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PERSPECTIVE

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Factors Affecting Protein Thiol Reactivity and Specificity in Peroxide Reduction

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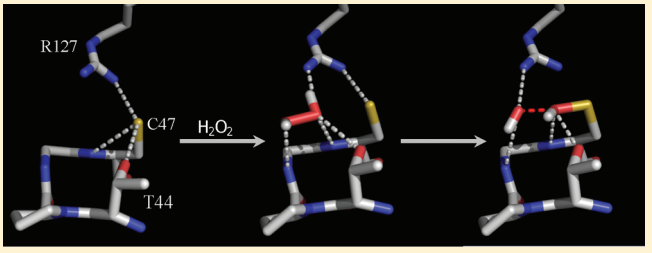
Supporting Information

ABSTRACT: Protein thiol reactivity generally involves the nucleophilic attack of the thiolate on an electrophile. A low pK_a means higher availability of the thiolate at neutral pH but often a lower nucleophilicity. Protein structural factors contribute to increasing the reactivity of the thiol in very specific reactions, but these factors do not provide an indiscriminate augmentation in general reactivity. Notably, reduction of hydroperoxides by the catalytic cysteine of peroxiredoxins can achieve extraordinary reaction rates relative to free cysteine.



The discussion of this catalytic efficiency has centered in the stabilization of the thiolate as a way to increase nucleophilicity. Such stabilization originates from electrostatic and polar interactions of the catalytic cysteine with the protein environment. We propose that the set of interactions is better described as a means of stabilizing the anionic transition state of the reaction. The enhanced acidity of the critical cysteine is concurrent but not the cause of catalytic efficiency. Protein stabilization of the transition state is achieved by (a) a relatively static charge distribution around the cysteine that includes a conserved arginine and the N-terminus of an α-helix providing a cationic environment that stabilizes the reacting thiolate, the transition state, and also the anionic leaving group; (b) a dynamic set of polar interactions that stabilize the thiolate in the resting enzyme and contribute to restraining its reactivity in the absence of substrate; but upon peroxide binding these active/binding site groups switch interactions from thiolate to peroxide oxygens, simultaneously increasing the nucleophilicity of the attacking sulfur and facilitating the correct positioning of the substrate. The switching of polar interaction provides further acceleration and, importantly, confers specificity to the thiol reactivity. The extraordinary thiol reactivity and specificity toward H_2O_2 combined with their ubiquity and abundance place peroxiredoxins, along with glutathione peroxidases, as obligate hydroperoxide cellular sensors.

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INTRODUCTION

Hydrogen peroxide reacts with free cysteine with a rate constant of 26 M^{-1} s^{-1},[1] whereas the same reaction with Cys51 of human peroxiredoxin 2 (Prx2) occurs nearly 4 million times faster ($k = 1 \times 10^8$ M^{-1} s^{-1},[2]) and close to the diffusion-control limit (unless otherwise indicated, pH-independent rate constants are used throughout this perspective for the comparison of reactivities). Is C51 of Prx2 a highly reactive thiol? Other oxidants and thiol-specific reagents seem to argue against that since C51 of human Prx2 is a thiol of ordinary reactivity for almost every reagent except peroxides.[3,4]

Given the importance of protein cysteine residues in redox biochemistry and their key role in diverse physiopathological processes where oxidative stress is involved, it is important to understand the factors affecting protein thiol reactivities. Thus, with focus on peroxiredoxins and particularly human Prx2 and Prx5, we attempt to explain how factors dependent on thiol chemistry, protein structure, reaction mechanism, and substrate recognition contribute to some exceptional thiol reactivity while preserving the selectivity of the enzymatic system (throughout this perspective, the term reactivity is used in its kinetic sense, generally described by rate constants). We will also discuss additional extra chemical factors determining which thiols may play a role in reactions with oxidants, with implications in antioxidant protection and oxidant-dependent signaling.

EXPECTED THIOL REACTIVITY IN LOW-MOLECULAR WEIGHT COMPOUNDS

More Acidic Thiols Are Less Reactive. Protein thiol reactivity is a complex subject, as it involves a large diversity of functions such as catalysis, structural or metal-chelating, aside from thiols with no known function.[5] The variety of reactions is so wide that it should be clear that trying to define general protein−thiol reactivity is pointless and that a reaction-specific approach would be much more constructive. Even so, two facts underlie the reactions of thiols in biochemistry:

- most reactions involve the nucleophilic attack of the thiolate on an electrophile, and
- most thiols have pK_as within two units of neutral or "physiological" pH.

