CHEMICAL REVIEWS

Review

Cite This: *Chem. Rev.* 2019, 119, 10829–10855

pubs.acs.org/CR

Catalysis of Peroxide Reduction by Fast Reacting Protein Thiols

Focus Review

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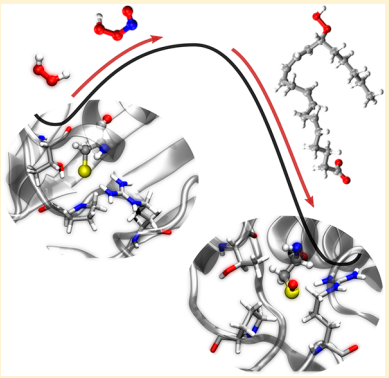
ABSTRACT: Life on Earth evolved in the presence of hydrogen peroxide, and other peroxides also emerged before and with the rise of aerobic metabolism. They were considered only as toxic byproducts for many years. Nowadays, peroxides are also regarded as metabolic products that play essential physiological cellular roles. Organisms have developed efficient mechanisms to metabolize peroxides, mostly based on two kinds of redox chemistry, catalases/peroxidases that depend on the heme prosthetic group to afford peroxide reduction and thiol-based peroxidases that support their redox activities on specialized fast reacting cysteine/selenocysteine (Cys/Sec) residues. Among the last group, glutathione peroxidases (GPxs) and peroxiredoxins (Prxs) are the most widespread and abundant families, and they are the *leitmotif* of this review. After presenting the properties and roles of different peroxides in biology, we discuss the chemical mechanisms of peroxide reduction by low molecular weight thiols, Prxs, GPxs, and other thiol-based peroxidases. Special attention is paid to the catalytic properties of Prxs and also to the importance and comparative outlook of the properties of Sec and its role in GPxs. To finish, we describe and discuss the current views on the activities of thiol-based peroxidases in peroxide-mediated redox signaling processes.



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1. INTRODUCTION

For many years, peroxides in biology were considered toxic byproducts of aerobic metabolism. These peroxides include hydrogen peroxide (H_2O_2), peroxynitrous acid (ONOOH), and organic hydroperoxides (such as lipid hydroperoxides). Along the evolution, organisms developed efficient systems to reduce toxic peroxides. The biological function of peroxides was thought to be confined to cells of the immune system to fight against pathogen invasion. Even in these cells, after the infection is controlled, inflammation needs to be resolved and again, the levels of peroxides need to be decreased. In aerobic organisms,

Received: June 11, 2019
Published: September 9, 2019

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829–10855

an enzyme specialized to eliminate hydrogen peroxide was found first: catalase, in general included in organelles specialized for that task, the peroxisomes.[1,2] Catalase effectively disproportionates H_2O_2 into water and oxygen and has a very large turnover number. The mechanism of catalysis of H_2O_2 elimination depends on the heme group of the enzyme, which is also present in other peroxidases such as ascorbate peroxidase.

A different peroxidase activity was detected in red blood cells in 1957[3] and later characterized as a selenoprotein in the early 1970s, as the classical glutathione peroxidase (GPxs).[4−6] GPxs possess a particular amino acid residue, a selenocysteine (Sec) that specifically reacts with peroxides with high rate constants ($k \sim 10^5-10^7$ M^{-1} s^{-1}) and is oxidized to selenenic acid. The enzyme is reduced back by two consecutive reactions with glutathione (GSH) with the formation of a mixed selenenylsulfide intermediate.

More recently, enzymes first described as thiol-specific antioxidants (TSA), and later named peroxiredoxins (Prxs), were found to be capable of reducing peroxides using only one or two cysteine (Cys) residues (no heme, and no Sec is required) and a reducing substrate such as thioredoxin (Trx).[7−10] [The IUBMB enzyme nomenclature committee acknowledges several abbreviations for referring to peroxiredoxins (https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC1/11/1/15.html), Prx and PRDX being the most widely used.] They are very abundant in most organisms and cell compartments. Because the peroxidatic Cys residue of Prxs (C_P) reacts with peroxides with rate constants in the 10^4-10^8 M^{-1} s^{-1} range, Prxs are able to detect very low levels of peroxides, act as sensors and, importantly, provide specificity to the signaling process through particular protein−protein interactions. Indeed, these thiol peroxidases are not only implicated in directly decreasing the levels of peroxides to diminish oxidative damage but also in regulating redox signaling toward adaptive response to oxidative stress.[11] In addition to Prxs, other peroxidases that depend on thiols for catalysis have been described, such as thiol-based GPx and organic hydroperoxide resistance protein (Ohr).[12,13]

In this review, we will focus on the mechanisms of catalysis of hydroperoxide reduction by thiols with special emphasis on the specialized protein thiols such as the peroxidatic Cys of Prxs.

2. PEROXIDES IN BIOLOGY

According to the IUPAC Gold Book (https://goldbook.iupac.org/html/H/H02905.html), hydroperoxides are monosubstitution products of hydrogen peroxide (HOOH, or H_2O_2) having the ROOH skeleton, in which R is any organyl group.[14] Through this review, however, we preferred to utilize the term hydroperoxide in a broader sense, to include H_2O_2 itself as well as other ROOH compounds irrespective of the organic or inorganic nature of R (thus including also peroxyacids or peracids). Utilizing this broad definition of hydroperoxide, many of them are formed and play crucial roles in biological systems.

2.1. Hydrogen Peroxide

Life on Earth appeared and evolved in the presence of H_2O_2 because it is abiotically produced by ultraviolet radiation of water.[15,16] Additionally, H_2O_2 is formed in vivo, where it plays a role as a key mediator in redox signaling and regulation of cellular functions.[17−20] In activated phagocytic cells, such as macrophages or neutrophils, it is formed in the phagosome through the dismutation of superoxide radical ($O_2^{\bullet-}$), in turn arising from the one-electron reduction of oxygen catalyzed by NADPH oxidase (NOX 2) during the "respiratory burst". In addition, several members of the NOX family expressed in nonphagocytic cells produce H_2O_2 (either directly or through $O_2^{\bullet-}$ dismutation) in response to different signaling molecules including growth factors and cytokines.[21] Mitochondria are also important sources of $O_2^{\bullet-}$ that after Mn superoxide dismutase (SOD)-catalyzed dismutation form H_2O_2.[22−24] Superoxide can be reduced to H_2O_2 either nonenzymatically[25] or in reactions catalyzed by superoxide reductases (SOR) expressed in some bacteria.[26,27] Finally, H_2O_2 can also be formed from the direct two-electron reduction of oxygen catalyzed by oxidases present in different cellular compartments or in the extracellular space, such as xanthine oxidase and amino acid oxidases, among others.[28−32]

H_2O_2 can permeate biological membranes, in a process that is facilitated and regulated by specific aquaporin isoforms.[33−36] With $E^{o\prime}_{(H2O2,H2O)} = 1.349$ V,[37] H_2O_2 is a strong oxidant but kinetic barriers limit its reactivity mostly to thiol-, selenol-, or heme-dependent peroxidases and a few other transition metal centers.[20] The former reactions are in the basis of H_2O_2-dependent redox signaling (see section 4), while reaction with some reduced transition metal centers may promote the formation of the highly oxidant hydroxyl radical ($^{\bullet}OH$) or metal-oxo complexes through Fenton chemistry.[38−40] $^{\bullet}OH$ is a nonspecific oxidant due to its extremely high reactivity and corresponding short half-life (<μs). However, it is worth noting that localized generation of $^{\bullet}OH$, resulting from the reaction with either metal binding to DNA or from the presence of reduced transition metal cofactors in proteins, promotes site-specific DNA damage[41] or selective one-electron amino acid oxidation.[42]

2.2. Peroxymonocarbonate

The equilibrium of H_2O_2 with CO_2/HCO_3^- forms peroxymonocarbonate ($HOOCO_2^-$ or HCO_4^-, reaction R1), which is an anion at physiological pH.[43,44] Kinetic and mechanistic analysis indicate that HCO_4^- formation involves CO_2 and either H_2O_2 ($k = 0.02$ M^{-1} s^{-1}) or its conjugate base, HOO^- ($k = 280$ M^{-1} s^{-1}) as the reactive species, i.e., it is a perhydration reaction.[43] From the pK_a of H_2O_2 and the rate constants mentioned above, it was calculated that both pathways contribute ~60% and 40%, respectively, to HCO_4^- formation at pH 7.4.[45]

$$HCO_3^- + H_2O_2 \rightleftarrows HCO_4^- + H_2O \quad \text{(R1)}$$

The reported K_{eq} of reaction R1 (0.33 M^{-1} at 25 °C) and the slow k_{app} of HCO_4^- formation (0.034 M^{-1} s^{-1} at pH 7.4) in comparison with those of other biologically relevant targets of H_2O_2 caused this route not be considered of biological relevance unless accelerated by carbonic anhydrase or by protein or lipid environments.[45] Particularly relevant is the formation of peroxymonocarbonate at the active site of metal-containing enzymes, such as xanthine oxidase[46] or CuZnSOD,[47] that reduce HCO_4^- to yield carbonate radical ($CO_3^{\bullet-}$). This reaction is in the basis of the increase of CuZnSOD peroxidase activity in bicarbonate buffers:[47]

$$HCO_4^- + M^{n+} \rightarrow OH^- + CO_3^{\bullet-} + M^{(n+1)+} \quad \text{(R2)}$$

Peroxymonocarbonate is a two-electron oxidant ($E^{o\prime} = 1.80$ V),[48] and in general, the S_N2 reactions involving HCO_4^- as electrophile are ~10^2 faster than those of H_2O_2 because the CO_3^{2-} formed as a product is a better leaving group than OH^-.[48,49] In cells, in addition to the metalloproteins indicated above, thiol-containing compounds and particularly GSH due to its high abundance are considered preferential targets for

DOI: 10.1021/acs.chemrev.9b00371
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peroxymonocarbonate. Although kinetic data regarding protein thiols is still scarce, the peroxidatic thiol of the Prx AhpE from *Mycobacterium tuberculosis* was reported to react with HCO_4^- with a rate constant of 1.1×10^7 M^{-1} s^{-1}, suggesting that Prxs and probably other thiol peroxidases could be relevant targets.[45,50,51] Because of its negative charge, diffusion of HCO_4^- through different cell compartments is considered to be impaired unless facilitated by anion channels.

2.3. Peroxynitrous Acid

Another hydroperoxide of biological interest is peroxynitrous acid (ONOOH), the conjugated acid of peroxynitrite anion ($ONOO^-$), which is formed from the fast recombination reaction between $O_2^{\bullet-}$ and nitric oxide ($^\bullet NO$).[52,53] [IUPAC recommended names for peroxynitrite anion ($ONOO^-$) and peroxynitrous acid are oxoperoxonitrate (1−) and hydrogen oxoperoxonitrate, respectively. The term peroxynitrite is used to refer to the sum of $ONOO^-$ and ONOOH. In turn, the recommended name for nitric oxide is nitrogen monoxide or oxidonitrogen($^\bullet$).] Because of the differences in membrane permeation between $^\bullet NO$ and $O_2^{\bullet-}$,[54−56] peroxynitrite is mostly formed at sites of $O_2^{\bullet-}$ generation, particularly in the phagosomes of activated phagocytes and mitochondria.[57,58] Peroxynitrite formation in the macrophage phagosome is involved in pathogen clearance.[59] In turn, several diseases are associated with increased mitochondrial peroxynitrite formation.[60−62] Peroxynitrite can act either as a one-electron ($E^\circ_{(ONOOH,H+/\bullet NO2,H2O)}$ = 1.6 V) or two-electron oxidant ($E^\circ_{(ONOOH,H+/NO2-,H2O)}$ = 1.3 V).[63] Thiols or selenols, particularly those of thiol or selenol-dependent peroxidases (see section 4), some transition metal centers, mainly in heme peroxidases, and CO_2 are the main targets of peroxynitrite in biological systems. Considering the reported rate constants of reactions and target concentrations, the half-life ($t_{1/2}$) of peroxynitrite has been estimated to be less than 1 ms, depending on the cellular compartment as well as metabolic conditions, which translates into travel distances ($TDt_{1/2}$) in the μm range.[53,64] Oxidation of some transition metal centers by peroxynitrite can lead to the formation of secondary oxidizing compounds, such as compound I and II of heme peroxidases and nitrogen dioxide radical ($^\bullet NO_2$).[65] In turn, the reaction of peroxynitrite with CO_2 forms the nitrosoperoxocarboxylate adduct, whose rapid (<1 μs) homolysis generates $^\bullet NO_2$ and carbonate radical ($CO_3^{\bullet-}$) in ∼30% yields.[66−68] These secondary species can promote one-electron oxidations and nitration processes through radical pathways.[69] Peroxynitrous acid (pK_a = 6.8)[70] can diffuse through lipid membranes with an estimated transmembrane diffusion constant of 2×10^3 s^{-1},[53,71,72] while peroxynitrite anion can cross biological membranes through anion channels.[71] Thus, peroxynitrite is in principle able to reach different compartments from its initial formation site (e.g., escape from mitochondria[64] or reach phagocytosed pathogens[73]), provided the distances are not much larger than $TDt_{1/2}$.

