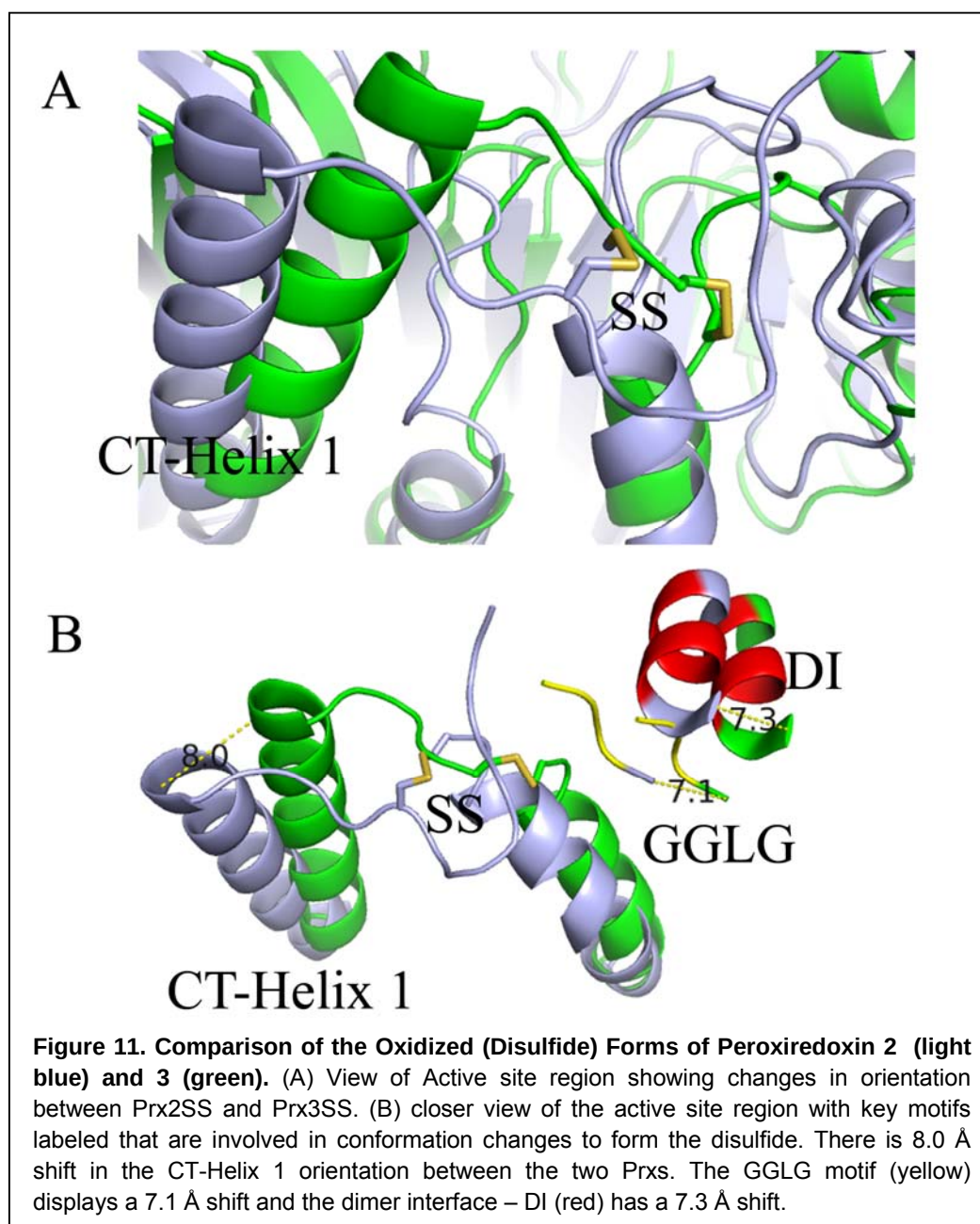
A closer inspection of this region also reveals four additional unique Prx3 residues (**SPAA** in green) when compared to Prx2 (**NVDD** in black) (Fig. 10C). These latter four residues belong to CT-Helix 2 and the former four are part of the Srx backside interface. These residues are arranged strategically around the resolving cysteine (Cys-SR), therefore influencing its range of motion and affecting the number of conformational states this region adopts prior to forming the disulfide bond. Within the active site region around Cys-SP, the conserved Prx2 residues (R127 and E54) and the conserved Prx3 residues (R184 and E111) are similarly oriented around Cys-

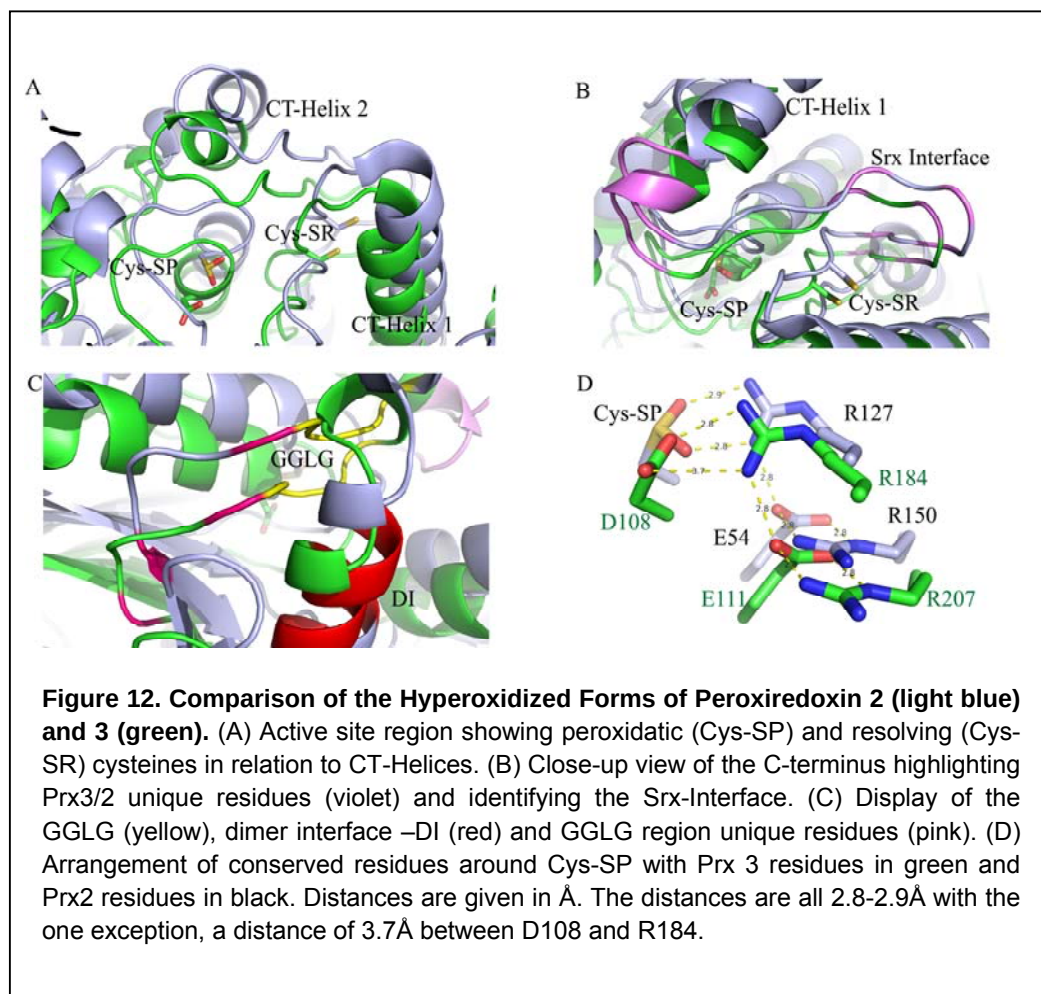
SP (Fig. 10D). The major difference observed is between the conserved Prx2 R150 and Prx3 R207, where the latter is oriented away from the conserved glutamate. Within Prx2 these three conserved residues form an essential hydrogen bonding network that restricts the movement of Cys-SP, thus preventing a quick transition to the locally unfolded conformation leaving it properly oriented for a second H_2O_2 molecule to attack and hyperoxidize it. Prx3 appears to have this hydrogen bonding network only partially in place, with the orientation of Arg207 away from the glutamate. We hypothesize that the loss of this Arg-Glu interaction introduces less restriction on the movement of Cys-SP, allowing it to transition faster to the locally unfolded state that prevents reaction with a second H_2O_2 molecule. Moreover, the rapid transition to the unfolded state could allow facile disulfide formation with the Cys-SR residue. As a result, the Cys-SP residue of Prx3 is protected from hyperoxidation.