Having a pK_a not far from 7 implies that when studying a thiol reaction we must bear in mind that only a fraction is available as thiolate, and thus, only part of the reactivity is detectable. Comparing apparent rate constants of different low-molecular weight thiols obtained at the same pH is common in the literature[6−9] but may lead to the erroneous conclusion that more acidic thiols are more reactive in general.[10]

We should be able to discern the fact of having a series of apparent rate constants increasing with decreasing thiol pK_a from the possibility of extrapolating the trend to more acidic thiols without a supporting mechanism. In fact, one drawback of using low-molecular weight thiols as models of protein-cysteine reactivity is the absence of low-molecular weight *aliphatic* thiols having pK_a < 6.5 and that *aromatic* thiols are very poor models since they pertain to a different trend in reactivity.[11] To propose a mechanism of reactivity considering that the thiolate is the reactive species, the fraction of available thiolate should be accounted for in order to compare reactivities. Thus, pH-independent rate constants (k_{RS^-}), corresponding to the general reaction (eq 1) are better descriptors of reactivity trends regardless of the experimental conditions employed to obtain the rate constants.

$$RS^- + \text{Target} \rightarrow \text{Products} \quad (1)$$

The numeric value of k_{RS^-} is obtained by dividing the apparent rate constant at a given pH (k_{app}^{pH}) by the fraction of available thiolate at the same pH as follows:

$$k_{RS^-} = k_{app}^{pH}\left(\frac{K_a^{RSH} + [H^+]}{K_a^{RSH}}\right) \quad (2)$$

where K_a^{RSH} is the ionization constant of the thiol. Better yet, k_{RS^-} can be extrapolated by determining k_{app}^{pH} experimentally in a pH range around the pK_a value and fitting the results to eq 2.

Using data from the literature,[7−9] we have prepared the plots of Figure 1A−B to illustrate this point. It can be seen that for low-molecular weight thiols, a higher pK_a usually implies a higher reactivity; i.e., on a molecule-per-molecule basis, more basic thiolates are better nucleophiles. The exception in Figure 1C corresponds to the reduction of hydrogen peroxide, for which there is no change in reactivity with K_a^{RSH}. Additionally, there is an empirical linear relationship between nucleophilicity (k_{RS^-}) and basicity (pK_a), usually represented by a Brønsted relationship having the form of eq 3.

$$\log k_{RS^-} = \beta_{Nuc} pK_a^{RSH} + C \quad (3)$$

The nucleophilicity constants (β_{Nuc}) are the slopes of the plots in Figures 1A−C, for the reactions of thiolates with taurine chloramine, peroxynitrous acid, and hydrogen peroxide, respectively.

These Brønsted relationships are common in the literature for diverse reactions involving thiols as nucleophiles,[12−17] with β_{Nuc} values in the range of 0 to 1, meaning that k_{RS^-} remains constant or increases with thiolate basicity, but never decreases. We will get back to the significance of β_{Nuc} when dealing with the factors depending on the reaction mechanism. In order to predict the apparent rate constants for more acidic thiols, we can combine eqs 2 and 3 into eq 4 as previously reported.[18]

$$\log k_{app}^{pH} = pK_a^{RSH}(\beta_{Nuc} - 1) + C - \log([H^+] + K_a^{RSH}) \quad (4)$$

Choosing a pH value, one can extrapolate k_{app}^{pH} for hypothetical thiols of any desired pK_a. Such calculations yield the continuous lines of Figure 2.

The downward trend is evident for thiols with pK_a < 7.4 in Figure 2A and B as the pH-dependent fraction of available thiolate tends to one, and the decreasing trend of the Brønsted relationship prevails. This tendency has been previously noticed for thiol−disulfide exchange reactions,[19] but seldom considered