2.4. Lipid Hydroperoxides

Polyunsaturated fatty acids, either free or bound to phospholipids, glycolipids, or cholesterol esters in biological membranes or in lipoproteins, are prone to nonenzymatic as well as enzymatic lipid peroxidation that form the corresponding hydroperoxides as first stable products.[74−76] Formation of fatty acids hydroperoxides (FA-OOH) is involved in aging and various diseases.[77] They can lead to cellular membrane damage.[77] In the presence of some transition metals, they can form alkoxyl ($LO^\bullet$) and peroxyl ($LOO^\bullet$) radicals, which further propagate lipid peroxidation or modify other biomolecules.[78,79] Furthermore, peroxidized lipids can condense with amino groups in proteins, leading to the formation products that affect protein function.[80] In nonenzymatic lipid peroxidation, unsaturated fatty acids are oxidized nonspecifically. On the contrary, enzymes that catalyze peroxidation of fatty acids, such as lipoxygenases and cyclooxygenases, display high regio- and stereoselectivity and produce particular hydroperoxides which have been frequently associated with signaling actions.[81−83] Because of the low solubility of FA-OOH in aqueous media, synthetic organic and more water-soluble compounds like *tert*-butyl hydroperoxide (*t*-BHP) and cumene hydroperoxide (CHP) have been frequently used as their analogues. However, results obtained using these artificial compounds should be treated with caution, in light of reports indicating that enzymes involved in hydroperoxide metabolism are not equally active toward these artificial short-chain compounds compared with the natural substrates.[13,50,84] In addition to free or lipid-bound fatty acid, cholesterol and other sterols can react with singlet oxygen to form hydroperoxides, that may affect cellular functions, either directly or through degradation products.[85,86] Sterols can also participate in lipid peroxidation reactions that involve peroxyl radicals that may evolve to hydroperoxides and other oxysterol species.[87]

2.5. Other Hydroperoxides

Several amino acids (particularly tyrosine, cysteine, tryptophan, histidine, and proline), either free or as protein residues, can be modified to hydroperoxides by oxidants such as $^\bullet OH$ and singlet oxygen.[88,89] These amino acid hydroperoxides are unstable and can further propagate the initial oxidative damage.[90,91] Other biologically relevant hydroperoxides include urate hydroperoxide[92] and hydroperoxides of DNA.[93] In general, these hydroperoxides are nonenzymatically generated, through initial one-electron oxidation of the organic compound followed by oxygen addition to form a peroxyl radical, which is then reduced to the corresponding hydroperoxide.[94] An alternative route of formation, which is particularly favored at inflammation sites, is through the fast recombination reaction between the initial organic radical with $O_2^{\bullet-}$.[92,95]

3. REDUCTION OF HYDROPEROXIDES BY THIOLS

Cysteine is one of the least abundant amino acids and one of the most chemically intriguing and functionally diverse. The integration of Cys in the genetic code permitted redox flexibility and cellular fitness under the evolving conditions of environmental and endogenous oxidative challenge.[96−98] The thiol group in Cys can lose a proton and become an anionic thiolate (reaction R3). This fact is responsible for most of its nucleophilic reactivity in the form of oxidation or modification by electrophiles, leading to a wide variety of derived species.[99,100]

$$RSH + H_2O \rightleftarrows RS^- + H_3O^+ \quad (R3)$$

The electronic structure of sulfur atom, together with the relatively low dissociation energy of the S−H bond, greatly determines its ability to accomplish very different functions. The pK_a of thiols are often close to physiological pH (Cys pK_a is ∼8.3−8.5).[101,102] Nevertheless, this property is extremely sensitive to the surrounding microenvironment, so actual protein thiol pK_a values span a very wide range. (see section 4).

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

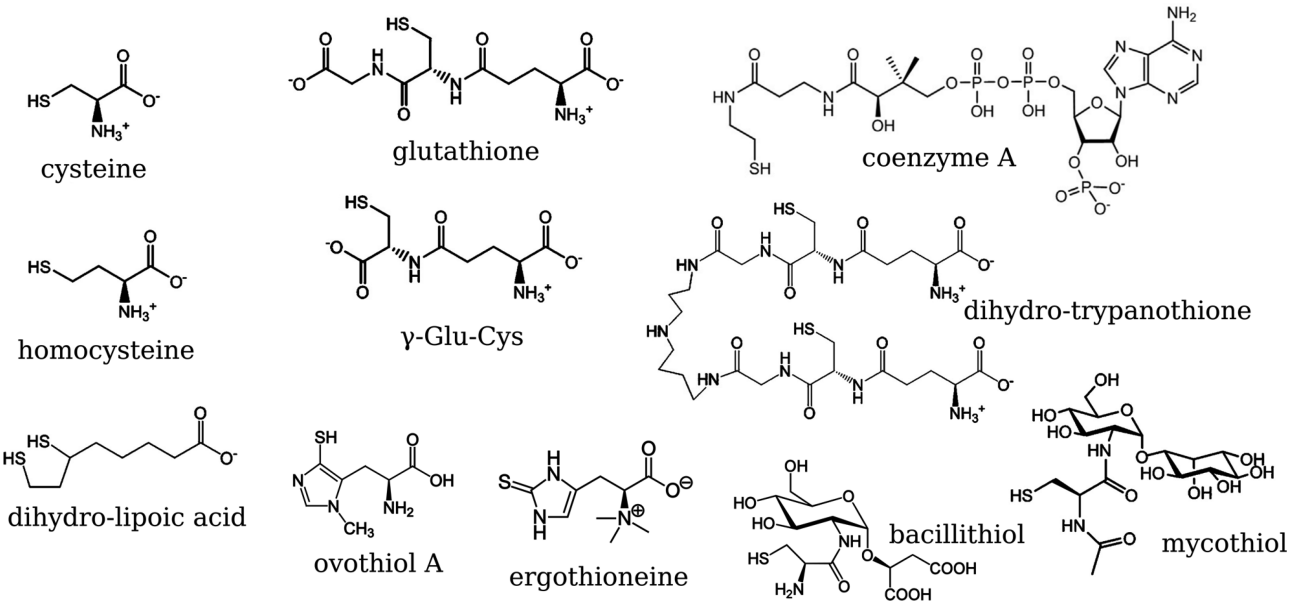

Figure 1. Relevant low molecular weight thiols in biological systems.

Biological thiols are either part of protein Cys, or functional groups of low molecular weight (*lmw*) compounds. The latter species are important in cellular redox processes, but their identity and abundance differs over the entire biome. The tripeptide glutathione (γ-glutamylcysteinylglycine) is the most widespread *lmw* thiol, its concentration is in the millimolar range in most cells. However, some organisms use other *lmw* thiols that may be as abundant or even more than GSH. Figure 1 presents some important *lmw* thiols for most of cell types or for specific organisms. For example, *Actinomycetes* (such as *Mycobacteria*) synthesize mycothiol (1-D-myo-inosityl 2-(*N*-acetylcysteinyl) amido-2-deoxy-α-D-glucopyranoside); in turn, trypanosomatids synthesize trypanothione (bis(glutathionyl) spermidine) from GSH. In every case, these species are involved in a multiplicity of metabolic and regulation pathways.[103,104]

3.1. Reactivity with Low Molecular Weight Thiols

Among the diversity of chemical modifications that thiols may suffer in a cellular context, the oxidation of thiols by hydroperoxides is a central biochemical process in which the thiol is oxidized to the corresponding sulfenic acid with the concomitant reduction of the hydroperoxide to the resultant ROH:[105,106]

$$RS^- + R'OOH \rightarrow RSO^-/RSOH + R'OH/RO^- \qquad (R4)$$

The reactive species in this process are thiolates and hydroperoxides,[106,107] meaning that reactants pK_as and pH have a strong influence on the observed rate constants for this reaction (see next section).

Taking into account the rate constants and physiological concentrations of different cellular thiols, hydroperoxides would almost not react with *lmw* thiols in a cellular context but mainly with specialized protein thiols and/or other non- Cys-dependent peroxidases.[108−110] Table 1 presents an updated compilation of the reported rate constants of *lmw* thiols with H_2O_2 and ONOOH. From the limited data available, the reactivity of HCO_4^- with *lmw* thiols is ~10^2 higher than that of H_2O_2.[45,49] It is worth mentioning that systematic information about the reactivity of thiols with organic and/or FA-OOH is still lacking, and from the few available examples in the literature, the nature

Table 1. Rate Constants of Oxidation of Thiols by H_2O_2 and ONOOH

LMW thiol	pK_a [a]	k^{app}_{H2O2} [b] (M^{-1} s^{-1})	refs	k^{app}_{ONOOH} [b] (M^{-1} s^{-1})	refs
cysteine ethyl ester	7.5	1.40[c]	102	6830	112
penicillamine	7.9	4.5	106	6420	112
cysteine	8.3	2.9	106	4500	105
dihydro-trypanothione	7.4	5.37[d]	113	3600	112
mycothiol	8.8	0.6[e]	114	1670[f]	115
glutathione	8.8	0.87	106	1360	116
homocysteine	9.1	0.18[c]	102	700	112
N-acetyl cysteine	9.5	0.16	106	415	112
dihydrolipoic acid	10.7	0.27	117	250	112

[a]In the case of aminothiols, the reported pK_a values are macroscopic ionization constants for the SH group[106] or the microscopic pK_a of the ammonium thiol, which is the predominant species at neutral pH.[102] [b]Values reported at pH 7.4 and 37 °C unless otherwise indicated. [c]At pH 7.06 and 25 °C. [d]At pH 7.2 and 27 °C. [e]At pH 7.4 and 25 °C. In the case of the dithiols trypanothione and dihydrolipoic acid, indicated values are per thiol group, assuming a similar reactivity for both thiols. [f]Estimated from Brønsted correlations.

of the R-group in the R-OOH may affect significantly its reactivity properties.[111] Nevertheless, from a mechanistic perspective, it is reasonable to expect that many aspects can be extrapolated from the knowledge on the H_2O_2 reactions

As described in Table 1, the reaction of *lmw* thiols is significantly faster with ONOOH than with H_2O_2 (apparent second-order rate constants are approximately 2−3 orders of magnitude higher at physiological pH). These differences were well captured by reaction free energy profiles determined using multiscale quantum classical (QM/MM) computer simulations that show the greater capacity of ONOOH compared to H_2O_2 in oxidizing *lmw* thiols (Figure 2). As stated before, the direct oxidation of thiols by hydroperoxides occur by a nucleophilic substitution mechanism (S_N2), in which thiolates and protonated hydroperoxides are the reactive species (reaction R5).[106,112] In the case of thiol oxidations by H_2O_2, QM/MM molecular dynamics simulations indicated that the classical S_N2 reaction mechanism is modified by the proton transfer of one

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

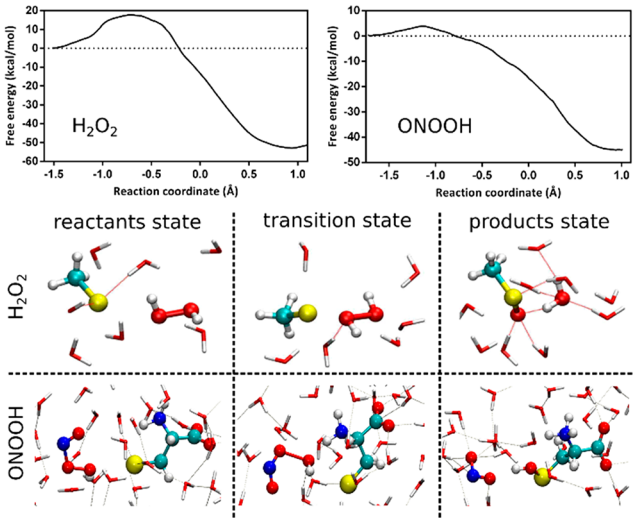

Figure 2. Mechanistic analysis of the reaction between *lmw* thiols and H_2O_2 or ONOOH. (upper panel) Free energy profiles of the reaction of the model compound CH_3S^- with H_2O_2 (left) and $CysS^-$ with ONOOH. (lower panel) Typical snapshots extracted from the simulations of reactants, transition state, and products of both reactions.[121,122]

peroxidic oxygen to the other so that water and sulfenate are the direct products of the reaction as indicated in reaction R5 (Figure 2, upper panel). However, this transfer occurs after the transition state in the reaction coordinate, and therefore lacks significant kinetic consequences.[118−121] In the case of ONOOH, the reaction proceeds directly to sulfenic acid and nitrite as indicated in reaction R6 (Figure 2, lower panel).[122]

$$RS^- + H_2O_2 \rightarrow RSO^- + H_2O \quad (R5)$$

$$RS^- + ONOOH \rightarrow RSOH + NO_2^- \quad (R6)$$

In both cases, the solvent plays a key role in positioning the reactants and assisting the significant charge redistribution that takes place in the first stages of the reaction.[121,122]

Because protonated hydroperoxides and thiolates are the reactive species, the determined apparent rate constants of the reactions are affected by pH as given by

$$k^{app} = k\frac{K_a^{RSH}}{K_a^{RSH} + [H^+]} \times \frac{[H^+]}{[H^+] + K_a^{ROOH}} \quad (1)$$

where k^{app} is the apparent rate constant of the reaction, k is the pH-independent rate constant, and K_a^{RSH} and K_a^{ROOH} are the ionization constants of the thiol and the hydroperoxide, respectively;[112] thus the terms $\frac{K_a^{RSH}}{K_a^{RSH} + [H^+]}$ and $\frac{[H^+]}{[H^+] + K_a^{ROOH}}$ indicate the availability of thiolate and of protonated hydroperoxide, at a given pH. As the pK_a of H_2O_2 is 11.6,[123] more than 99.9% is protonated in any biochemically relevant environment. However, in the case of ONOOH, with a pK_a close to physiological pH,[124,125] the fraction of protonated hydroperoxide is importantly affected by pH. Additionally, because thiol pK_a values are usually relatively close to physiological pH, the fraction of thiolate at a given pH also affects apparent rate constants. It can be shown that eq 1 describes a curve with a maximum at pH midway between pK_a^{ROOH} and pK_a^{RSH}, as *lmw* thiols have pK_a^{RSH} above 7.5, the determined k^{app} (usually at pH 7.4), is far below the maximum. Thus, the higher the pK_a^{RSH} the lower k^{app} value determined at pH 7.4. This lower k^{app} is not related to the reactivity of each thiol but to the availability of the thiolate at the pH of the measurement.