A comparison of the disulfide forms of Prx2 (light blue) and Prx3 (green) also reveal global structural differences (Fig. 11A). A closer inspection reveal a shift in the C-terminal regions with the CT-Helix 1 of Prx2, situated 8 Å away from that of Prx3 (Fig. 11B). The GGLG (yellow) and the dimer interface-DI (red) motifs are located 7.1 Å and 7.3 Å respectively, between Prx2 and Prx3. These observed differences in the locally unfolded state between Prx2 and Prx3 result from the different conformational states through which the disulfide is formed. This supports the idea that Prx3 evades hyperoxidation through forming the disulfide differently from Prx2.

A structural alignment of the hyperoxidized fully folded states of Prx2 (light blue) and Prx3 (green) show quite subtle global changes between the two (Fig. 12A). When the CT region around Cys-SR is carefully inspected with the unique residues between Prx2 and 3 highlighted in pink, differences are observed in the Srx interface region and in the CT-Helix 2 (Fig. 12B). An important observation is that at the beginning of Prx3 CT-Helix 2 where A246 and A247 (SPAA residue group) are part of a canonical helical turn. In contrast, Prx2 residues in the same region, D188 and

D190 (NVDD residue group), are partially unfolded. This lends credence to the hypothesis that a greater range of motion is built into the Prx2 C-terminus in this region to allow more conformational states to be sampled prior to forming the disulfide, thus enabling the sulfenic acid to persist longer and in the correct orientation (i.e. the fully folded state) to be hyperoxidized.





Further studies are needed to acquire a more detailed understanding of the hyperoxidation and the structural features that are necessary to confer susceptibility to hyperoxidation. Additionally a crystal structure is needed of the Srx-Prx3-SO₂⁻ complex, since Prx3 possesses the unique property of hyperoxidation resistance and may have a novel interaction with Srx, due to the differences in its C-terminal region.

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CHAPTER FOUR

REDUCTION OF CYSTEINE SULFINIC ACID IN EUKARYOTIC, TYPICAL 2-CYS PEROXIREDOXINS BY SULFIREDOXIN

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Running title: Cysteine sulfinic acid reduction by Srx

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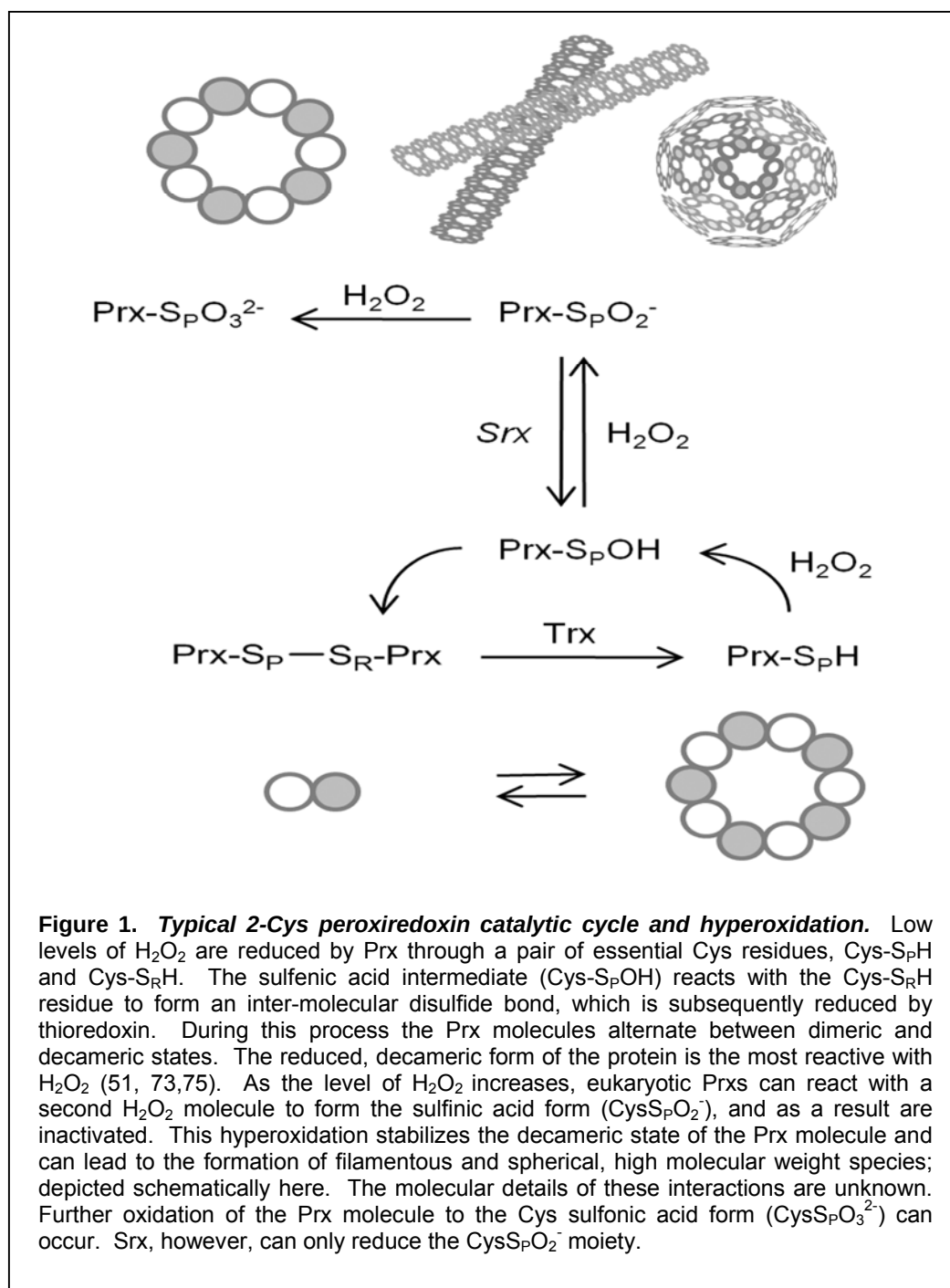
ABSTRACT

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 using an unusual ATP-dependent mechanism. In this process the Prx molecule undergoes dramatic structural rearrangements to enable repair. Structural, kinetic, mutational, and mass spectrometry-based approaches have been used to dissect the molecular basis for Srx catalysis. The available data supports the direct formation of Cys sulfinic acid phosphoryl ester and protein-based thiosulfinate intermediates. This review will discuss 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 oligomeric state will also be discussed.