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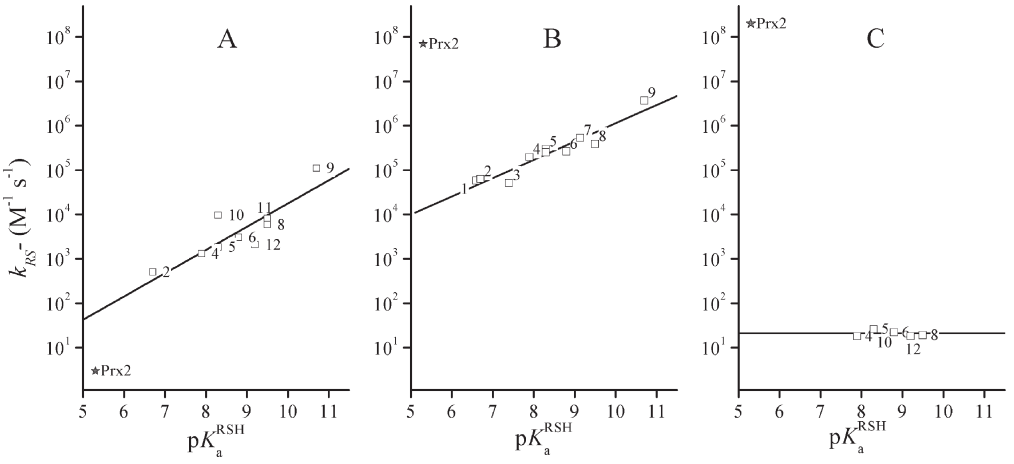

Figure 1. pH-independent rate constants for taurine chloramine reduction (A), peroxynitrous acid reduction (B), and H_2O_2 reduction (C) vs thiol pK_a. Data obtained from refs 1−4, 7, 8, 177, 178. Key: (1) cysteine ethyl ester; (2) cysteine methyl ester; (3) trypanothione; (4) penicillamine; (5) cysteine; (6) glutathione; (7) mercaptoethylguanidine; (8) *N*-acetylcysteine; (9) dihydrolipoic acid; (10) cysteamine; (11) 2-mercaptoethanol; (12) dithiothreitol. Prx2 is human peroxiredoxin 2 and is not considered for the linear regressions. Linear fits are: $\log k_{RS^-} = 0.52\ pK_a^{RSH} - 0.99$ for taurine chloramine reduction; $\log k_{RS^-} = 0.41\ pK_a^{RSH} + 1.92$ for peroxynitrous acid reduction; $\log k_{RS^-} = 0\ pK_a^{RSH} + 1.33$ for H_2O_2 reduction.

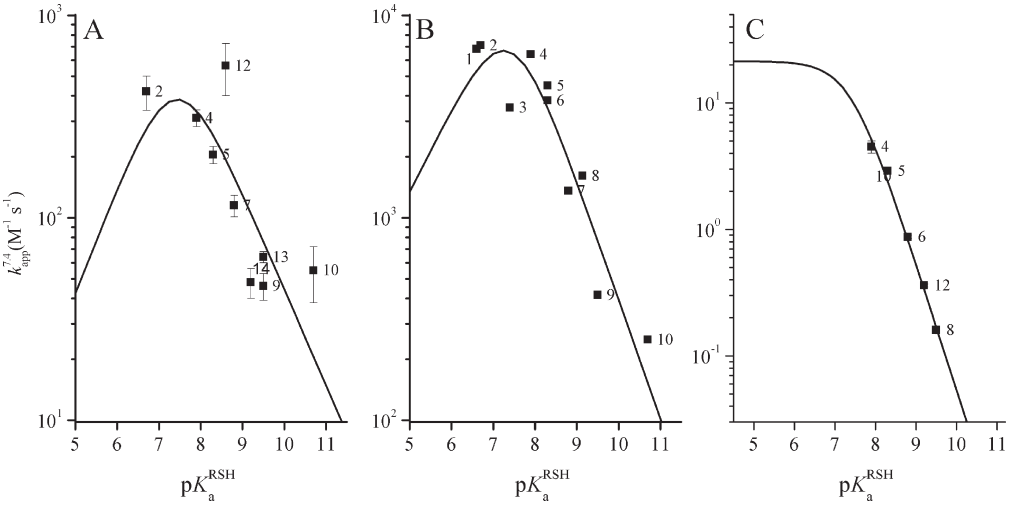

Figure 2. Apparent rate constants at pH 7.4 for taurine chloramine reduction (A), peroxynitrous acid reduction (B), and H_2O_2 reduction (C) vs thiol pK_a. Data obtained from refs 1, 7, 8, 177, 178. Codes for thiols are the same as for Figure 1. The continuous lines represent the calculated $k_{app}^{7.4}$ values for hypothetical thiols having pK_as in the range 5−11 using the corresponding linear regression of Figure 1 and eq 4. Note that for the $k_{app}^{7.4}$ in panel B, the result of eq 4 is multiplied by 0.137, i.e., the available fraction of ONOOH at pH 7.4 (pK_a = 6.6).

in the discussion of reaction mechanisms of thiol biochemistry. The fact that only a few commercially available thiols have pK_as below 7.4 has contributed to conceal this trend, but the downward trend is more evident if the k_{app}^{pH} values are measured at higher pH values (see Figure S1, Supporting information).