However, pH-independent rate constants follow a linear Brønsted correlation with the pK_a of the thiol as

$$\log k = \beta_{nuc} pK_a^{RSH} + A \quad (2)$$

where β_{nuc} is the nucleophilic constant of the reaction. As β_{nuc} is positive, the relationship indicates that the more basic the thiolate the more reactive it is. The slopes are 0.27 and 0.4 for H_2O_2 and ONOOH, respectively.[102,126] These β_{nuc} are considered indicators of the degree of charge transfer from the nucleophile to the electrophile in the transition state, which are consistent with the data obtained in our QM/MM simulations.[121,122] pH-independent rate constants of thiols reacting with H_2O_2 are in the $\sim 10^1-10^2$ M^{-1} s^{-1} range, while with ONOOH are in the $\sim 10^4-10^6$ M^{-1} s^{-1} range (Table 1). As expected for a S_N2 mechanism, they also follow a Brønsted relationship correlating with the pK_a of the leaving group.[49] Indeed, that was the case for glutathione as well as the single thiol group of bovine serum albumin oxidation, that react with H_2O_2, peroxymonocarbonate, and ONOOH with pH independent rate constants of $\sim 10^1$, $\sim 10^3$, and $\sim 10^5$ M^{-1} s^{-1}, respectively.[45,49,112,127]

Protein Cys residues are often involved in the coordination of metal ions, many of which can be subject to redox changes, thus further complicating the interpretation of results involving reactions with hydroperoxides. In the case of Zn-thiolates, however, the metal is not oxidized. Hydroperoxides oxidize Zn-thiolate complexes by a nucleophilic substitution mechanism of the S_N2 type, that through an initial sulfenate formation leads to disulfide bridges and release of Zn^{2+}. Intriguingly, several reports indicate that Zn-thiolate proteins act as redox switches, allowing cell response to increased levels of H_2O_2.[128−130] However, the rate constants of Zn-thiolate oxidations are frequently unknown, and for those that have been determined, they are lower than for the corresponding free thiolates,[131−134] which argues against a general role as potential sensors for this oxidant in cells.[135]

Alcohol dehydrogenase from yeast is inactivated by peroxynitrite through reaction with a Zn^{2+}-coordinated thiolate with an average rate constant of 3.9×10^5 M^{-1} s^{-1} at pH 7.4 and 37 °C. Inactivation by hydrogen peroxide occurred at much lower rates (k = 1.3 M^{-1} s^{-1}).[136]

4. CYSTEINE-BASED PEROXIDASES

In eukaryotes, two protein families comprise the so-called Cys-based peroxidases, Prx and GPx, while some prokaryotes contain additional thiol-based peroxidases. As it will be discussed later, GPxs may be Cys-based or Sec-based peroxidases. Both Prx and GPx are members of the Trx superfamily, thus they share the folding architecture known as Trx fold, whose minimal information is given by a structural motif consisting of a central core of four-stranded β-sheets and three surrounding α-helices.[137] The basic shared catalytic mechanism is that the so-called peroxidatic Cys/Sec at the active site of these enzymes reduces hydroperoxides, yielding the sulfenic/selenenic acid intermediate, with rate constants that are 3−7 orders of magnitude higher than those of *lmw* thiols. However, the fate of the sulfenic acid and the molecular determinants of such extraordinary rates, are quite different for both protein families. We will now present the most important features of Prxs and GPxs biochemistry and then offer some examples of other proteins with reactive Cys residues toward hydroperoxides.

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

Elementary differences when comparing *lmw* thiols versus protein Cys reactivity will be discussed. Finally, we will discuss the importance of Cys-based peroxidases in redox regulation and signaling processes.

4.1. Peroxiredoxins

Prxs (EC1.11.1.15) are a family of ubiquitous broad-spectrum peroxidases that catalyze the reduction of hydroperoxides. They were first identified at Sue Goo Rhee's lab as a factor protecting glutamine synthetase from oxidation and described at that moment as thiol-specific antioxidant (TSA) enzymes because a thiol was required as reductant to observe activity of that 25 kDa protein.[7−10] During the last 25 years, great effort was dedicated to understand every aspect of the biochemistry, biology, and biomedicine of these enzymes. Nowadays, it is well recognized that Prxs are a widely distributed family that have evolved from an ancestor protein having the Trx fold (Figure 3a and 3c),[138]

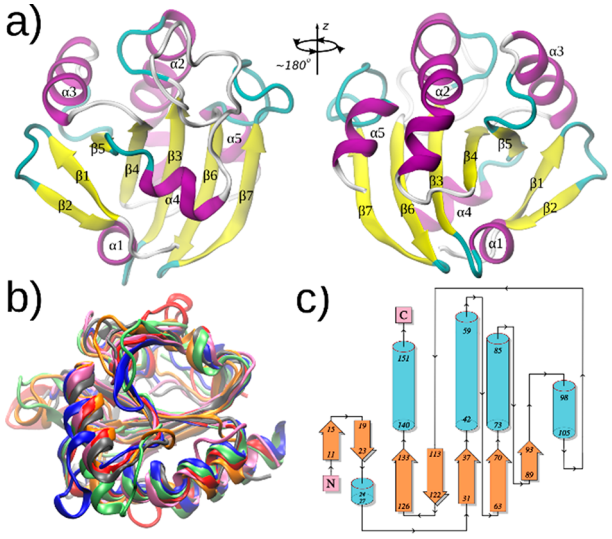

Figure 3. Structural overview of Prxs. (a) Two different views of the typical Prx fold. The structures are colored by the secondary structure characteristics. (b) Three-dimensional alignment of one member of each six Prx subfamilies: *Hs*PrxII, blue (PBD 1QMV); *Xc*PrxQ, red (PDB 3GKK); *Ec*Tpx, gray (PDB 3HVV); *Hs*PrxV, orange (PBD 1HD2); *Py*PrxVI, green (PDB 1XCC); *Mt*AhpE, violet (PDB 4X0X). (c) Topological diagram of Prx folding using *Mt*AhpE as Prx model.

that many Prxs have high expression levels (~1% of mammal cellular proteins),[108] and that they exhibit extremely fast reactions with most hydroperoxides, with rate constants in the 10^4−10^8 M^{-1} s^{-1} range.[127,139,140] Considering these facts, kinetics competition analyses have predicted that eukaryotic Prxs will be responsible for the reduction of most mitochondrial and cytoplasmic H_2O_2 and peroxynitrite.[53,64,141,142] Accordingly, Prxs are considered key players protecting cells from oxidative stress.

Since the discovery and functional characterization of the first Prxs from mammals, yeast, bacteria, plants, and other organisms, a battery of different roles, cellular processes as well as relationship with different human diseases have been described, meaning that the primary antioxidant function of these enzymes is not the only one they have. It is worth to underscore their role in regulating H_2O_2 concentrations, as key sensors and regulators of H_2O_2-related signaling processes (see below),[143−145] their role as protein chaperones,[146,147] their participation in the regulation of the circadian rhythm,[148−150] or their importance in innate immunity and inflammation processes acting both as pathogen-associated molecular patterns or host-derived damage-associated molecular patterns,[151] among many others. These relationships between Prxs and redox associated cellular processes have been the topics of excellent recent reviews compiled in a journal issue.[152] Furthermore, important links between Prxs and the evolution of different types of cancers and other diseases have been also described and/or reviewed recently.[153−160]

Regarding primary structure, all Prxs known share common patterns. They all rely in the presence of a Cys residue that is essential for Prx catalytic activity, the so-called peroxidatic Cys (C_P). At least two different criteria for classifying Prx family members have been proposed. The first is related to the presence or absence and the location of a second Cys residue that participates in the catalytic mechanism forming a disulfide with the newly oxidized C_P, known as resolving Cys (C_R). Hence, 1-Cys Prxs are those that do not contain a C_R in its sequence, atypical 2-Cys Prxs represent the subgroup in which C_R and C_P are in the same subunit, and those that present the C_R in an adjacent subunit within the oligomeric assembly of the enzyme are called typical 2-Cys Prxs. This classification is based on the fate of the sulfenic acid formed at C_P upon hydroperoxide reduction (see next section) and also gives an indication on the possible reductants that may interact with the oxidized enzyme during the catalytic cycle. More recently, a novel clustering of Prx sequences and structural properties led to a new Prx classification into six major subfamilies and a systematically organized Prx database (http://csb.wfu.edu/prex/).[161,162] The authors proposed to name these subfamilies after a "canonical" member of each one of them: Prx1, Prx6, Prx5, Tpx, PrxQ, and AhpE (Figure 3b). This classification is likely to contain more information between subgroups, regarding important properties

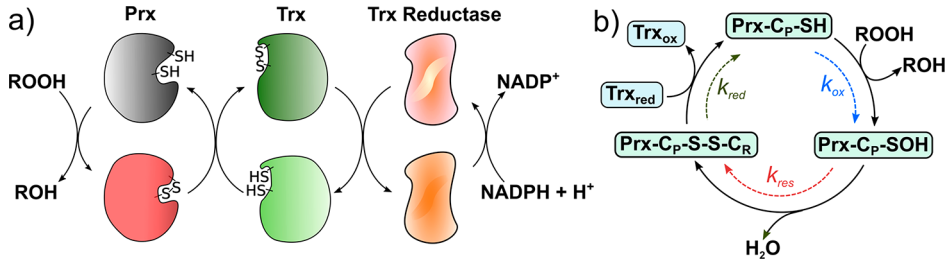

Figure 4. Catalytic cycle of Prxs. (a) Complete overview of electron flux during Prx catalyzed hydroperoxide reduction. Note that the most usual Prx reductant partners are shown, although not every Prx relies on the Trx/Trx reductase/NADPH system (see text). (b) Catalytic cycle: oxidation, reaction of C_P with hydroperoxides, yielding the sulfenic acid; resolution, disulfide formation between C_P and C_R (this process differs in 1-Cys Prxs where the resolving thiol is not present and a mixed disulfide bond is formed); reduction by Trx to complete the cycle.

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

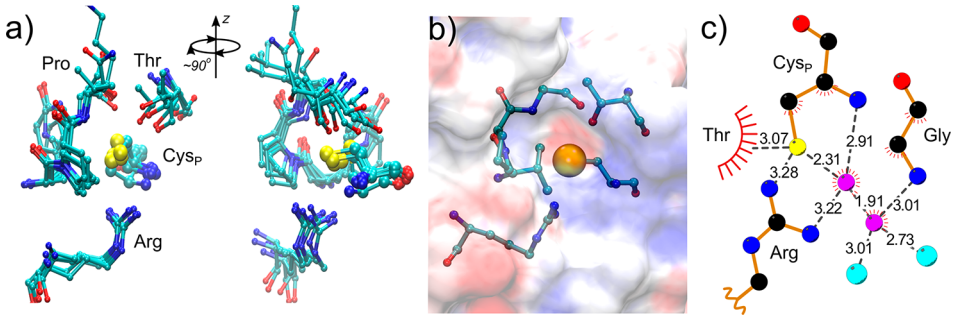

Figure 5. Prx active site structure. (a) Two views of the catalytic tetrad of Prxs. The structures derive from the same alignment shown in Figure 3b to highlight the conserved properties of the active site architecture. (b) "Solvent view" of the electrostatic potential generated by active site's residues. Positive and negative regions are depicted in blue and red scales, respectively. (c) 2D representation of the predicted structure of the transition state of the reaction. The structure corresponds to an average configuration obtained from QM/MM molecular dynamics simulations using H_2O_2 as oxidant (violet circles) and *Mt*AhpE as Prx model.[191] 2D representation was prepared using the LigPlot$^+$ software.[200] Distances showed correspond to the average value sampled during simulation. Water molecules are represented as light-blue circles.