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 biological contexts and disease states. Given their high abundance within cells and reactivity with H_2O_2 (10^5 - $10^7 \text{ M}^{-1}\text{s}^{-1}$), 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 intra-molecular disulfide bonds are formed with the adjacent monomer of the dimer during the normal catalytic cycle (21). The “peroxidatic” Cys (Cys- $\text{S}_\text{P}\text{H}$) of the typical 2-Cys or Prx1 subclass attacks a H_2O_2 molecule (Fig. 1) to form a Cys sulfenic acid (Cys- $\text{S}_\text{P}\text{OH}$) intermediate. An inter-molecular disulfide bond is then formed with the “resolving” Cys (Cys- $\text{S}_\text{R}\text{H}$), 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.* 5 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- $\text{S}_\text{R}\text{H}$ residue to approach the Cys-SOH moiety, and therefore decreases the rate of disulfide bond formation. As a result, the Cys- $\text{S}_\text{P}\text{OH}$ can react with a second H_2O_2 molecule and become hyperoxidized to the Cys sulfinic acid (Cys- $\text{S}_\text{P}\text{O}_2^-$) (70,77). Under conditions of extreme oxidative stress, this latter species can be further oxidized to the Cys sulfonic acid (Cys- $\text{S}_\text{P}\text{O}_3^{2-}$).



The hyperoxidation of 2-Cys Prxs can lead to the formation of spherical aggregates (Fig. 1) of very high molecular weight (>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 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_PO₂⁻ moiety can be reduced and the peroxidase activity restored by an enzyme known as sulfiredoxin (Srx) (6,69,71). Another enzyme called sestrin was 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 flood-gate hypothesis whereby H₂O₂ levels can rise 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.

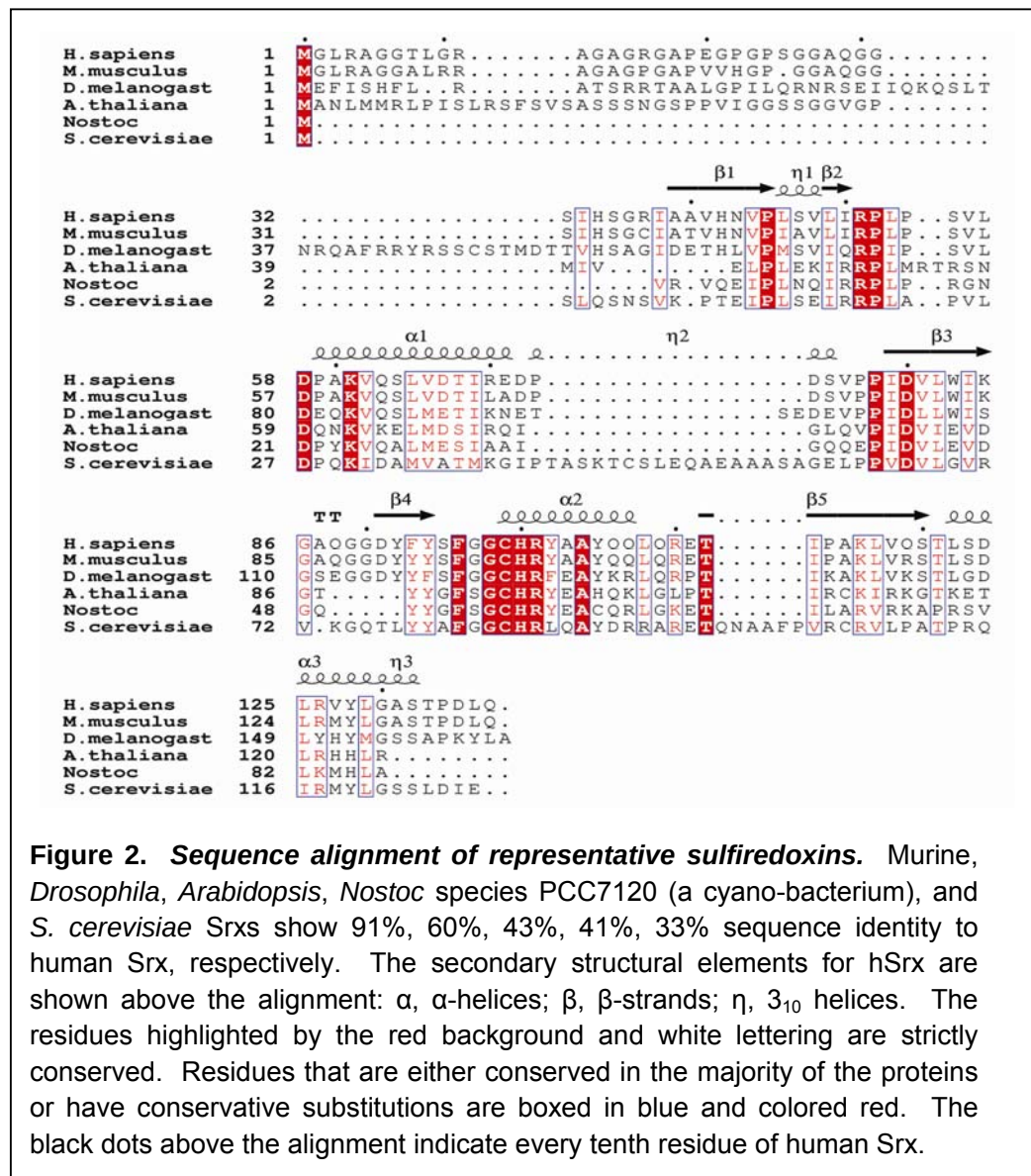
Humans have four typical 2-Cys Prx isoforms with different cellular compartmentalization and susceptibilities to hyperoxidation and inactivation (13,21,42,54,75). This inactivation can have serious systemic consequences, as evidenced by the increased oxidative stress found in the knockout mice of PrxI, PrxII, and PrxIII and their development of anemia, splenomegaly, hypersensitivity to lipopolysaccharide challenge, and arterial thickening (12,20,40,46). Moreover, the hyperoxidation of Prxs is a biomarker for oxidative stress associated with adriamycin treatment leading to “chemobrain”, Alzheimer’s disease, Parkinson’s 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, an AP-1 and Nrf2 target gene, in skin

cancer, immuno-stimulated macrophages, synaptic NMDA receptor activity, cigarette-induced emphysema, and cardiac dysfunction (15,60-62,64,67).