Despite the fact that low-molecular weight acidic thiols are generally less nucleophilic on a molecule-per-molecule basis, acidic protein thiols are the rule in the active site of many enzymes. Thioredoxins, glutaredoxins, peroxiredoxins, peptidases, phosphatases, and disulfide isomerases all have critical cysteine residues with pK_as well below that of free cysteine (Table 1).

Thiolate Availability Is Not Sufficient to Explain Reactivity in Protein Thiols. Why have some thiols with theoretically decreased reactivity been naturally selected as specialized residues of catalytic relevance? Availability of the nucleophile appears as the obvious answer and is often mentioned in the literature; thiols with low pK_a are mostly deprotonated and thus available for nucleophilic attack at neutral pH, providing a kinetic advantage.

To what extent is the apparent reactivity of an acidic thiol augmented with respect to free cysteine in a nonspecific reaction? Calculations using the Henderson−Hasselbalch equation show that this availability factor is rather small and would provide an acceleration of less than 1 order of magnitude for reactions occurring at pH 7.4 (Table 1). Therefore, availability of the thiolate is not the main argument in cases where accelerations of several orders of magnitude are observed. Moreover, as we saw in Figure 2, for many reactions, enhanced acidity implies a penalty in reactivity, which diminishes even further the kinetic advantage of availability. At least, that is the case for low-molecular weight thiolates. Many protein thiols depart from the trends of low

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Table 1. Thiolate Availability for Free Cysteine and Selected Proteins at pH 7.4

thiol	pK_a	ionized fraction at pH 7.4	thiolate availability relative to cysteine
cysteine	8.3	0.1118	1
glutaredoxin 1[165]	3.2	0.9999	8.94
papain[11]	3.4	0.9999	8.94
DsbA[31]	<4	>0.9996	8.94
protein disulfide isomerase[166]	4.2	0.9900	8.93
protein tyrosine phosphatase 1B[21]	4.7	0.9981	8.93
peroxiredoxin 5[20]	5.1	0.9950	8.90
peroxiredoxin 2[a]	5.3	0.9921	8.87
DJ-1[167]	5.4	0.9901	8.85
creatine kinase[47]	5.7	0.9804	8.77
thioredoxin[36]	6.7	0.8337	7.46

[a] Denicola, A., unpublished results.

molecular weight thiols as previously reported[20] and as illustrated for peroxiredoxin 2 in Figure 1B and C.

■ ACIDITY AND NUCLEOPHILICITY OF PROTEIN THIOLS CAN HAVE EXTRAORDINARY VALUES

The acidity of low-molecular weight thiols in aqueous solution is solely dictated by the inductive and electrostatic effects of functional groups a few bonds away from the sulfur atom. In proteins, other effects come into play, such as diffuse electric fields arising from charged amino acids and dipolar secondary-structure features, solvent exclusion, and specific hydrogen bond networks.

Some protein thiols are conspicuously acidic, as can be seen in Table 1, but somehow their reactivity toward specific targets is not diminished as expected from plots like those in Figure 2. Some of the effects of protein environment seem to diminish pK_a without decreasing nucleophilicity. For instance, it has been shown that a considerable decrease in pK_a (4.6 units) can be achieved by charge−charge interactions that act through space at the active site of papain without diminishing its nucleophilic reactivity.[11] Another study on protein tyrosine phosphatase shows that the mutation of a neighboring histidine alters both the pK_a of the catalytic cysteine and its nucleophilicity, but there is no single trend in the changes, suggesting that the problem is more complex than a simple Brønsted relationship.[21]

In a recent article on thiol-dependent glutathione peroxidases,[22] the authors present the case of an active-site mutant that increases the calculated cysteine pK_a in 2.8 units (from 7.2 to 10), which they use to explain why the apparent rate constant at pH 7.4 decreases by ca. 3 orders of magnitude (1.3×10^6 to 4.0×10^3 M^{-1} s^{-1}). What they failed to mention is that the k_{RS^-} is about the same for both variants of the protein (1.6×10^7 vs 1.9×10^7 M^{-1} s^{-1}, calculated by us on the basis of their data), implying that the decrease in pK_a is achieved without sacrificing nucleophilicity.