Table 2. Reaction Constants of Oxidation of Prxs by H_2O_2 and ONOOH

Prx subfamily	Prx	k^{app}_{H2O2} (M^{-1} s^{-1})	k^{app}_{ONOOH} (M^{-1} s^{-1})	ref
Prx1	*Hs*Prx1	3.8×10^7	1.1×10^7	169−171
		1.1×10^8		
	*Hs*Prx2	1.3×10^7	1.4×10^7	172,173
		1×10^8		
	*Hs*Prx3	2.0×10^7	1.0×10^{7a}	64,174
	T. cruzi mTXNPx	1.8×10^7	6.0×10^6	175
	T. cruzi cTXNPx	3.0×10^7	1.0×10^6	175
	S. cerevisiae Tsa1	2.2×10^7	7.4×10^5	168
	S. cerevisiae Tsa2	1.3×10^7	5.1×10^5	168
	S. typhimurium AhpC	3.7×10^7	1.5×10^{6b}	167,176
Prx6	*Hs*Prx6	3.4×10^7	3.7×10^5	177
	A. marina Prx6	1.1×10^7	2.0×10^6	178
Prx5	*Hs*Prx5	$(3–4) \times 10^5$	1.2×10^8	102,126,179
	P. falciparum	3.2×10^7	1.5×10^7	180
Tpx	*M. tuberculosis* Tpx	$>1 \times 10^5$	1.5×10^7	181
PrxQ	*X. fastidiosa* PrxQ	4.5×10^7	1.0×10^6	182
	M. tuberculosis PrxQ B	6.0×10^3	1.4×10^6	183
	C. glutamicum	9.5×10^3	1.3×10^6	184
AhpE	*M. tuberculosis* AhpE	8.0×10^4	1.9×10^7	50,185

Apparent rate constants (k^{app}) were reported at physiological pH except for [a]pH = 7.8 and [b]pH = 6.8.

like oligomerization interface, hydroperoxide, and electron donor specificity, and sensitivity toward inactivation during turnover. Most important features and substantial differences among Prx subfamilies had been thoroughly presented in recent reviews,[162−164] thus, we will focus on the chemical and mechanistic aspects of Prx catalysis.

4.1.1. Mechanism of Catalysis. The majority of Prxs are 2-Cys Prxs that rely on the Trx system as physiological reductant. So, the overall reaction catalyzed by Prxs may be described as

$$Trx_{red} + ROOH \rightarrow Trx_{ox} + ROH + H_2O \quad (R7)$$

where the oxidized Trx (Trx_{ox}) can be restored to the reduced form (Trx_{red}) by Trx reductase using NADPH as an electron source (Figure 4a). The global process showed in reaction R7 involves a three-step catalytic cycle: (1) reduction of the hydroperoxide with the concomitant formation of a sulfenic acid on C_P (oxidation), (2) condensation of C_P sulfenic acid with C_R to form a disulfide (resolution), and (3) reduction of the disulfide by Trx (Figure 4b). Although recent evidence suggests the formation of an enzyme−substrate complex during the oxidation process,[165,166] the existence of a Michaelis-type complex needs further experimental confirmation. During the next sections, we will describe the most important structural and kinetic aspects of these three steps, along with the biochemical implications of each of them.

4.1.1.1. Oxidation. All Prx family members share the first step of the catalytic cycle, in which the thiolate in C_P is oxidized to sulfenic acid. As described in section 3, the reactive species in this reaction are the anionic form of the C_P and the protonated form of the hydroperoxide. A very important aspect of the architecture of Prx active site is that presents two strictly conserved Arg and Thr/Ser residues (see Figure 5a) that are

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

Table 3. Kinetics and Activation Parameters for the Reduction of H_2O_2 by free Cys and *Mt*AhpE[191,201]

	k_{H2O2} (M^{-1} s^{-1})[a]	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal K^{-1} mol^{-1})	$\Delta G^{\ddagger}$ (kcal mol^{-1})[b]
free Cys	14.9	16.4 ± 0.3	1.7 ± 1.1	15.9
*Mt*AhpE-C_P	(8.0 ± 1.5) × 10^4	4.8 ± 0.5	−19.1 ± 1.9	10.5

[a]pH-independent rate constants. [b]Calculated at 25 °C.

responsible for establishing a network of polar interactions stabilizing the thiolate and lowering its pK_a significantly.[127,139] Consequently, pK_a values of the C_P are in the range of 5−6.3, so at physiological pH more than 90% of the thiolate is available.[126,167,168]

The oxidation step is typically the reaction of the catalytic cycle with highest rate constants, close to the diffusion limit in several cases. For example, second-order rate constant for the reduction of H_2O_2 are up to 10^7 times and up to 10^4 times higher for ONOOH reduction compared to the same reactions with free Cys in aqueous solution. Table 2 presents second-order rate constants for H_2O_2 and ONOOH reduction by different Prxs. For a discussion on substrate specificities, see section 4.4.

How this acceleration is achieved? This question has been matter of debate, and any attempted answer needs to integrate information from biochemical experiments such as site directed mutagenesis and kinetics, structural insights coming mostly from X-ray structure determinations, and theoretical chemistry computer simulations. Prx active site is extremely conserved (Figure 5a), and besides small structural variations that may account for the observed reactivity variability among different Prxs, the fundamental aspects of catalytic determinants are common for the entire family. C_P is positioned at the N-terminus of α2-helix (Figure 3), at the bottom of the cave-shaped active site. As mentioned above, the stabilizing network of C_P thiolate involves the conserved Thr/Ser and Arg residues, that together with a Pro residue had been recognized as the "catalytic tetrad" of Prxs. The alcohol group of the Thr/Ser is placed to form a hydrogen bond (*hb*) with the thiolate, and the Arg guanidinium group acts as an *hb* donor to form a salt bridge stabilizing the thiolate and maintaining the architecture of the active site.[102,166,186−190] The presence of these groups near the thiolate of C_P lays out a nanoheterogeneous environment that yields a partially positive C_P surroundings as depicted in Figure 5b that assist the effective binding of the partially negative oxygen atoms of the hydroperoxide moiety.[102,186,188,190−192]

Once the hydroperoxide enters the active site, the reaction occurs as expected for S_N2 reactions; the transition state is linear with a (S−O−O) angle of ∼180°, the peroxide O−O bond is practically broken, and the newly S−O bond is being formed.[102,191] The process is aided by improved interactions between the hydroperoxide and the active-site residues, which is highlighted by the decrease in the activation enthalpy (see Table 3).[191] The *hb* network of the C_P sulfur is disrupted by the entrance of the ROOH, resulting in a destabilization of the thiolate that increases its nucleophilicity prior to the transition state (Figure 5c).[102,191] The proton transfer from the reactive oxygen to the leaving water oxygen atom that is observed in the simulations of the reaction using *lmw* thiols (see section 3.1) is also observed here, probably assisted by the amide group of the preceding residue to C_P or by the guanidinium of the arginine.[102,191] The reaction then proceeds yielding the sulfenate of C_P. The conformations and/or interactions of this C_P−SO^- are also under debate, as significant differences on C_β−S_γ−O_δ angles and C_α−C_β−S_γ−O_δ dihedrals are present when comparing the few available structures of these intermediates states.[188,193−199]

Recently, we implemented a different strategy to shed new light in the characterization of this critical step. By means of temperature dependence fast kinetics experiments, we were able to determine the activation parameters of the reaction using *Mt*AhpE as a Prx model[191] and compare them with already reported parameters for the reaction with free Cys.[201] Results are summarized in Table 3. Remarkably, the results show that the $\Delta G^{\ddagger}$ decrease is explained by means of two opposing contributions; while the enzyme is capable to diminish $\Delta H^{\ddagger}$ by a factor of ∼4, the unfavorable entropic term ($-T\Delta S^{\ddagger} = 5.7 \pm 0.7$ kcal mol^{-1}) implies that this term represents more than 50% of the total $\Delta G^{\ddagger}$ at 25 °C. This may indicate that Prxs active sites are capable of significantly improve the interaction network at the transition state of the reaction, with a concomitant entropic cost that yields a net decrease in $\Delta G^{\ddagger}$.[191]

In summary, the evidence reported so far supports the idea that the extraordinary acceleration factors performed by Prxs can be interpreted as the sum of two effects related to the organization and rearrangement of the *hb* network during the reaction within the active site; namely, the destabilization of the nucleophile at the reactants state and the stabilization of the transition state. Also, the conserved Arg and Thr/Ser residues, together with an amide group of an adjacent residue and one or two water molecules are essential to explain the effect.[102,127,191,192]

4.1.1.2. Resolution. The condensation of sulfenic acid with thiol to yield a disulfide is extremely fast for *lmw* thiols.[201−204] The most favorable protonation states of the reactants are RSOH and RS^-, which usually do not coexist because sulfenic acids tend to be more acidic than thiols.[102] This fact could reduce 2 or 3 orders of magnitude the apparent with respect to the pH-independent rate constant, depending on the pH and the pK_a of the species involved.

The resolution step, as the formation of a disulfide bond between C_P and C_R, is exclusive of 2-Cys Prx. It involves a conformational change and a redox reaction, and the overall process is relatively slow. C_P−SOH and C_R−SH sit typically at 15 Å of each other, which equals to an effective molarity of approximately 0.94 M assuming they move in a 7.5 Å radius sphere. Assuming a diffusion-controlled rate for condensation, an apparent rate constants of $\geq 10^8$ s^{-1} would be expected, considering the effective molarity and in the absence of steric hindrance.[170] The rate constants of resolution measured by us[64,126,170,171] and other groups[169,205] are much slower, in the range of 10^{-1}−10^2 s^{-1}. As the resolution also includes the so-called fully folded (FF) to locally unfolded (LU) conformational transition, a slow FF to LU change would explain a slow resolution. Nevertheless, as long as the conformational equilibrium favors the FF conformation, even very rapid rates of conformational exchange can explain slow resolution rate constants.[170]

The slow resolution step is a factor contributing to the sensitivity of the Prx to be inactivated by hyperoxidation, i.e., the oxidation of the sulfenic acid to a sulfinic acid by a second

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

hydroperoxide molecule. Resolution and hyperoxidation are parallel reactions of the C_P sulfenic acid. However, even if hyperoxidation is very easily verifiable in vitro, it is hard to reconcile with in vivo conditions as a major contender. In Table 4 the calculated concentration of H_2O_2 causing 1% hyperoxidation per catalytic cycle is compared with the GSH concentration, causing 1% glutathionylation in Prx2. Two important facts are apparent, first, the $C^{1\%}_{H2O2}$ is very high compared to normal intracellular concentrations of H_2O_2,[19] and second, glutathionylation (at least in the case of Prx2) seems a much more likely outcome because intracellular concentration of GSH is typically in the millimolar range. It is worth noticing that these calculated $C^{1\%}_{H2O2}$ are in good agreement with recent determinations of this property.[206] As a general consideration, even if slow, given that the resolution reaction is unimolecular, it will outcompete most bimolecular reactions unless they are very fast because of a large rate constant and/or an elevated concentration of reactant. This topic was recently approached in depth in the context of the transcription factor STAT3 acting as an alternative reductant intercepting the C_P sulfenic as a means to relay the redox sensing of Prx2.[207]

Table 4. Comparison between Resolution, Hyperoxidation, and Glutathionylation as Reactions in Kinetic Competition

peroxiredoxin	k_{res} (s^{-1})	k_{hyp} (M^{-1} s^{-1})	$C^{1\%}_{H2O2}$ (μM)[a]
Prx1	12.9[170]	1770[171]	72.9
Prx2	0.2[171]	1970[171]	1.0
Prx3	22.0[205]	12000[205]	18.3
	k_{res} (s^{-1})	k_{GSH} (M^{-1} s^{-1})	$C^{1\%}_{GSH}$ (μM)
Prx2	0.6[170]	500[208]	12.8

[a] $C^{1\%}_{H2O2}$ is the $[H_2O_2]$ causing 1% hyperoxidation per catalytic cycle and is calculated as $[H_2O_2]$, making resolution 100 times faster than hyperoxidation thus $C^{1\%}_{H2O2} = k_{res}/(100 \times k_{hyp})$. Accordingly, $C^{1\%}_{GSH}$ is the [GSH] causing 1% glutathionylation through condensation with the C_P sulfenic acid.

4.1.1.3. Reduction. The reduction reactions as part of the catalytic cycle can be divided according to the number of Cys involved. In 1-Cys Prx sulfenic acid is the oxidized intermediate in the catalytic cycle, and the enzymes do not seem to have a universal reducing partner. The literature provides examples of sulfur-based reductants such as Trx, H_2S, and GSH, with or without the intervention of additional enzymes,[209−213] and ascorbate has also been shown to reduce 1-Cys Prx of the Prx6 family.[214] In turn, dehydroascorbate can be nonenzymatically reduced by GSH, but the reaction is catalyzed by several enzymes in plants and animals.[215,216] The equilibrium reaction of dehydroascorbate and ascorbate yields monodehydroascorbate, which can be enzymatically reduced by the flavoenzyme monodehydroascorbate reductase in plants.[215] The lack of a universal reductant has been shown for the Prx 6 from *Arenicola marina* that (unlike other members of the Prx 6 subfamily)[209] is not reducible by H_2S, dihydrolipoic acid, or GSH/Trx reductase.[178]

The kinetics of reduction of 1-Cys Prx are only partially characterized. The best studied reaction is that of mammalian Prx6 reduced by GSH and catalyzed by glutathione transferase π. The reported specific activity of 5 μmol min^{-1} (mg Prx6)$^{-1}$ can be translated to a k_{cat} of at least 2 s^{-1},[217] although it is not clear how efficient this system could result under in vivo conditions. Another well studied example is that of *Mycobacterium tuberculosis* AhpE, that can be reduced by mycothiol/mycoredoxin-1/mycothiol reductase system,[218] and also by H_2S.[213]

Two-Cys Prx have a relatively simpler collection of reducing partners, they are usually proteins of the Trx superfamily (such as tryparedoxins in trypanosomatids or plasmoredoxin in *Plasmodium*), or proteins containing Trx domains, such as AhpF[219] in bacteria and NTRC[220] in plants. The reaction consists in two consecutive thiol disulfide exchanges, in the first the *N*-terminal Cys of the Trx attacks the Prx disulfide in the C_R, yielding the thiolate of C_P and a mixed disulfide, the second step is the attack of the *C*-terminal Cys of the Trx active site on the *N*-terminal Cys to release the dithiol Prx and the disulfide Trx. The sequence of reactions has been studied in several systems,[221,222] and the intermediate disulfide has been trapped using a mutant Trx lacking its *C*-terminal Cys. However, the structural characterization is incomplete and based solely in low resolution electron microscopy and structural modeling.[223−225] It seems that the reduction step of 2-Cys Prx can be accomplished by any Trx, independently of the species, thus the standard Prx activity assays usually employ the systems of Trx coupled to TR from *Escherichia coli*[226] or *Saccharomyces cerevisiae*.[227]

The kinetics of reduction of 2-Cys Prx are also understudied, only a few systems of partner Prx and Trx have been thoroughly studied, most notably the reduction of Prx5 by Trx2, both human mitochondrial enzymes,[126] the reduction of TPx by Trx1 from *E. coli*,[228,229] and the reduction of PrxQ B by TrxC from *Mycobacterium tuberculosis*.[230] The reductions of tryparedoxin peroxidase by tryparedoxin (a Trx-like protein) from trypanosomatids,[175] and of AhpC by AhpF from *Salmonella typhimurium*, a flavo-enzyme present in several bacteria,[219,231] have been also characterized.