This review will focus 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 oligomeric state. The reader is directed to the following manuscripts for insight into the role 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 H₂O₂ 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 upon the addition of ATP-Mg²⁺, 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 μ M (11,27,32,71). GTP, dATP, and dGTP also support the reaction, but the relevance of these nucleotide forms have not been investigated (11). The K_M values for human Trx1 and glutathione (GSH) (1.2 μ M and 1.8 mM, respectively) suggest that either could be the physiological reductant for the Srx reaction (11). As described in more detail below, 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 *A. thaliana* Srx range from 0.1–1.8 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, H₂O₂-mediated signaling events can be potentiated.

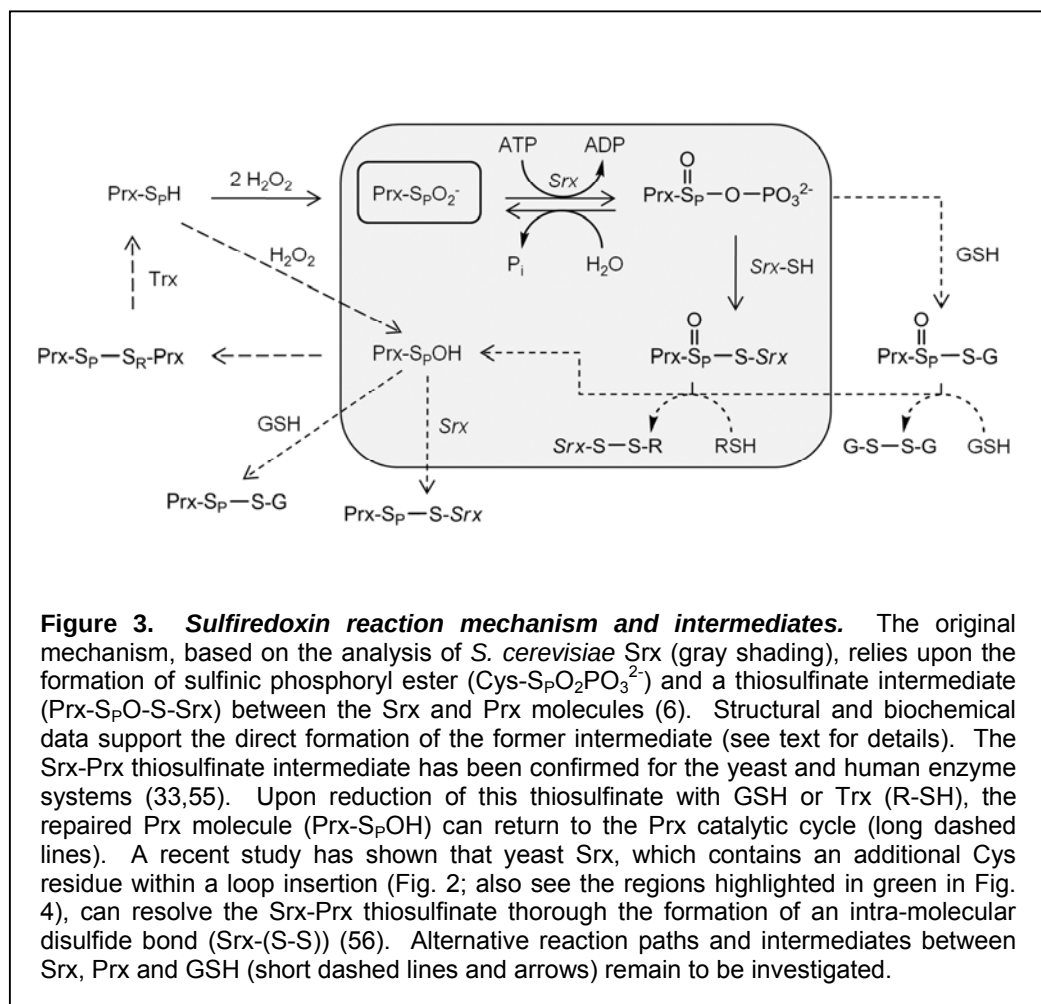


Srx is highly conserved between species (Fig. 2) and found only in eukaryotic organisms, with *C. elegans* as a notable exception currently without explanation (30). Bacteria apparently do not need Srx as their Prxs are not readily hyperoxidized (74). Human Srx exhibits a ubiquitous tissue distribution, although the expression level varies greatly (11). Srx is localized predominantly in the cytosol and can repair 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 utilizes an intramolecular disulfide bond during catalysis, and the 1-Cys PrxVI. Srx also cannot repair glyceraldehyde-3-phosphate dehydrogenase. As described below, 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