Finally, an interesting case is illustrated by cytosolic glutathione *S*-transferases (GST), a family of detoxication enzymes that catalyze the conjugation of glutathione with electrophiles. One of the main features of GST is that the observed pK_a of the glutathione thiol decreases upon binding to the enzyme, and this has been proposed as part of the substrate activation.[23] The use of mutant GST has enabled the modulation of glutathione pK_a along with the measurement of its reactivity. The results indicated that β_{Nuc} are either small (≤ 0.25[24]) compared to low-molecular weight thiols, or even negative,[25] indicating that protein structure and binding can modulate the acidity of a thiol independently of its nucleophilicity.

Protein Structural Factors That Influence Acidity. Protein structure can influence thiol acidity in a number of ways. The most evident effects originate from charge and dipole distribution around the cysteine residue. Ion-pairing is the usual way of producing particularly acidic thiols, thus histidinium−thiolate ion pairs produce low pK_as in papain and other thiol peptidases,[11,26,27] glyceraldehyde-3-phosphate dehydrogenase,[28] yeast pyruvate decarboxylase,[29] protein tyrosine phosphatases,[21,30] and DsbA;[31] guanidinium groups from arginine are partially responsible for the low pK_as of peroxiredoxins,[32,33] while ammonium from lysine plays a role in the acidity of glutaredoxin,[34] α1-antitrypsin,[35] and thioredoxin[36] thiols. On the other end, anionic residues in the vicinity cause thiols to behave as weaker acids; such is the case of C461 of cDsbD (pK_a = 10.5),[37] of C406 in arginine deiminase (pK_a = 9.6),[38] and of C14 of glutaredoxin 3 (pK_a > 10.5).[39]

In addition to charged residues, the local electric field is complemented by α-helix dipoles;[40] thus, most *N*-terminal cysteines in proteins having a thioredoxin fold have increased acidities in part due to the α-helix immediately following the redox motifs.[41−44]

Weak interactions with uncharged residues also play a role in the stabilization of protein thiolates; description of hydrogen bond (or ion−dipole interactions) networks supporting thiolates are numerous for the catalytic cysteines of many thiol enzymes.[39,45−47] These ion−dipole interactions act by differentially stabilizing the thiolate because the protonated thiol is a very poor hydrogen bond acceptor,[48] thus promoting lower pK_as.

Desolvation is another protein factor affecting thiol acidity as water molecules are almost always excluded from the catalytic site of peroxiredoxins. A quick survey of the structures deposited in the Protein Data Bank for reduced and cysteine-to-serine mutants in the peroxidatic cysteine reveals that 20 structures have no water molecules at less than 4.5 Å from the sulfur atom (PDB IDs: 1E2Y, 1HD2, 1N8J, 1NM3, 1URM, 1UUL, 1X0R, 1XIY, 1XXU, 1Y25, 1YEX, 1ZYE, 2A4V, 2H01, 2I81, 2PWJ, 2YZH, 2Z9S, 3A5W, 3HVV)[32,49−63] and that only 6 structures (including three cysteine-to-serine mutants) have at most one water molecule at 4.5 Å or less (PDB IDs: 1TP9, 1XCC, 2PN8, 2V2G, 2WFC, 3DRN).[49−52]

As a result of this desolvation, the apparent dielectric constant should decrease from the value of bulk water (ε = 78.5), causing the enhancement of all electrostatic interactions. Thus, ion-pairing and ion−dipole attractions become stronger, but also the basicity of the thiolate increases, as protonation implies the mutual neutralization of two charged species. In fact, low-molecular weight thiols have pK_as that increase with decreasing dielectric constant. For instance, *n*-butyl mercaptan has a pK_a of 10.9 in water and 17 in DMSO (ε = 49.1).[53]

Protein Factors That Influence Nucleophilicity. According to classical studies, nucleophilicity depends on the following:

(a) Structural and molecular properties of the nucleophile such as its basicity, polarizability, and the presence of nonbonding electrons in the atom adjacent to the

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Table 2. Acidity and Hydrogen Peroxide Reactivity of Several Protein Thiolates

		reaction with H_2O_2	
RSH	pK_a	k_{RS^-} (M^{-1} s^{-1})	k_{RS^-}/k_{Cys}
cysteine[1]	8.3	26	1
papain[123,168]	3.4	62	2.38
PTP 1B[169]	4.7	9	0.35
Prx5[20]	5.2	3.0×10^5	11538
Prx2[2]	5.3	1.0×10^8	3800000
AhpC[33,99]	5.8	3.9×10^7	1500000
GAPDH[76]	8.3	2.8×10^3	110

nucleophilic atom.[54] This implies that OH^- is a better nucleophile than Cl^-, RS^- is more nucleophilic than RNH_2, and ROO^- exceeds the nucleophilicity of RO^-, respectively. In the case of protein thiolates, these factors cannot be invoked since in every case the nucleophile is a cysteine.