4.1.1.4. Considerations on the Catalytic Cycle. From the standpoint of kinetics, there is one striking characteristic of the catalytic cycle of 2-Cys and is the combination of the apparently very fast step of oxidation with the rather slow step of resolution. The presence of the slow step is clearly seen through the turnover number, a quick view of Brenda-enzymes.org shows that k_{cat} is rather small for Prxs, with most of the values falling below 100 s^{-1} and several below 1 s^{-1}. Compare these values with that of catalase 5.5 × 10^5 s^{-1}.[232]

It is apparent that while Prx are extremely fast in the stoichiometric reduction of hydroperoxides (high k^{app}_{ROOH}, Table 2), they slow down when the complete catalytic cycle needs to be involved. This would not make sense if the sole function of Prx were the fast reduction of H_2O_2 because resolution becomes rate limiting at low H_2O_2 concentrations,[170] but it is a potential indicator that reveals whenever H_2O_2 concentration reaches a threshold. Naturally, a rate-limiting step causes the accumulation of the reactants for that reaction. In the case of resolution, it would be the C_P−SOH, which is a potentially very reactive species toward condensation with thiols. Of course, not every thiol would react with it, but given the right molecular recognition 2-Cys Prx can form heterodisulfides with specific targets, constituting a signal of "high H_2O_2". Most interestingly, we have recently seen[170] that this signaling can happen in a stepwise manner in compartments with more than one Prx differing in k_{res}, thus allowing such compartment to provide different responses to increasing fluxes of H_2O_2.

A point that has attracted the attention of many studies is the question of oligomerization and its potential effect on the peroxidase and signaling activity of Prx. The subject is vast and still not settled and exceeds the scope of this review. We would only like to propose a few experiments that need to be done in

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

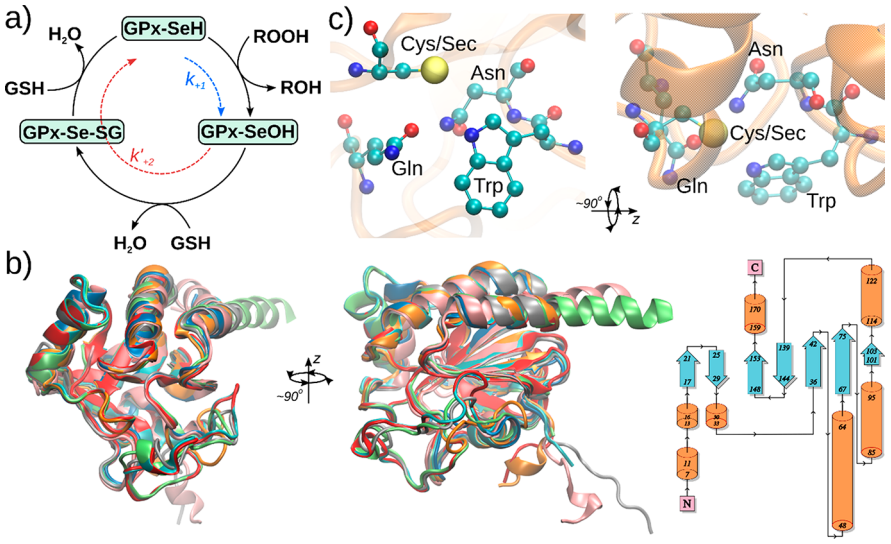

Figure 6. Catalytic cycle and structural features of GPxs. (a) GPxs "canonical" catalytic cycle scheme. Note that k_{+1} correspond to the bimolecular rate constant, whereas k'_{+2} correspond to an apparent rate constant that involves a series of forward and reverse processes. Adapted with permission from ref 239. Copyright 2013 Elsevier. (b) Structure alignment of several human GPx: GPx1, cyan (PDB 2F8A); GPx2, red (PDB 2HE3); GPx3, green (PDB 2R37); GPx4, orange (PDB 2OBI); GPx5, gray (PDB 2I3Y); GPx7, blue (PDB 2P31); GPx8, pink (PDB 3CYN). A topological diagram of GPx4 as a representative family member is also shown. (c) Catalytic tetrad of GPx: besides the reactive Cys/Sec residue, the conserved critical Gln, Trp, and Asn residues are highlighted.

order to better understand the dynamics of oligomerization and its effects on the reactions (if any). Oligomerization experiments need to be done in systems where the concentration is constant, methods such as size exclusion chromatography or ultracentrifugation may distort the results. Because electrostatic interactions are very important, the experimental approach has to emphasize strict conditions of pH and ionic strength comparable with those the protein would encounter in vivo. Finally, tagging, introducing mutations or otherwise modifying the protein structure may have effects both on the quaternary structure and the activity and such effects may well be independent from each other.

4.2. Glutathione Peroxidases

GPxs comprise members of a thiol/selenol peroxidases family that are phylogenetically related (EC1.11.1.9). Gpx1 was the first enzyme described as a selenoprotein at Flohé's[4] and Hoekstra's[233] laboratories. The discovery started an essential field in biology, redox selenium-biochemistry was born. Through the last decades, different mechanisms of selenium uptake and metabolism were characterized, together with the description of the complex machinery that makes Se insertion in proteins possible (Sec). Furthermore, 25 selenoproteins have been predicted to be present in humans,[234] so it is a very prolific research field nowadays.[235−238] The chemical implications of the selenol presence in Sec versus thiol in Cys are discussed in the next section.

Mammalian GPx1−4 are selenoproteins with a Sec in the catalytic center, GPx6 is also a selenoprotein in humans but not in rats or mice,[234] however, GPx5, GPx7, and GPx8 contain a Cys residue instead of Sec and are so-called CysGPx. Indeed, hundreds of CysGPx-homologous sequences were identified over all domains of life, and selenoproteins GPxs are not the most abundant,[239] nor the most ancient.[240] Furthermore, most CysGPxs rely on redoxins like Trxs as reductants instead of GSH,[241] thus the historical term *glutathione peroxidase* is strictly correct only for a small subgroup of the GPx family.[242] From an evolutionary perspective, the GPx family comprises three phylogenetically related clusters that share a Cys-containing ancestor: GPx1/GPx2, GPx3/GPx5/GPx6, and GPx4/GPx7/GPx8, in which GPx7 and GPx8 (CysGPxs) evolved from a GPx4-like ancestor.[12,240,241]

Structurally, they belong to the Trx superfamily, therefore, as Prxs, they present the basic Trx scaffold with a few differential structural motifs as shown in Figure 6b. Moreover, an analysis of the available structures of GPxs, indicates that the general fold is very conserved through the entire family, and structural variability is limited to two regions, the oligomerization loop and the functional helix, which are implicated in the oligomerization state of the enzymes and their specificity regarding the reducing substrate.[240,242,243] The quaternary structure of GPxs is another important feature of this protein family, GPx1, GPx2, GPx3, GPx5, and GPx6, are homotetrameric, whereas GPx4, GPx7, and GPx8 are monomeric.[240]

Although in principle, every GPx appears to have an antioxidant role as its main function, they present a quite complex cell localization and tissue-specific expression patterns, particularly in mammals. GPx1 is ubiquitously expressed and is present both in the cytosol and mitochondria,[244] GPx2 is expressed preferably in the intestinal epithelium,[245] GPx3 in plasma,[246] GPx5 is secreted in the epididymis,[247] and GPx6 in the olfactory epithelium,[248] whereas GPx7 is located in the lumen of the endoplasmic reticulum of a wide variety of tissues,[249] each of these GPxs being soluble enzymes. Nevertheless, GPx4 and GPx8 are membrane or membrane-associated proteins.[249] Of particular interest is the expression and translation patterns of GPx4, which exists in three different isoforms, a cytosolic (cGPx4), a mitochondrial (mGPx4), and a sperm nuclear GPx4 (snGPx4). While cGPx4 is ubiquitously expressed in cells, mGPx4 and snGPx4 are mainly expressed in testis, and it has been related with male fertility, apoptosis, and ferroptosis.[250,251] A controversial feature in GPx field is the role of GPx1 against peroxynitrite-mediated cytotoxicity. Different experiments showed that the hepatocytes of GPx1 knockout mice are more resistant to peroxynitrite-mediated injury than

DOI: 10.1021/acs.chemrev.9b00371
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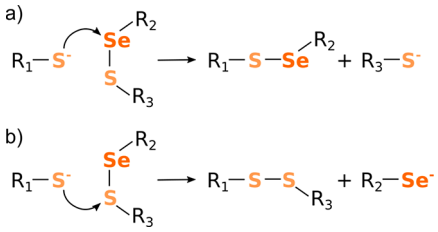
wild-type mice,[252−255] most probably because the enzyme could interfere with signaling actions of peroxynitrite, as also described for *lmw* selenium compounds.[256] The role of GPxs in plants is another exciting and active topic that has recently comprehensively reviewed.[257]

4.2.1. Cysteine versus Selenocysteine in Peroxidases. Having a selenocysteine in the active site of an enzyme is more energy demanding than having a Cys residue, as it requires the participation of several specialized biomolecules determining that a small group of specific UGA stop-codons can be recognized by the specific tRNA ($tRNA^{[Ser/Sec]}$), allowing Sec incorporation.[258−262] The presence of Sec in a small number of enzymes (usually in redox reactions) suggests an inherent advantage of Sec over Cys.[238,263−265]

Several hypotheses have been put forward, many of them based on the chemical differences between selenium and sulfur. Being Se larger than S, it can better stabilize a negative charge as selenolate which produces a lower pK_a of Sec compared to Cys as free amino acids (5.2 vs 8.3).[266] RSe^- are also more polarizable and less solvated than RS^-, both properties making it a better nucleophile in water solution. In a comparison pertinent to this review, selenocysteamine reacts with H_2O_2 with a rate constant nearly 3 orders of magnitude larger than cysteamine (970 vs 1.1 M^{-1} s^{-1}).[267,268] However, as we will discuss below, extrapolation of reactivity of *lmw* compounds in solution yields poor results in trying to predict the chemical behavior of active site residues.

Beyond the comparison of acidity and nucleophilicity, Sec has also been proposed as a better electrophile and a better leaving group when making part of a selenosulfide bond attacked by a nucleophile. These properties are somehow opposite if said selenosulfide is an oxidized intermediate in the catalytic cycle of a peroxidase. If Sec is a better electrophile, it will be attacked preferentially by the nucleophile (e.g., thiolate), yielding a different selenosulfide and a leaving thiolate (Scheme 1a). On

Scheme 1. Two Modes of Thiolate Attack on a Selenosulfide



the other hand, if Sec is a better leaving group, the thiolate will attack the sulfur in the selenosulfide yielding a disulfide and a leaving selenolate (Scheme 1b), this is the favored reaction in selenium GPx.[239]

Another difference that may provide advantages to Sec vs Cys-dependent peroxidases is the relative difficulty of forming higher oxidation states of selenium (seleninate, $RSeO_2^-$, and selenonate, $RSeO_3^-$) compared with their sulfur counterparts and the possibility of seleninate being reduced non enzymatically by *lmw* thiols.[251,269] These hyperoxidation reactions can inactivate Prxs and have been proposed to play an important role in a model of signaling, see section 4.5.

Finally, other "enhanced" properties of Se vs S, such as the ability of forming additional covalent bonds (hypervalency), the tendency of forming weaker π-bonds, and the relative difficulty of one-electron oxidation,[238,263] seem further removed from the scope of this review.

Artificial mutants in which Sec has been substituted by Cys show a significant decrease in activity that has been observed in thioredoxin reductase (TrxR)[270] and formate dehydrogenase.[271] The Sec to Cys mutant of pig GPx4 show a marked decrease in rate constants of about 2 orders of magnitude in k_{+1} and nearly 5000-fold in k'_{+2}.[272,273] It is not surprising that active site mutants are less active than their *wt* counterparts; what might be more interesting would be comparing natural variants that use one or the other amino acid in the active site. In this case, TrxR provides an example of a Cys variant (*Drosophila melanogaster* TrxR) that is as fast as a Sec variant (human TrxR) using diverse Trxs as substrates.[274] In this case, the flanking residues in the C-terminal XCC(U)X sequence play a predominant role in modulating the different reactivities.[274] A very interesting and closer comparison would be between mammal GPx6 that is a selenoenzyme in a number of species (human, cow, dog, horse, ferret) but naturally uses cysteine in mice and rats.