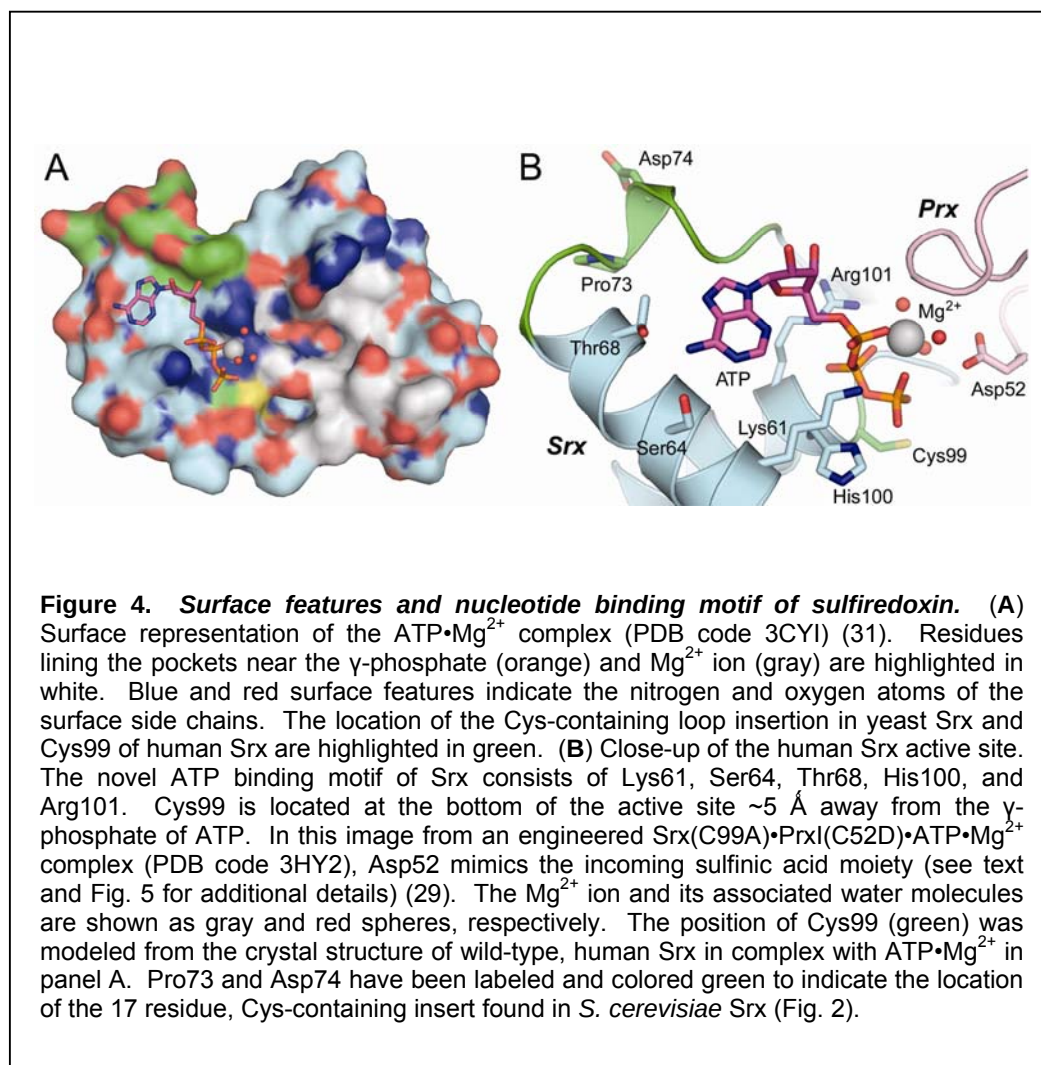
In the first step of the original mechanism proposed by the Toledano laboratory (Fig. 3, gray shaded region), the Cys-S_PO₂⁻ moiety (Cys52 in human PrxI, hPrxI) is phosphorylated by the γ-phosphate of ATP to form the sulfinic phosphoryl ester (Cys-S_PO₂PO₃²⁻) (6). This type of ATP-mediated activation is reminiscent of the activation of carboxyl groups in a variety of biological processes, but is novel for sulfur chemistry (7,16,17). A thiosulfinate intermediate (Prx-S_PO-S-Srx) is then formed, following the attack of a conserved Cys residue in Srx (Cys99 in hSrx). GSH or Trx could then facilitate the collapse of the thiosulfinate to release the repaired Prx molecule in the Cys-S_POH state, which can return to the Prx catalytic cycle. Subsequent studies by several laboratories have utilized 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 regards 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 if these proteins bind ATP or have reductase activity. The ATP•Mg²⁺ and ADP complexes of Srx (Fig. 4) reveal a unique nucleotide binding motif that is generated by the following residues: Lys61, Ser64, Thr68, His100 and

Arg101. Cys99 interacts with Arg51 (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 His100 and Arg101 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 Cys99 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 sections below, phosphorylation of the C99S Srx variant is possible to a minor extent (27). This finding resulted in an alternative proposal where 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 $\text{Srx} \cdot \text{ATP} \cdot \text{Mg}^{2+} \cdot \text{PrxI}$ complex, however, support a direct in-line attack by the Prx Cys- $\text{S}_\text{P}\text{O}_2^-$ moiety (27,29,31).

THE SRX-PRX EMBRACE: ACTIVE SITE AND BACKSIDE INTERFACES

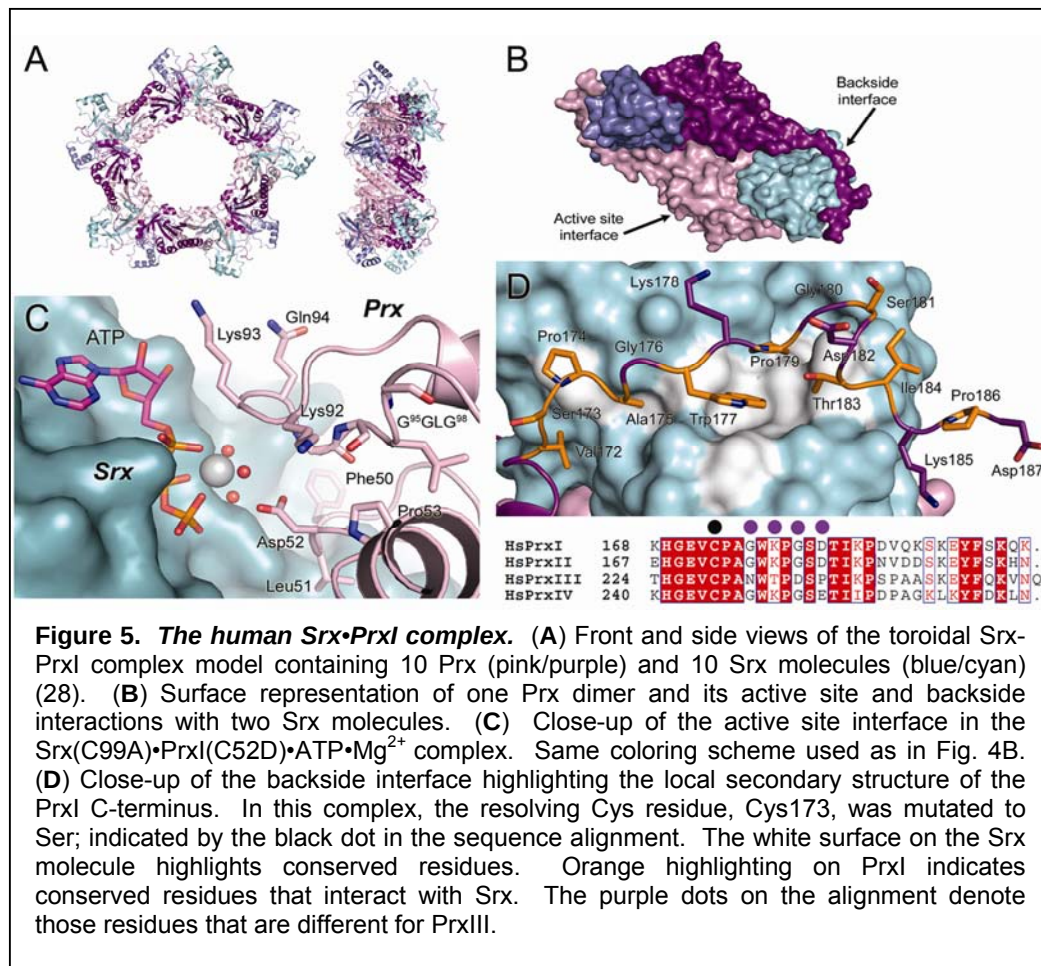
One of the conundrums of Srx-mediated repair is exemplified by the crystal structure of hPrxII in the hyperoxidized state (30,58). In this structure, the Cys- SO_2^- moiety is not accessible to Srx because of its stable interaction with a conserved Arg residue and the presence of the overlaying GGLG and YF motifs. Therefore, the helix containing Cys- SO_2^- must partially unfold, an attribute already known to occur during normal catalysis, to enable 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 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 computationally predict 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). In order to form the disulfide between Cys99 of Srx and Cys52 of PrxI, the remaining Cys residues of PrxI were mutated to Ser in order 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. In order to increase the diffraction quality of the crystals, a mutation was also made at the dimer-dimer interface. The mutation of Cys83 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-SO₂⁻ 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_PH residue did unfold to establish the disulfide bond with Cys99 of Srx (28). This change placed Phe50 of PrxI within the primarily hydrophobic surface pocket (Fig. 4B) generated by Leu53, Asp80, Leu82, Phe96, Val118, Val127, and Tyr128 of Srx. Analysis of the toroid model (Fig. 5A) also indicates that Phe26, Phe82 and Leu85 of PrxI may also contribute to this pocket. In order 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 below, between residue 43 of Srx and residue 185 of PrxI (29).