(b) Factors involving the environment of the nucleophile, such as solvation, steric hindrance, hydrogen bonding, and formation of cyclic transition states, which are more likely to be affected by the protein environment.

Indeed, the same protein factors that affect acidity have an influence on the nucleophilicity of the thiolate. The previously discussed charge and dipole interactions are usually considered in the literature as a means of *lowering the pK_a and stabilizing the thiolate*,[55] but from a purely catalytic viewpoint, thiolate stabilization would be counterproductive. A stabilized thiolate would need a larger energy increase to reach the transition state. In Table 2, we have collected a few examples of protein thiolates reacting with hydrogen peroxide. We can see every possible combination of high and low pK_as and high and low rate constants, and we can understand the lack of correlation as an illustration of specificity. While low pK_a seems to be the norm among catalytic cysteines, fast reactions only manifest specifically with the proper substrates. We could conclude that the extraordinary acidity of thiols is more a byproduct than a goal in the natural selection of catalytic cysteines.

From an enzymological perspective, having an indiscriminate nucleophile makes no sense as it would result in the inactivation of the enzyme due to the reaction with a nonsubstrate electrophile. Selective pressure then prompts for the evolution of specialized catalysts; therefore, it is not surprising that the most reactive thiols in Table 2 are those from proteins specializing in H_2O_2 reduction, regardless of their pK_a.

Finally, water and its exclusion by the protein can account for part of the catalysis of thiolates.

Activation and Stabilization of Thiolates via Weak Interactions. The remarkable reactivity of the thiolate as a nucleophile is often mentioned, mainly based on classical works of the second half of the past century.[54,56] The trend that places thiolates among the best nucleophiles holds in water and other protic solvents because it arises partially from solvation effects. In gas phase, and even in polar aprotic solvents, the trend is reversed,[57,58] and charge concentration or "hardness" is the prevalent factor of nucleophilicity. In water and other protic solvents, the solvation energy affects nucleophilicity because the solvation energy lowers the ground-state energy relative to the transition state, in which the charge is more diffuse. This causes an increase in activation energy. Viewed from another perspective, the solvation energy affects nucleophilicity because the solvation shell must be disrupted to arrive at the transition state; then, thiolates with their diffuse charge and poorer solvation suffer to a lesser extent the solvent blunting of their reactivity. Even so, water solvation is an important factor in slowing nucleophilic attacks by thiolates in comparison with other media. Thiol−disulfide exchange in dimethylsulfoxide and dimethylformamide occurs up to 3 orders of magnitude faster than in water.[58]

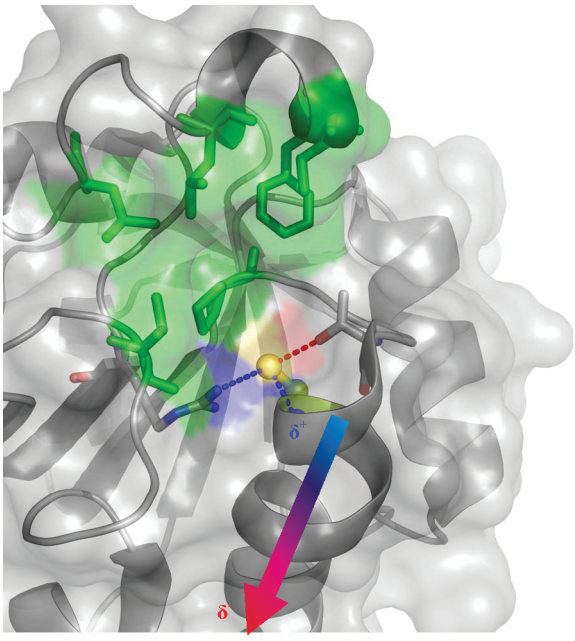


Figure 3. Active site structure of peroxiredoxin 5 (PDB ID 1HD2) showing dipolar interactions of the thiolate (yellow) with R127 (left), T44 (right), and C47 amide (down). The macrodipole of the α-helix is depicted as an arrow, and the "wall" of hydrophobic residues that flanks the active site appears in green.