A remarkable phenomenon observed in GPxs is that the nonselenium enzymes are actually Trx peroxidases with GSH being a very poor reducing substrate. This was originally observed in *Plasmodium falciparum*[275] and later in *Drosophila melanogaster*,[276] *Saccharomyces cerevisiae*,[277] and many other species.[242] Besides the evident need of surface changes between the two kinds of GPx to accommodate very different reducing substrates, this evolutionary change could also be read as a difference in the chemical reactivity between Se and S. Namely, for Sec-GPx, no enzyme is needed to reduce the oxidized form, two successive reactions with GSH are sufficient, whereas Cys-GPx need a thiol disulfide oxidoreductase. In other words, selenosulfides are labile enough to be reduced by GSH, whereas disulfides in peroxidases need an enzymatic reducing pathway.

4.2.2. Catalytic Mechanism of Hydroperoxide Reduction by Glutathione Peroxidases. As presented in the previous section, most members of the GPxs family are not strictly "glutathione peroxidases" as their physiological reductant substrate is not GSH but Trx or other redoxin-like enzyme.[242,278−280] These enzymes mimic the reaction sequence of Prxs, and we will not discuss them herein, nonetheless we will focus on the chemical and structural features of hydroperoxide reduction by "canonical" GPxs.

In general terms, the global reaction catalyzed by GSH-dependent GPxs is

$$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O \quad (R8)$$

in which a hydroperoxide is reduced at the expense of two GSH equivalents, yielding GSSG (see Figure 6a). The observed kinetic pattern, that was first studied for GPx1,[281,282] resulted in a "ping-pong" mechanism with infinite V_{max} and infinite Michaelis constants, as the reaction involves two independent events: the oxidative part in which reduced enzyme is oxidized by a hydroperoxide (ROOH) and the reductive part that comprise reduction of the oxidized enzyme by GSH. It is worth noticing that the characterization of GPx1 catalytic mechanism represented the first experimental evidence of the previously described Dalziel's mechanism IVii, which does not comply with the Michaelis−Menten hypothesis, as "since there are no enzyme−substrate compounds, the initial rate increases indefinitely with increase of substrate concentration".[283] In principle, the latter is true for the oxidative part of the catalytic cycle, as no saturation kinetics is observed for different

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

Table 5. Apparent Oxidation Second-Order Rate Constants of Selected Mammal GPxs by Various Hydroperoxides[a]

GPx	hydroperoxide	k_{+1} (M^{-1} s^{-1})	reductant	k'_{+2} (M^{-1} s^{-1})	ref
human GPx1	H_2O_2[b]	4.1×10^7	GSH	2.3×10^5	296
	t-BHP[b]	4.2×10^6			
bovine GPx1	H_2O_2[c]	1.5×10^7	GSH	4.2×10^5	282
	ONOOH[b]	8.0×10^6			63
human GPx3	H_2O_2[b]	4.0×10^7	GSH	7.9×10^4	296
	t-BHP[b]	2.3×10^6			
human GPx4	PCHP[b]	1.5×10^7	GSH	5.7×10^4	296
porcine GPx4	H_2O_2[d]	3.0×10^6	GSH	$\sim 1 \times 10^5$	272
	t-BHP[d]	1.2×10^6			
	PCHP[d]	1.4×10^7			
mouse GPx7	PCHP[d]	9.5×10^3	GSH	13	297
			*Hs*PDI	3.5×10^3	
human GPx8	H_2O_2	95	*Hs*PDI	ND	249

[a]Adapted and updated from Orian et al.[298] *t*-BHP, *tert*-butyl hydroperoxide; PCHP, phosphatidylcholine hydroperoxide; HsPDI, *Homo sapiens* protein disulfide isomerase. [b]Values reported at pH 7.4. [c]Values reported at pH 6.7. [d]Values reported at pH 7.6. ND: not determined.

hydroperoxides, i.e., the selenolate in the active site reacts very rapidly with the oxidant yielding the corresponding selenenic acid, without the need of the formation of a previous enzyme−substrate complex. This step is very fast, with second-order rate constants (k'_{+1}) in the order of $\sim 10^7$ M^{-1} s^{-1} (Table 5). Furthermore, as this step is not reversible, the apparent rate constants may be interpreted as the microscopic rate constants ($k'_{+1} \cong k_{+1}$, see Figure 6a). However, the reductive part of the cycle is much more complex, comprising two stepwise bindings and chemical reactions with GSH molecules, together with the final release of GSSG and reduced enzyme, meaning that first a selenosulfide is formed between the oxidized GPx and GSH,[284,285] that is then reduced by the second GSH molecule. The series of steps involved in the reductive part are usually encompassed in an apparent reductive rate constant (k'_{+2}, see Figure 6a and Table 5). In summary, the sequential series of chemical reactions and equilibria involved in GPxs catalytic mechanism may be detailed as follows:

$$\text{GPx−SeH} + \text{ROOH} \rightarrow \text{GPx−SeOH} + \text{ROH} \quad \text{(R9)}$$

$$\text{GPx−SeOH} + \text{GSH} \rightleftarrows \text{GPx−SeOH:GSH} \quad \text{(R10)}$$

$$\text{GPx−SeOH:GSH} \rightleftarrows \text{GPx−SeSG} + H_2O \quad \text{(R11)}$$

$$\text{GPx−SeSG} + \text{GSH} \rightleftarrows \text{GPx−SeSG:GSH} \quad \text{(R12)}$$

$$\text{GPx-SeSG:GSH} \rightleftarrows \text{GPx−SeH} + \text{GSSG} \quad \text{(R13)}$$

The molecular determinants that leads to Sec (or Cys) activation toward hydroperoxides in GPxs are quite different than those of Prxs, although the chemical effect is comparable, as the rate constants for the oxidative part of the cycles are in similar ranges (see Tables 2 and 5). The most significant difference between the general aspects of Prxs and GPxs active sites is that there is no net positive charge nearby the Se (or S) atom in GPxs. The catalytic thiol/selenol of GPxs is located at a conserved NVAxxC(U)G motif near the *N*-terminus of the enzyme. In the active site structure, $C_P(U_P)$ is directly interacting with a very conserved Gln and with the Trp and Asn residues of a strictly conserved C-terminal WNF motif (Figure 6c). The current view in the GPxs literature is that these four residues, Sec(Cys), Gln, Trp, and Asn, shape the GPxs active site tetrad, participating in positioning the reactive $C_P(U_P)$ via the strong interaction with the Trp residue, reducing thiol/selenol pK_a through a proton shuttle that involves the carboxamide group of the Asn, and activating the peroxyl-bond by polarizing it through mainly the *hb* interaction with the Gln carboxamide group.[240,286,287] The role and relevance of these residues has been assessed through the impact on the kinetic properties of several mutants and also via computational calculations in different Sec and/or Cys GPxs.[240,243,264,278,287−290]

Although the stoichiometry of the reaction between the selenol and the hydroperoxide imposes the reaction product to be the selenenic acid, this reaction intermediate has been extremely elusive. In the presence of GSH it becomes rapidly reduced, and excess ROOH leads to overoxidized forms such as seleninic acid, as is the case of the first GPx1 X-ray structure.[291] Furthermore, mass spectrometry attempts to identify this intermediate showed a product with a −2 mass compared to the reduced enzyme (instead of the expected +16 mass of the selenenic acid).[285] This is in agreement with the fact that a selenylamide was observed when oxidizing the GPx mimic ebselen, being the selenylamide that is more stable than the selenenic precursor and also, very importantly, being prone to reduction by thiols such as GSH.[292] These facts led to hypothesize that a selenylamide may be an intermediate during GPx catalysis.[280,286] Recently, this was corroborated in a mass spectrometry and QM calculations combined study, using rat GPx4 as a model, where an eight-membered ring (selenylamide) linking the Se and the nitrogen of the peptide bond two amino acids downstream the Sec was identified.[293] There is no report detecting a sulfiredoxin-like activity for GPxs, neither sulfenamide homologue has been identified in any CysGPx observed for SecGPxs, so authors had hypothesized that the selenylamide formation in SecGPxs may protect the selenenic acid of being further modified to overoxidized irreversible forms of Sec such as seleninic or selenonic acids.[286,293]

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

Very recently, another variant of GPx catalytic cycle was suggested on the basis of the X-ray structure determination of *Hs*GPx4.[294] The authors observed that the U_P was in the seleninate form (SeO_2^-) even when tris(2-carboxyethyl)-phosphine was present in the crystallization solution. The same oxidation state was observed several years ago during the determination of GPx1 structure.[295] This led the authors to hypothesize that if cellular reductants may reduce SeO_2^- back to SeO^-, this may be interpreted as a new possible pathway of GPxs catalytic cycle. It is worth noticing that this recent idea needs experimental confirmation, and the authors were cautious discussing it.[294]

4.3. Other Examples of Protein Thiols Prone to Oxidation by Hydroperoxides

In addition to Prxs and GPxs, there are other protein thiols that react with hydroperoxides faster than expected according to Brønsted correlations with their thiol pK_a. Some are also members of the Trx superfamily. Examples are *Saccharomyces cerevisiae* glutaredoxins 1 and 2 or several glutathione transferases which reduce different hydroperoxides very rapidly (rate constants in the 10^6 M^{-1} s^{-1} range).[299−302] More notably, there are proteins structurally unrelated with the Trx superfamily that rapidly reduce hydroperoxides. Among them, a particular case is that of the organic hydroperoxide reductase (Ohr), whose primary function is the reduction of organic hydroperoxides. Ohr belongs to a protein family that also includes the osmotically induced protein (Osm), the Ohr/Osm family, which have a distinctive structural α/β fold as cylindrically shaped homodimers.[303,304] The genes codifying for this protein family were first described in bacteria but later also detected in eukaryotes, particularly in fungi.[305] Although members of the Ohr subfamily are mainly induced by organic hydroperoxides while those of the Osm subfamily by osmotic stress,[306,307] both are active in the reduction of organic hydroperoxides by a two-Cys mechanism, and use lipoyl-dependent proteins as reducing systems.[308] Reduction of peroxynitrite and FA-OOH by *Xylella fastidiosa* Ohr is rapid, with rate constants of ~10^7 and ~10^7−10^8 M^{-1} s^{-1}, respectively, at pH 7.4 and 25 °C.[13] More notably, Ohr protected *Pseudomonas aeruginosa* from FA-OOH- and peroxynitrite-dependent cytotoxicity, oxidants that are involved in host−pathogen interactions.[13] A detailed molecular explanation for the oxidizing substrate specificity of Ohr is still lacking. The preference for FA-OOH was structurally inferred from the fact that *Xf* Ohr cocrystallized with polyethylene glycol bound in the active site, which would reflect the ability of the enzyme to accommodate long chain hydrophobic substrates.[309] Furthermore, docking analysis indicated that FA-OOH would fill a cavity of the enzyme through a large hydrophobic contact area, with the hydroperoxide group in close proximity with the peroxidatic thiolate.[13] The active site of Ohr proteins possess, in addition to the peroxidatic Cys, highly conserved Arg and Glu residues that are important for catalysis.[310] In particular, polar interactions among these three residues are important to stabilize a "closed" conformation of the reduced protein and to activate the thiolate for the reduction of hydroperoxides. Oxidation to the intramolecular disulfide form triggers changes in the interactions of the conserved Glu and in the dynamics of the conserved Arg which favor the "open" conformation which is thought to facilitate reduction.[303,311]

Other proteins with thiols highly reactive toward hydroperoxides include the bacterial transcription factors OxyR and OhrR that control bacterial transcriptional responses to hydrogen peroxide (rate constant of ~10^5 M^{-1} s^{-1})[312,313] and organic hydroperoxides, respectively,[312,314,315] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic pathway enzyme that has been recently proposed to be involved in DNA repair and heme binding and delivery to target proteins, among other functions,[316,317] that reacts with H_2O_2 with rate constant in the 10^2−10^3 M^{-1} s^{-1} range.[318] An important case of protein inactivation due to Cys oxidation by hydroperoxides are the protein tyrosine phosphatases (PTP), enzymes that catalyze the hydrolysis of phosphotyrosine from specific signal-transducing proteins. In particular, the catalytic Cys of human PTP1B react with hydroperoxides and the sulfenic acid converts to a sulfenylamide through the formation of a covalent bond with the peptide nitrogen of a neighboring residue.[319,320] The direct reaction with H_2O_2 is slow (9.1 M^{-1} s^{-1} at pH 7),[321] thus it has remained puzzling how such oxidation can occur. It was demonstrated that PTP1B reacts with HCO_4^- much faster than with H_2O_2, suggesting the former as a possible route for PTP1B oxidation during cell signaling.[322] Indeed, a recent work indicated that HCO_4^- was able to oxidize the phosphatase in the presence of Prx2 and its reductant system in vitro and in an adenocarcinoma cell line.[323]

4.4. Comments on Hydroperoxide Specificities

As a S_N2 reaction, the oxidation of thiolates is expected to be faster for those hydroperoxides with better leaving groups, i.e., those with less basic leaving groups.[127,324,325] Indeed, that was the case for glutathione as well as the single thiol group of bovine serum albumin oxidation that reacted with H_2O_2, HCO_4^-, and ONOOH with pH independent rate constants of ~10^1, ~10^3, and ~10^5 M^{-1} s^{-1}, respectively.[45,49,112,127] However, the protein microenvironment can notably affect the reactivity of cysteine residues.[127,326,327] In the case of peroxidatic cysteine residues of thiol-dependent peroxidases, preferred oxidizing substrates vary and do not follow the above-mentioned trend with the leaving group pK_a.[50,126] Instead, the different families or subfamilies of enzymes have particular specificities.