In an effort to approximate the Cys-SO₂⁻ moiety, Cys52 was mutated to Asp, *i.e.* substitution of the sulfur atom for a carbon atom (R-SO₂⁻ vs. R-CO₂⁻). These modifications enabled crystals to be soaked with ATP and Mg^{2+} . The resulting complex (Fig. 5C, PDB code 3CYI) recapitulated the docking of Phe50 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, Gln92, Arg93 and Arg94, may play a role in the Srx-Prx interaction.

Upon 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 c-Abl, c-Myc, MIF, phospholipase D1, and the PDGF receptor also raise the possibility that the binding of the 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 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.* mutated the catalytic Cys99 of hSrx to Ser and Ala, known to inactivate the protein and to still allow for ATP binding (27). Analysis of the reactions including [γ -³²P]-ATP by SDS-PAGE and autoradiography revealed that less than 1% of Ser99 had been phosphorylated when incubated for four hours with wild-type, hyperoxidized hPrxI, but not reduced hPrxI. This data was taken as evidence for the phosphorylation of Cys99 of Srx prior to the

phosphorylation of the sulfinic 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 His100. A different study using ^{18}O -labeled PrxI-S $^{18}\text{O}_2^-$ also showed that the phosphorylation of the Prx molecule is readily reversible ($k = 0.35 \text{ min}^{-1}$) (33). Further support for this notion comes from studies where 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 was also dependent upon 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 Asp52 relative to the γ -phosphate of ATP within the $\text{ATP}\cdot\text{Mg}^{2+}$ 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.* Cys172 in hPrxII; Cys171 in *S. cerevisiae* Tsa1) and other Cys residues not required for catalysis (*i.e.* Cys70 in hPrxII; Cys48 and Cys106 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 above in place, both studies readily observed the formation of a thiosulfinate intermediate between the Srx and Prx molecules (Prx-S_PO-S-Srx; $k = 1.2\text{--}1.4 \text{ min}^{-1}$). This rate is similar to the overall rate of the reaction $0.1\text{--}1.8 \text{ min}^{-1}$ (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 ($k = 0.14 \text{ min}^{-1}$ for hSrx). This 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). Since the Cys-S_RH residue has been mutated and an inter-molecular 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. In fact, the former does occur with wild-type, yeast Srx, but in this case Cys48, uniquely present within a surface loop (Fig. 4), attacks the Prx-S_PO-S-Srx species to form an intra-molecular disulfide, *i.e.* Cys48-Cys84 (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 reduce 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 Cys99 of hSrx to the ATP molecule and the formation of a Prx sulfinic phosphoryl intermediate (Fig. 4B & 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, has found that the deglutathionylating activity of Srx is specific for typical 2-Cys Prxs, when compared to glutaredoxin 1 (Grx1) (49). Srx was able to remove GSH from Cys83 and Cys173 of hPrx1 *in vitro* to a greater extent than the peroxidatic Cys52, which was readily removed by Grx1. The reaction resulted in

the glutathionylation of Srx on Cys99. 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 following H₂O₂ exposure. Overexpression of Srx had the opposite effect. Based on the Srx-PrxI complex structure and the ability of the proteins to readily form a disulfide linkage (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 Pro174 and Pro179 of PrxI and Tyr92 of Srx at the backside interface decreased the deglutathionylating activity. Why the mutation of these residues would impact 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 impacts the sulfinic acid reductase activity of Srx will need to be conducted with caution.