The environment of thiolates in proteins is partially aqueous but mostly proteic, and as already discussed, protein residues provide the charge, dipole, and hydrogen bond interactions that surround the thiolate, with one huge catalytic advantage over thiolates in solution: the interactions can be altered by protein conformation, providing a switch for turning the reactivity of the catalytic residue on and off.

The idea that the network of hydrogen bonds and polar interactions results in the stabilization of the catalytic thiolate of many enzymatic systems is well supported by structural data. However, from the viewpoint of catalysis the least stable nucleophile will be the most reactive and thus the fastest in the nucleophilic attack. In fact, hydrogen bonding *decreases* the nucleophilic reactivity of thiols[59] as evidenced in Zn-thiolate compounds. It has been observed that even a single hydrogen bond can reduce the reactivity of a thiolate by 2 orders of magnitude.[60]

Protein Factors with Mechanism-Dependent Effects on Catalysis. For discussing the catalytic factors related to reaction mechanisms, we will use our reaction of interest, i.e., the reduction of hydroperoxides by peroxiredoxins.

Oxidation of thiol groups was the first biochemically relevant reaction of peroxynitrous acid (ONOOH) to appear in the

Table 3. Rate Constants for the Reactions of Peroxiredoxin Thiolates with H_2O_2 and ONOOH in Comparison with Cysteine[a]

		k_{S^-} (M^{-1} s^{-1})		k_{S^-}/k_{Cys}		
RS^-	peroxiredoxin subfamily	H_2O_2	ONOOH	H_2O_2	ONOOH	$k_{RS^-}^{ONOOH}/k_{RS^-}^{H_2O_2}$
cysteine[1,61]		26	290 000	1	1	11 000
AhpC (*St*)[62,99]	AhpC-Prx1	39 000 000	11 000 000	1 500 000	5.1	0.28
Prx2 (*Hs*)[2]	AhpC-Prx1	100 000 000	70 000 000	3 800 000	240	0.7
Tsa1 (*Sc*)[67]	AhpC-Prx1	22 000 000	5 300 000	850 000	18	0.24
Tsa2 (*Sc*)[67]	AhpC-Prx1	13 000 000	3 900 000	500 000	13	0.3
TXNPx (*Tc*)[70]	AhpC-Prx1	30 000 000	7 300 000	1 150 000	2.5	0.24
Prx5 (*Hs*)[20,64]	Prx5	300 000	510 000 000	11 500	1700	1700
AhpE (*Mt*)[121]	AhpE	82 000	140 000 000	3200	480	1700
Prx Q (*Xf*)[171]	BCP-PrxQ	45 000 000	6 300 000	1 730 000	22	7.1

[a] Species in parentheses: *St, Salmonella tiphymurium*; *Hs*: *Homo sapiens*; *Sc, Saccharomyces cerevisiae*; *Tc, Trypanosoma cruzi*; *Mt, Mycobacterium tuberculosis*; *Xf, Xylella fastidiosa*.

scientific literature.[61] Nearly 10 years after, it was shown that some thiol proteins might be the principal way that cells have to dispose of this oxidant.[62] The extremely high reactivity of peroxiredoxins toward ONOOH came as a surprise because the reported rate constant was 3 orders of magnitude larger than that of low-molecular weight thiols. Since the year 2000, evidence has accumulated supporting the important role of peroxiredoxins in ONOOH reduction.[63−71] Additionally, kinetic and enzymatic data reveal that peroxiredoxins have thiol groups that react surprisingly fast with hydrogen peroxide and organic hydroperoxides.[72]

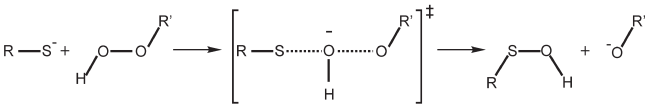
However, some questions related to the reaction between peroxiredoxin thiol and the ROOH groups remain unanswered, for instance, what makes the peroxidatic cysteine so fast in its reaction with peroxides? Are there detectable factors in the structure of peroxiredoxins that reveal specialized reactivity toward different peroxides? Is the diversity and apparent redundancy of peroxiredoxin isoforms related to their peroxide specialization, or is there some other role for them?