Starting with the Prx family, the six different subfamilies differ in this respect.[161] The members of the AhpC-Prx1 subfamily (or typical two cysteine Prxs) usually react extremely fast with H_2O_2, with rate constants that are 10^7−10^8 M^{-1} s^{-1}.[168,173] Additionally, the reaction is faster than with peroxynitrous acid (k = 10^6−10^7 M^{-1} s^{-1}), for which AhpC-Prx1 subfamily members show a discrete 10^1−10^2 acceleration relative to free Cys.[127,167,168,173] The reactivity of some members of the subfamily with organic hydroperoxides, including *t*-BHP, CHP, FA-OOH, and amino acid or protein hydroperoxides, have also been reported and are usually lower than with H_2O_2.[328] Furthermore, phospholipid hydroperoxides seem to be very poor substrates and lead to a rapid enzyme inactivation.[84,329] Members of the Prx6 subfamily show a similar kinetic specificity.[209] While human Prx5 is not so reactive with H_2O_2, it rapidly reduces organic hydroperoxides and ONOOH.[126] Special attention deserves the fact that only members of the Prx6 subfamily possess Ca^{2+}-independent phospholipase A2 activity, which allow them to participate in the repair of peroxidized cell membranes.[330] The fast reactivity of Prx5 as well as Tpx subfamily members toward organic hydroperoxides such as *t*-BHP and CHP, ~10^1−10^2 higher than with hydrogen peroxide, have been ascribed to a series of apolar residues surrounding the C_p.[181,189] It should be noted, however, that antioxidant protein AOP from *Plasmodium falciparum*, which also belongs to the Prx5 subfamily, shows similar reactivity toward the different oxidizing substrates, which

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

Table 6. Solvent Parameters and Kinetic Rate Constants for the S_N2 Reaction between Chloride and Methyl Bromide

solvent	dipole moment (Debye)[341]	ε_r	hydrogen bonding	k (M^{-1} s^{-1})[342]	acceleration relative to H_2O
water	1.86	78.36	yes	4.9×10^{-6}	1
methanol	2.88	32.66	yes	6.6×10^{-6}	1.3
dimethylformamide	3.81	36.71	no	0.4	8.2×10^{4}
acetone	2.70	20.56	no	3.2	6.5×10^{5}
gas phase	0	1	no	1.3×10^{10}	2.6×10^{15}

is in the $\sim 10^6-10^7$ M^{-1} s^{-1} range.[180] FA-OOH are the preferred oxidizing substrates for PrxQ subfamily members that also rapidly reduce peroxynitrite but show modest reactivity toward H_2O_2.[182,183] On the basis of the crystal structures of different oxidizing states of PrxQ from *Xanthomonas campestris* and cocrystallized moieties, a model for interactions of the alkyl chains of hydroperoxides and a hydrophobic pocket of the enzyme has been proposed.[331] Finally, AhpE from *Mycobacterium tuberculosis*, the only subfamily member to be kinetically studied to date, is extremely reactive toward different FA-OOH.[50,185] Computational studies in combination with experimental activation parameters determinations indicated that these type of substrates can be anchored close to the enzyme's active site via hydrophobic interactions between the aliphatic side chain of the FA-OOH and a hydrophobic patch at the entrance of the active site.[192]

In the case of GPxs, reported rate constants of H_2O_2 reduction are higher for selenium-dependent enzymes (such as mammalian GPx1, GPx3, GPx4) than for cysteine-dependent ones (such as mammalian GPx7 and GPx8). Furthermore, most GPxs rapidly reduce artificial organic hydroperoxides such as *t*-BHP, and those that have been investigated were able to reduce free FA-OOH. Indeed, both GPx1 and GPx4 inhibit the formation of lipid mediators derived from fatty acid peroxidation that participate in the regulation of different physiological processes.[332] On the contrary, among the eight GPxs subfamilies expressed in mammals, only GPx4, the phospholipid hydroperoxide glutathione peroxidase (PHGPx), efficiently reduces hydroperoxides in fatty acids bound to phospholipids, with rate constants in the 10^7 M^{-1} s^{-1} range. This is a unique property which is probably in the basis of the lethal phenotype of GPx4 knockout mice. Other members of the GPx family with PHGpx activity have been reported in other organisms such as *Drosophila melanogaster* and trypanosomatids.[287,333,334] To note, the latter are Cys-dependent GPxs. The molecular basis of the PHGPx activity of GPx4 and related PHGpx enzymes have been related to the accessibility and electrostatic environment of the peroxidatic (seleno)cysteine residue.[335] Finally, both the Sec-dependent GPx1 and the Cys-dependent Poplar GPx5 reduce peroxynitrite, with rate constants in the $\sim 10^6$ M^{-1} s^{-1} per subunit.[252,336]

The thiol-dependent peroxidase Ohr, in turn, rapidly reduces FA-OOH, with rate constants in the range of 10^7-10^8 M^{-1} s^{-1}, while reactivity toward *t*-BHP and CHP were slower and hydrogen peroxide was still less reactive.[13] Indeed, in silico studies indicated that the FA-OOH docked well into the active site of Ohr and suggested that hydrophobic interactions are the major factors for the binding and orientation of these substrates. The enzyme also reduced peroxynitrite, with a rate constant of 10^7 M^{-1} s^{-1}. Of note, when studying the sensitivities of *Pseudomonas aeruginosa* strains lacking Ohr expression toward different hydroperoxides, a trend that correlated with the reactivity determined in vitro was obtained.[13]

4.5. Limitations of Comparing Low Molecular Weight Thiols and Protein Thiol Reactivity

As presented in the previous sections, the chemical properties of protein Cys compared with *lmw* thiols reveal enormous differences from the much wider ranges of pK_a[337] to the exquisitely specific nucleophilic reactivity that determine specialization of different Cys toward hydroperoxide reduction, disulfide reduction, addition to aldehydes, or peptide hydrolysis, among others. Although some variety is present among *lmw* thiols, as their pK_a can be modulated by nearby charged functional groups and the nucleophilicity of the thiolate is consistently higher for less acidic thiols, the range of reactivities is much narrower than those found in protein Cys.

The most important difference is that the reactivity of *lmw* thiols studied in the biochemical literature is mostly evaluated in aqueous solution. Water is a relatively polar solvent and one of the best at forming hydrogen bonds. Such combination defines that water presents one of the highest dielectric constants (ε_r = 78.36), thus being an excellent solvent for charged and/or polar solutes. In the case of the central reaction of this review (reaction R4), solvation plays a major role in decreasing the nucleophilicity of the thiolate. The comparison of rate constants of S_N2 reactions with a thiolate nucleophile in gas phase vs methanol reveals the 14 orders of magnitude difference imposed by solvation:[338,339]

$$CH_3S^- + CH_3Br \rightarrow CH_3SCH_3 + Br^- \quad (k = 8.4 \times 10^{11}\ M^{-1}\ s^{-1},\ \text{gas phase}) \tag{R14}$$

$$C_4H_9S^- + C_4H_9Br \rightarrow C_4H_9SC_4H_9 + Br^- \quad (k = 7.4 \times 10^{-3}\ M^{-1}\ s^{-1},\ \text{methanol}) \tag{R15}$$

Furthermore, by studying the classic S_N2 reaction of chloride with methyl bromide in different solvents and in gas phase, a number of factors are evident (Table 6). First, water slows down S_N2 reactions, suggesting that there is plenty of opportunity for making the reaction faster by modifying the environment. Second, the polarity of the solvent or its dielectric constant have ambiguous effects. Finally, the ability of the solvent to form hydrogen bonds appears as the crucial factor in slowing down a S_N2 reaction with an anionic nucleophile. It has long been recognized that solvents that impede the interaction of the active center with the reactant through hydrogen bonding can suppress the reactivity of that reactant.[340] Water stabilizes the thiolate group by a number of polar and hydrogen bond interactions resulting in a remarkable decrease in nucleophilicity. Burying a thiolate into a protein environment with less efficient solvation and excluding water molecules will partially destabilize the thiolate, elevating its energy closer to the corresponding transition state. Of course, transition states are also partially destabilized by being partially buried in the protein structure, but they usually present more diffuse charge distribution that is less prone to specific interactions with water molecules. There is

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

a substantial caveat to the previous rationale: moving the S_N2 reaction from an aqueous to a less hydrogen bonded environment will increase the nucleophilicity of the thiolate *toward all neutral electrophiles*. As discussed before, increased reactivity is only one factor and specificity also needs to be addressed in order to obtain a proficient enzyme. In that regard, we must consider that the dynamic protein environment of the nucleophile, transition state, and leaving group, can be substantially altered by relatively minor structural changes that can tune down or up reactivity and stability by establishing transient weak interactions.[102]

The sulfur atoms in the active sites of reduced Prxs are remarkably buried, an analysis of solvent accessible surface area using the GETAREA server[343] of the C_P sulfur of six reduced Prx (one of each subfamily) shows between 80 to >99% of the surface buried relative to free Cys (Table 7). How polar is the

Table 7. Solvent Accessible Surface Area (SASA, Å^2) Calculated for Different Peroxidatic Cys of Different Prxs

thiol	PDB	sulfur SASA (Å^2)[a]
cysteine		69
*Hs*PrxIV (C124)	3TJF	0.7 ± 0.1
*Ap*PrxQ (C49)	2CX4	13 ± 17
*Hs*PrxV (C47)	1HD2	1.6
*Py*PrxVI (C47)	1XCC	0.7 ± 0.5
*Mt*AhpE (C45)	4X0X	0.5 ± 0.9

[a]Mean and standard deviation are shown when more than one protein chain is present in the crystal structure.

active site and what is the local dielectric constant in its surroundings are two interesting and difficult questions. The electrostatic potential map around the C_P (Figure 6c) reveals a quite polar environment composed by the ion pair between the thiolate and the conserved arginine, together with contributions from the conserved threonine and the backbone. With dimensions of a few angstroms the dielectric constant concept can be conserved, if it is assumed that in nanoheterogeneous environments it becomes a local function related to the protein structure. There have been efforts in computational chemistry to provide approximations to the dielectric distribution based on the protein structure that show a consistent pattern of keeping a low average ε (<20) up to the radius of gyration, this pattern being most notorious among compact globular proteins.[344] Then again, the average ε is a poor descriptor of the electrostatic properties in the proximity of the active site.

Aside from the presence of two/three water molecules in the vicinity of C_P,[188,191,192] Prxs active sites are not very much solvent exposed (Table 7), being nevertheless a very polar microenvironment. Then, broadly speaking, the C_P is buried in a mostly polar, almost water-free environment where hydrogen bonds can be provided by neighboring residues, with the added advantage that such hydrogen bonds can be subtly altered to enhance or diminish the nucleophilicity of the C_P, stabilize the transition state, and facilitate the protonation of the leaving group by slight conformational changes in the active site.

5. REDOX SIGNALING BY THIOL PEROXIDASES

Hydroperoxides, specially H_2O_2, function as signaling molecules. In bacteria, yeast, plants, and also in mammals, a significant number of protein redox modifications had been demonstrated to be associated with changes in hydroperoxides levels and linked to various physiological processes such as energy metabolism, the response to stress or growth signals, or even related with increased proliferation and malignant transformation, among many others. Also, H_2O_2 is not only a modulator of transcription factors and/or signaling molecules but has been involved in the specific regulation of individual genes.[345,346] Because of their extreme rate in reducing hydroperoxides and their relatively high concentrations, thiol peroxidases are key participants in these redox signaling processes mediated by hydroperoxides. In this section, we discuss the role of thiol peroxidases in redox signaling by

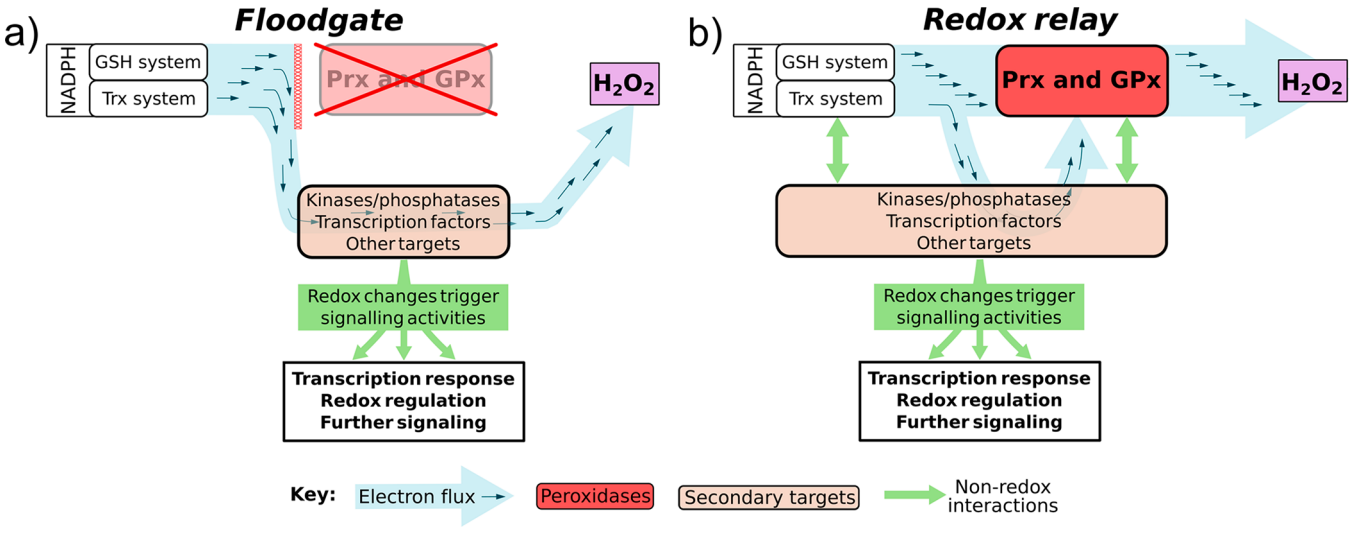


Figure 7. Two models for hydroperoxide (H_2O_2 in particular) signaling involving Prx and GPx. (a) The floodgate model posits that peroxidases are inhibited or inactivated, abolishing their reduction of hydroperoxides and resorting to secondary targets as the route of reduction; as a result, secondary targets become oxidized, act as a redox bypass, and give rise to signals through phosphorylation, transcription control, etc. (b) The redox relay model involves the secondary targets as constitutive redox partners of one or several peroxidases and the direct reduction of H_2O_2 by secondary targets becomes irrelevant. Prx and GPx that act as sensors that relay the signal through specific oxidation of protein thiols to the secondary targets. A further possibility involves the differential recognition of oxidized or reduced forms of the peroxidases (and the Trx system) not involving redox reactions.