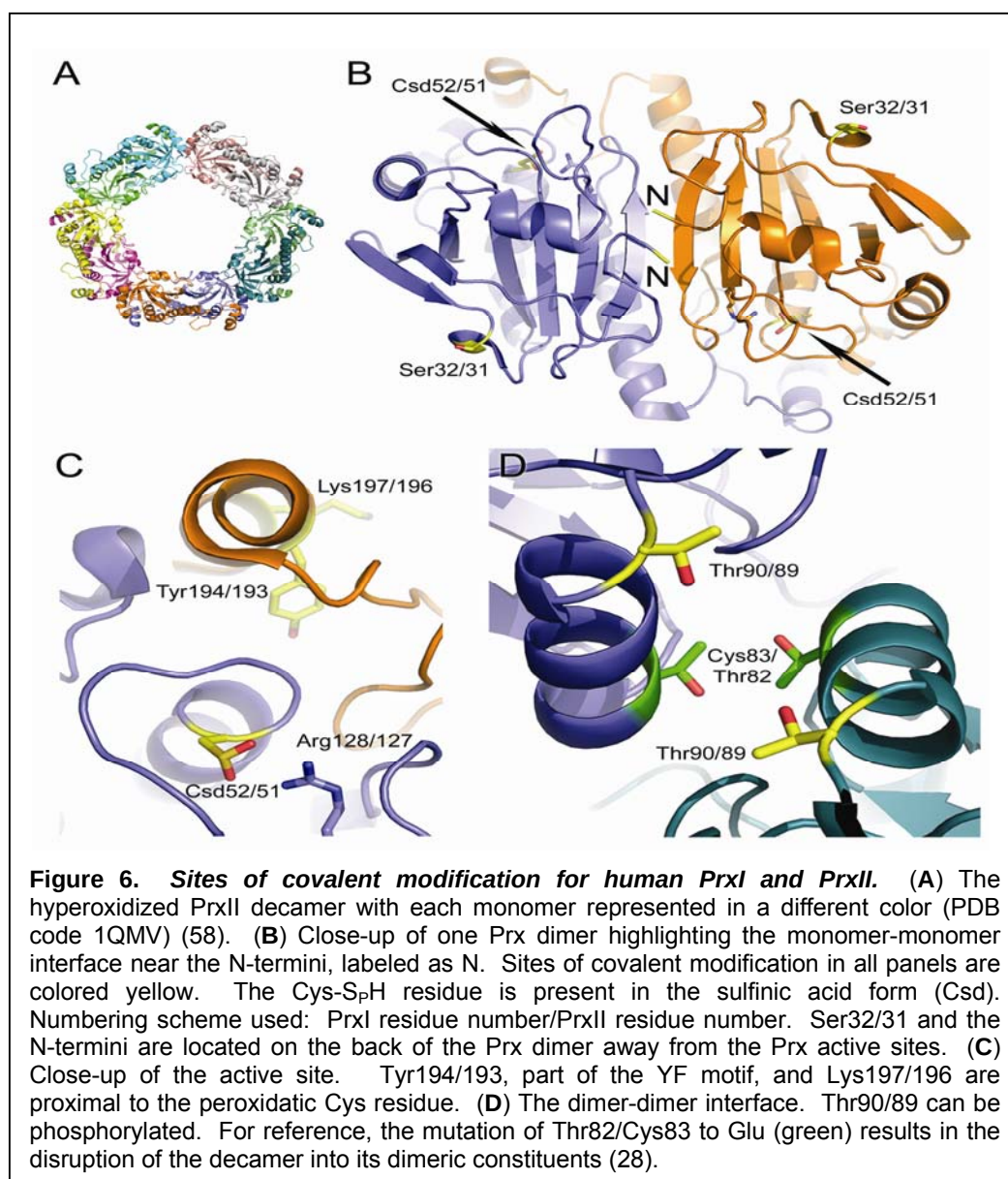
CONCLUSIONS AND ADDITIONAL OPEN QUESTIONS

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 upon novel interactions with the Prx molecule in order to juxtapose the sulfinic 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. In order 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. There appears to be a complex relationship 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 neuronal cell death in Parkinson's disease (18). Phosphorylation of PrxI/II leads to differential effects. Phosphorylation of Thr90/89 (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 Tyr194 (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 Lys196/197 acetylation, described above. Stimulation of peroxidase activity has also been observed when Ser32 (Fig. 6B) of PrxI is phosphorylated by TOPK (79).

From each of the brief examples above 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 Thr90 of PrxI should induce chaperone activity, as the Prx molecule must be able to initiate the catalytic cycle in order for hyperoxidation to occur. Moreover, any mutation or covalent modification that

stimulates peroxidase activity, e.g. Lys197/196 acetylation, could have been the result of an increased inter-molecular 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 opposite was observed. It also unclear how phosphorylation at Ser32, 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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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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CHAPTER FIVE

MAIN CONCLUSIONS AND FUTURE DIRECTIONS

Hyperoxidation: Hyperoxidation sensitivity is primarily mediated through the C-terminus of 'sensitive' typical 2-Cys peroxiredoxins. 'Robust' 2-Cys peroxiredoxins lack a key evolutionary adaptation that extends the C-terminus by 30-40 residues in 'sensitive' 2-Cys peroxiredoxins [1]. Residues unique to Prx3 within the C-terminus that also form the backside interface with the repair enzyme, Srx, confer resistance to hyperoxidation (Chapter 2) [2]. High resolution mass spectrometry studies (Chapter 2) not only confirmed the difference in sensitivity to hyperoxidation exhibited by Prx2, in contrast to Prx3, but also identified a new catalytic intermediate not previously observed in 2-Cys Prxs. This intermediate, a sulfenamide, was readily observed in Prx2 using time-resolved mass spectrometry. This intermediate is derived from the condensation of sulfenic acid with an adjacent nitrogen atom from a lysine, histidine or an arginine [3]. It can also be formed after the sulfenic acid condenses with the nitrogen of the backbone amide of an adjoining residue [4]. In the case of Prx2, the proposed residue is the conserved Arg127 stabilized by a conserved Glu54 and another conserved residue, Arg150. The sulfenamide was only observed in Prx3 after all the cysteines, with the exception of the peroxidatic cysteine, were mutated to serines. Prx3 also has an adjacent conserved residue Arg184 that is stabilized by Glu111. However, based on homology modeling (Chapter 3), the other conserved residue Arg207 is rotated away and does not interact with the conserved glutamate like in Prx2. This reduces the restriction on the movement of the peroxidatic cysteine in Prx3. Another interesting observation was the ready visualization of the sulfenic acid for Prx2, but not for Prx3 where it was only observed in the cysteine to serine mutant, previously described. This latter observation coupled with the formation of sulfenamide for Prx2 supports that the sulfenic acid intermediate is long-lived for Prx2. Thus, a more stable sulfenic acid intermediate increases the probability of hyperoxidation by a second H₂O₂ molecule.

It is fascinating that Prx3 appears to evade hyperoxidation by rapidly resolving its sulfenic acid intermediate to the inactive disulfide bonded state. The inactive disulfide state is preferable, as it can be quickly reduced by thioredoxin. In contrast, the hyperoxidized state requires a multi-step, retro-reduction process catalyzed by Srx in complex with magnesium ion and ATP. The faster the active, reduced state is recovered, the more efficient the enzyme would be at its peroxidase function. This is especially important for Prx3, as it is located within the mitochondria, one of the major sources of endogenous ROS. If excess H_2O_2 is not quickly removed from the mitochondria, it can be converted in the presence of iron to the more damaging hydroxyl radical by the Fenton-Harding reaction. At a certain critical level of ROS within the mitochondria, intrinsic cell death pathways are triggered which is deleterious to the tissues of the organ that these cells are a part of, such as the myocardium, leading to the development of pathologies such as cardiovascular disease.

On the other hand, an efficient peroxidase such as Prx3 can be upregulated to protect a cell that wants to evade apoptosis during oxidative stress conditions [5]. It can also be upregulated in a cell that proliferates rapidly using ROS-triggered growth cell signalling pathways without itself incurring ROS induced damage leading to its death [6]. Cancer treatment involving radiation and chemotherapy can kill cancer cells by producing deleterious amounts of ROS. However, cells that upregulate protective enzymes can generate a population of radiation and chemotherapy resistant cancer cells [7]. Therefore, it appears that cancer cells have evolved antioxidant-based mechanisms to evade cell death [8]. Prx3 remains an attractive enzymatic target for which an inhibitor could be designed and used in combination with radiation and/or chemotherapy to effectively induce death in cancer cells. Further studies are required to evaluate the efficacy of such an inhibitor for combination cancer treatment. However, due to the ubiquitous expression and highly

conserved active site of typical 2-Cys Prxs, like Prx3 and Prx2, there needs to be careful study of the potential cross reactivity and negative side effects of any inhibitor. If the compound cross reacts with Prx2, for example, extensive damage to other organs such as the liver, kidney and heart could occur. Currently there are two inhibitors for 2-Cys Prxs, thiostrepton and conoidin A (along with its derivatives) [9,10]. Additionally, siRNAs have been designed to knockdown Prx2 in cells [7]. Any inhibitor design process must incorporate detailed structural analysis of the Prx2 and Prx3.

Human Typical 2-Cys Peroxiredoxins 2 and 3 Structures: The strategy of using X-ray crystallography and homology modeling successfully generated structures for all three redox states of Prx2 and Prx3 (Chapter 3). Additionally, using homology modeling, different oligomeric states were generated for the reduced Prx2 to include monomer, dimer and decamer. For Prx3 reduced, monomer, dimer, decamer, dodecamer and two-ring catenane were modeled. These structures provided insight into the variability in susceptibility to hyperoxidation between Prx2 and Prx3. The hypothesis that Prx2 can engage in multiple conformations, thus slowing down disulfide bond formation and prolonging the sulfenic acid, was supported by the observation of less restrictions on movement within the N- and C-termini and the strong interactions around the peroxidatic cysteine maintaining the correct orientation for further reaction with H_2O_2 . Prx3 by contrast displayed an active site where the putative loss of a hydrogen-bonding interaction with Arg207 destabilizes the helix containing the peroxidatic Cys residue, hindering further reaction with H_2O_2 . In addition, the unique residues of the C-terminus, adjacent to the resolving Cys residue, of Prx3, appear to restrict the movement and ability to sample multiple conformations. These two features combine to enable a faster disulfide bond formation and thus a shorter lived sulfenic acid. These observations

were complementary to the results of the mass spectrometry experiments (Chapter 2).