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

describing and comparing the *floodgate* and the *redox relay* models.

It is worth pointing out that several lipid hydroperoxides and derived moieties participate in cell signaling and regulate a variety of physiological processes. The role of thiol peroxidases in the reduction of this kind of mediators has been recently thoroughly revised[332] and is beyond the scope of this review.

5.1. The Floodgate Model

"Eukaryotic 2-Cys peroxiredoxins (2-Cys Prxs) not only act as antioxidants, but also appear to regulate hydrogen peroxide–mediated signal transduction."[347] That is the opening line of a very influential article on the signaling mechanisms that involve Prx in which the authors first proposed the "floodgate model". In that model, Prxs act as floodgates that contain the oxidation mediated by H_2O_2 and the key to open such floodgates is the inactivation of Prx through hyperoxidation or other post-translational modification[159,348] (Figure 7a). The model was proposed very early in the study of Prx enzymology, mostly based on the structural comparison between eukaryotic and prokaryotic Prx, that differed (among other things) in their sensitivity to hyperoxidation in vitro. Only two examples of different Prx were used, namely the "robust" bacterial AhpC and the "sensitive" human Prx1. This was quickly (and unfortunately) extrapolated in the literature to "robust prokaryotic" vs "sensitive eukaryotic" Prx, avoiding the nuances and differences within each group and oversimplifying the complexities of structural and kinetic differences.

The kinetic support for the model was scarce at that time. Peroxidation rate constants were not determined until a few years later,[169,170,349] resolution and hyperoxidation rate constants had to wait more than a decade[171,205] and were only estimates in the case of AhpC until very recently.[350,351]

We can summarize the kinetic arguments against the floodgate model as follows:

- Prxs are very abundant and react extremely fast with H_2O_2 because reaction rates depend on both concentration and rate constant, Prx have a 10^5–10^6 fold advantage over other abundant targets (such as GSH or GAPDH) and even larger over less abundant targets such as phosphatases and transcription factors. This means that more than 99.999% of *all* Prx must be inactivated before other protein targets can become competitive. Even in this extreme case, other abundant and unspecific targets, such as GSH may compete for the reduction of H_2O_2.
- Not all fast peroxidases can be inactivated by hyperoxidation. Now we know that the sensitivity toward hyperoxidation in vitro is diverse even among eukaryotic Prx,[205] making some of them bad candidates as floodgates. Additionally, SecGPx and catalase have not been shown to be inactivated by elevated H_2O_2, thus constituting persistent floodgate.
- Hyperoxidation of the classical targets in the floodgate model (Prx1, Prx2, and Prx3) by H_2O_2 is not a very fast reaction, the initially measured rate constants of 1.2×10^4 M^{-1} s^{-1}[205] have been recently determined by more direct means and are in the range of $(1–2) \times 10^3$ M^{-1} s^{-1}.[64,82,171] In any case, GSH and even GAPDH given their high concentrations could prevent the hyperoxidation by scavenging H_2O_2.
- Finally, resolution and hyperoxidation are not the only two possible fates of Prx as sulfenic acids.[352] Glutathionylation is a very important alternative[208] and would be kinetically competitive as seen in Table 4. Also a potential fate is the relaying of oxidation to protein targets which may be a plausible signaling mechanism that simultaneously uses the sulfenic acid as oxidant and protects it from hyperoxidation.

Beyond the kinetic difficulties of the floodgate model, another question remains if the floodgate were eventually to be breached. How H_2O_2 would be directed to its specific signaling targets? The thermodynamics of reaction is very favorable for all thiols (e.g., the $\Delta G^{\circ\prime}$ of oxidation of GSH by H_2O_2 is −73.4 kcal/mol), so given opportunity and enough time *all* thiols would be oxidized, yielding an extremely unspecific "signaling" process. To circumvent this specificity problem, a number of researchers have proposed the idea of having H_2O_2 "locally confined" or "locally formed", but a few considerations can dispel the idea of "local" H_2O_2, for instance, H_2O_2 formed locally in a mammalian cell, in the absence of fast reacting targets, will be practically equilibrated in all the cell volume in a time scale of seconds,[353] even considering molecular crowding. In contrast, the $t_{1/2}$ of the reaction of 10 μM H_2O_2 with a slow target such as PTP1B is more than 2 h. Only membranes and fast reductants can (partially) prevent H_2O_2 from diffusing away from its site of formation. Of course, confinement by fast reductants does not result in accumulation of H_2O_2 but actually in its consumption.

Although the floodgate model predicts that both the overall oxidation of protein thiols and the H_2O_2 mediated signaling should be enhanced by decreases of the peroxidase activities, this is not what has been observed. Knockout cells for Prx1 and Prx2, the two main cytosolic Prx in mammalian cells, show lower overall formation of disulfides trapped by a modified Trx in cells exposed to H_2O_2.[354] The general assertion can also be seen, in particular cases, knockdown Prx1 represses the apoptosis signal-regulating kinase 1 (ASK1) disulfide formation and mitogen-activated protein kinase p38 phosphorylation[355] and Prx2 knockdown downregulates the oxidation of the signal transducer and activator of transcription 3 (STAT3).[145]

Despite all the potential hurdles presented in the previous paragraphs, the floodgate model cannot be simply disregarded. The involvement of hyperoxidation of Prxs of the Prx1 subfamily in signaling has been documented in numerous studies,[356] recently reviewed.[357] Perhaps a more nuanced approach with closer attention to quantitative factors can incorporate the possible gain of function of hyperoxidized Prxs, their potential as a recognizable signaling pattern, and the concomitant small increase in H_2O_2 levels into a novel model based in the floodgate, without the limitations imposed by the need to obliterate all peroxidase activity.

5.2. The Redox-Relay Model

This model of signal transduction proposes that the oxidized forms of a thiol peroxidase (sulfenic acid and disulfide) can oxidize secondary targets beyond their usual Trx substrates and, if such secondary targets have a role in transmitting a signal via post-translational modifications or transcription response, the signal can be switched on or off through the redox state of the secondary target (Figure 7b). The reaction sequence would involve the Prx C_P-SOH or disulfide reacting with a thiol in the target protein forming a transient intermolecular disulfide that leads to the oxidized target. The model was proposed very early based on the seminal study of the heterodisulfide formed between Orp1 (a Trx peroxidase of the GPx family, also known as GPx3) and the transcription factor Yap1 in *Saccharomyces cerevisiae*.[358] Also, in *S. cerevisiae*, it was found that all eight thiol

DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829–10855

peroxidases were essential for the activation and/or repression of genes in response to H_2O_2 but not by other oxidants.[359]

Although the model was proposed back in 2002, the name "redox relay" appeared a few years later[360] along with at least two more such as "facilitated oxidation" model[142] and "signal peroxidase" model.[355] Evidence for the redox relay involving individual Prx appeared in the H_2O_2-dependent activation of the apoptosis signaling kinase (ASK1) involving Prx1,[355] the activation of STAT3,[145] and the oxidation of protein deglycase DJ-1,[361] both involving Prx2 and perhaps Prx5,[362] and recently a large collection of putative targets identified through trapping with Prx1 and Prx2.[354]

So far, all identified intermolecular disulfides seem to arise from reactions of the C_P-SOH, which poses an interesting kinetic problem, we saw in section 4.1.1.2 that the slow resolution in some Prx may propitiate the reaction with GSH or other thiols but that the reactions needed to have a large rate constant and/or a high target concentration based in our simple calculations or in elaborate mathematical models.[207] One way to circumvent such restriction could be the existence of scaffolding proteins that could form ternary complexes with Prx and the target protein, thus making the reaction between C_PSOH, and the target thiol an essentially unimolecular reaction.[145] Again, this has been observed in the *S. cerevisiae* system of Orp1 and Yap1, in which the reaction is facilitated by the protein Ybp1 that acts as a scaffold.[363]

We have proposed that the differentially slow resolution of diverse Prx coexisting in the same compartment would provide a differential dynamic range of response to H_2O_2 levels,[170] whenever scaffolding proteins are involved, such dynamic range could be further modulated to lower or higher H_2O_2 concentrations, according to the state of the compartment at the moment. Additionally, diverse scaffolds and diverse Prx could provide specific signals responding to different types of stimuli. For instance, a sudden surge of ONOOH would be sensed by the most reactive Prx5 and the response would be mediated by Prx5-specific scaffolds and secondary targets, on the other hand, Prx2 would be most suited to sense variation in very low H_2O_2 concentrations and respond accordingly. There is also the possibility that a scaffold or selective mechanism could facilitate directed oxidation of secondary targets without the need of a peroxidase acting as a relay intermediate. As a matter of fact, the first evidence of such a mechanism may appear: the rates of PTP1B oxidation and Prxs hyperoxidation increase by the presence of physiological concentrations of CO_2/bicarbonate, redirecting H_2O_2 from Prxs to the phosphatase.[323,51]

Therefore, the redox relay model may explain a multifaceted system able to respond to the identity, flux, and persistence of hydroperoxides through the catalytic characteristics of the diverse Prx and subject to the needs and status of the specific compartment that would provide scaffolding proteins and secondary targets accordingly.

6. CONCLUSIONS AND PERSPECTIVES

It is interesting to note how at the early times of GPx biochemistry, its relative contribution to peroxide catabolism in mammalian cells and tissues as the only thiol-based peroxidatic enzyme was solely confronted with the already known catalase, a heme peroxidase.[364,365] The irruption of Prxs as ubiquitous and abundant thiol-based peroxidases has completely reshaped our view and understanding of hydroperoxide biology and chemistry and made more complex and diverse the enzyme peroxidatic network. The biological significance of the sometimes overlapping activities of Prxs and GPxs require further investigation.

As presented herein, several aspects of thiol-based peroxidases biochemistry contribute to their biological relevance, including human health and disease. From their significant role as pathogen virulence factors[58] to the involvement of Prxs and/or GPxs in the development of different pathologies,[157] just to name a few examples. Indeed, transcriptional modulation of their expression is related with the status and progression of different diseases and amenable of pharmacological intervention,[366] thus converting these enzymes in prospective therapeutic targets.

To accomplish their functions, thiol-based peroxidases developed unique microenvironments at their active sites to specifically enhance reactivity with hydroperoxides. In addition, they evolved flexible and plastic enough to respond to different redox states, post-translational modifications such as phosphorylation and other stimuli, changing their oligomeric state, interacting with secondary targets, and being part of larger molecular complexes. These properties convert these enzymes in intriguing and challenging systems, as very important questions remain open in the field related to hydroperoxide metabolism, signaling and detoxification.

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DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855

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ACKNOWLEDGMENTS

A.Z. is supported by a posdoctoral fellowship from Agencia Nacional de Investigación e Innovación (ANII), Uruguay. We are grateful for funding from Comisión Sectorial de Investigación Científica (CSIC I+D 2016 to MT, CSIC Grupos 2018 to A.D. and CSIC Grupos 2018 to R.R.) and Espacio Interdisciplinario (Centros_2015 to R.R.), Universidad de la República; Ministerio de Educación y Cultura (Fondo Vaz Ferreira 2018 to A.Z.), Uruguay. This work was also partially supported by the Universidad de Buenos Aires, CONICET, Centro de Biología Estructural Mercosur (CeBEM), Centro de Computación de Alto Rendimiento (CeCAR), and Agencia Nacional de Promoción Científica y Tecnológica de Argentina (PICT 2014-1022) and Programa de Desarrollo de Ciencias Básicas (PEDECIBA), Uruguay.

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DOI: 10.1021/acs.chemrev.9b00371
Chem. Rev. 2019, 119, 10829−10855