The homology models generated here could be used in the future to formulate successful strategies involving mutagenesis to obtain constructs for crystallization experiments of these redox states. Additionally, the models could be used as molecular replacement search models to solve the phases for any datasets obtained from these crystals. Currently a mutagenesis strategy based on the Prx3 C108D model, has produced a better crystallization construct, Prx3 C108DC2S, with the peroxidatic cysteine mutated to aspartate and the remaining cysteines mutated to serines. This is currently being optimized for diffraction screening. The crystals have a much better morphology than those derived from previous Prx3 crystallization constructs. When a dataset is acquired, Prx3 C108D homology model will be used as a molecular replacement search model to solve the phases.

The Prx2 and Prx3 homology models could also be potentially useful in virtual ligand docking and screening experiments during inhibitor development for Prx2 or Prx3. Protein-protein docking experiments could also be conducted using these models to understand how putative binding partners identified by future proteomic experiments interact. This can prove useful in elucidating several cell signaling pathways. There is a definite need within the redox cell biology field for structural data involving typical 2-Cys Prx binding partners that potentially have an impact on redox signaling pathways. Recent advances in high throughput biology have provided vast interaction networks needed to understand several processes within cells [11,12]. However, missing from these networks is the valuable information that can be gained from understanding the molecular basis that promotes these protein-protein interactions [13]. This has set a dangerous precedent where there are more identified interactions than 3D structures available for these interactions [14]. This has led to the pioneering development of a fully automated resource to identify

structural models for protein to protein interactions called Interactome3D [15]. This resource could prove invaluable in structurally modeling Prx-binding partner interactions involved in redox signaling networks.

An example of such a binding partner is STAT3, which has been hypothesized to interact with members of the typical 2-Cys Prx subclass and mediate resistance to cancer treatment [16-18]. Another such partner is Sestrin2, which was initially controversially identified as a Prx sulfinic acid repair enzyme, and then later shown not to have an effect using mouse models [19]. While the status of Sestrin2 as a sulfinic acid reductase remains controversial, there is no controversy concerning the position that this protein has an important role in redox based cell signaling pathways [20]. Sestrin2 could potentially be a binding partner of typical 2-Cys Prxs under oxidative stress conditions connecting it to genotoxic stress, p53 and mammalian Target of Rapamycin (mTOR) signaling pathways [21]. There remains a need for the structure of Sestrin2 and/or Prx-Sestrin2 complex. Homology modeling can be used to investigate these binding partners and to design constructs for successful crystallization experiments.

Srx Repair of Hyperoxidized Typical 2-Cys Prxs: Srx repair of the typical 2-Cys Prx sulfinic acid reductase is believed to function as an essential component of cell signaling pathways by reactivating the peroxidase function and terminating H₂O₂ based cell signaling [22,23]. There is sequence variation between Prx3 and the other members of this subclass (Prx1, 2 and 4) at the C-terminal region that forms an interface with Srx. This has led to the hypothesis that there should be a difference in the interaction of Srx with Prx3, and thus a difference in the repair rates when compared to the other 2-Cys Prxs. Further studies are needed to acquire clearer insight into the effects of this sequence variation. A combination of mass spectrometry and X-ray crystallography should be applied to analyze these claims. There is a need for a structure of the Prx3-Srx complex in the redox field. Extensive

analysis of the status of Srx and experiments needed within the field are dealt with in the review article presented in Chapter 4.

Typical 2-Cys Prxs Chaperones: There still remains a need within the field for further study of the chaperone function of typical 2-Cys Prxs. Detailed identification of the residues involved in the formation the higher MW chaperones and which surfaces bind the protein substrate is needed. Both X-ray crystallography and mass spectrometry could be used in these studies. Recently, a technique has been developed using an Orbitrap mass spectrometer to analyze intact macromolecular assemblies [24]. This method could go a long way toward elucidating biochemical and biophysical properties of Prx chaperones in their native states. This technique is so sensitive it can identify individual ions. It was able to produce a mass spectrum accurate to within 23 Da for the homogenous 801 kDa GroEL chaperonin from bacteria which is an oligomer consisting of fourteen identical subunits. Applying this technique to monitor substrate turnover and/or Srx repair leading to dissociation of these higher MW chaperones would provide a plethora of data needed to understand the role these chaperones play within cells under oxidative stress conditions.

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CURRICULUM VITAE

Educational/Training

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1. Undergraduate Summer Intern, Chemical Biology, Dr. Chunyu Wang, Rensselaer Polytechnic Institute (05/07 – 08/07)
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3. Ph.D. candidate , Biochemistry & Molecular Biology, Dr. W. Todd Lowther, Wake Forest School of Medicine (08/08 – current)

Academic and Professional Honors

Virginia Union University Presidential Scholar (2004-2008)

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B. Peer-reviewed Publications

1. Musayev, F. N., Di Salvo, M. L., Saavedra, M. A., Contestabile, R., Ghatge, M. S., **Haynes, A.**, Schirch, V., and Safo, M. K. (2009) Molecular basis of reduced pyridoxine 5'-phosphate oxidase catalytic activity in neonatal epileptic encephalopathy disorder, *J Biol Chem* 284, 30949-30956.
2. Lowther, W.T., and **Haynes, A.C.** (2011) Reduction of cysteine sulfinic acid in eukaryotic, typical 2-Cys peroxiredoxins by sulfiredoxin, *Antioxid & Redox Signal* 15, 99-109.
3. **Haynes, A.**, Qian, J., Reisz, J.A., Furdui, C.M., and Lowther, W.T. (2013) Molecular Basis for the resistance of human mitochondrial 2-Cys peroxiredoxin 3 to hyperoxidation. *J Biol Chem*, in review (submitted March 31, 2013).

C. Poster Presentations at Conferences:

May 2010 (Italy) Gordon Research Conference: Thiol-Based Redox Regulation & Signaling – Poster Presented (Title: Kinetic and Structural Analysis of Human Peroxiredoxin III (PrxIII), a Key Mitochondrial antioxidant enzyme).

September 2011 (Spain) ESF-EMBO Conference: Gluthathione and Related Thiols in Living Cells – Poster Presented (Title: Kinetic and Structural Analysis of Human Peroxiredoxin III (PrxIII), a Key Mitochondrial antioxidant enzyme).

August 2012 (U.S.A) Gordon Research Conference: The Molecular Underpinnings of Redox Regulation and Oxidative Stress – Poster Presented (Title: Mass Spectrometric Analysis of 2-Cys Peroxiredoxin Catalytic Cycle Intermediates and Hyperoxidation Phenomenon Using Human Peroxiredoxin 2 and 3).

E. Courses Attended:

April 2012 (Brookhaven National Lab, Upton, NY) RapiData 2012: Collection and Structure Solving – A practical course in Macromolecular X-ray Diffraction Measurement. (This course was funded by the 2011 Cowgill Fellowship).