Two conserved residues (Arg and Thr) and the amide group of the cysteine establish a network of polar interactions that stabilize the thiolate, thus lowering its pK_a[73] (see Figure 3). We saw that availability of the thiolate accounts for at most 1 order of magnitude, but overall, peroxiredoxins react with ONOOH up to 3 orders of magnitude faster than expected, and with H_2O_2, the reaction is 4 to 7 orders of magnitude faster than expected (see Table 3 below). Then, even in the absence of other effects, the availability of thiolate at neutral pH leaves most of the reactivity unaccounted for.

Hydroperoxide reduction by thiolates is a SN_2 or direct displacement reaction, as presented in Scheme 1. The reaction takes place via the nucleophilic attack of the thiolate on one of the peroxide oxygens; a transition state is reached in which the negative charge of the attacking group is *distributed among the two oxygen atoms and the sulfur*. To complete the reaction, the peroxide bond should break to release the leaving group. Therefore, any reagent able to stabilize the delocalized negative charge of the transition state would produce catalysis. The same structural features that have been assumed to fill the role of stabilizing the thiolate might just as well stabilize the anionic transition state, thus serving the purpose of catalysis much better.

Making a parallel with thiol−disulfide exchange reactions as discussed in the literature,[13] the Brønsted constants β_{Nuc}

Scheme 1. Reaction Mechanism of Hydroperoxide Reduction by a Thiolate[a]



[a] The reaction begins with the nucleophilic attack of the thiolate on the distal oxygen, partially forming a S−O bond and delocalizing the negative charge in the transition state among the two oxygens and the sulfur. The reaction proceeds to products by the rupture of the peroxidic bond with the leaving group, thereby retaining the negative charge.

determined in Figure 1 represent the degree of electron pair donation that the nucleophile effects to reach the transition state. Thus, $\beta_{Nuc} = 0$ means that the negative charge in the transition state remains practically in the attacking atom, as is the case of H_2O_2 reduction; and $\beta_{Nuc} = 1$ means that the electron pair resides in the leaving group atom. Intermediate values such as those for ONOOH and taurine chloramine reduction imply that the charge is shared among the three atoms. Then β_{Nuc} only describes the left half of the reaction as presented in Scheme 1, i.e., the nucleophilic attack of the thiolate on the peroxide oxygen. The right half, i.e., the breaking of the O−O bond depends on the stability of the leaving group, which can be in turn correlated with its Brønsted basicity, or pK_a. This relationship of the rate constant with the leaving group properties has been presented long ago for a number of nucleophilic displacement reactions on peroxides, none of which involves thiolates.[74] More recently, it has been shown that the rate constants of glutathione and the thiol of bovine serum albumin reacting with peroxides depend linearly on the pK_a of the leaving group.[75] Using the data for GSH,[75] this dependence can be expressed as follows:

$$\log k_{RS^-} = \beta_{Lg} pK_a^{R'OH} + 6.61 \qquad (5)$$

where β_{Lg} has a value of −0.32. Again, using data from the literature, a similar trend can be observed for the rate constant of the critical thiol of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reacting with hydrogen peroxide[76] and peroxynitrous acid.[77] Also, the rate constants of Prx5 with hydrogen peroxide and peroxynitrous acid parallels the trend of GAPDH, but the linearity is lost for other peroxides[20] (Figure 4).

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Remarkably, in the SN_2 mechanism represented in Scheme 1 the relative importance of the attacking thiolate and the leaving group is reflected in the nucleophilicity constants that multiply the pK_as, β_{Nuc} in eq 3 and β_{Lg} in eq 5. The similar absolute values obtained for the β_{Nuc} of peroxynitrous acid in eq 3 (0.41) and β_{Lg} of eq 5 (−0.32) suggest that the nucleophilic characteristics of both attacking and leaving groups are important for defining the rate constant of the reaction. The relative position of the three lines in Figure 4 shows the relative efficiency of each thiolate in reducing different peroxides. The catalysis (vertical displacement) can be understood as if each transition state was stabilized, and the result is an apparent lower pK_a of the leaving group. For example, the leaving group in the reduction of H_2O_2 is OH^- (pK_a = 15.7) and with glutathione as reductant k_{RS^-} is 22 M^{-1} s^{-1}; reaction with GAPDH can be understood simply as a 380-fold enhancement (vertical displacement) or as a stabilization of the transition state that translates in an apparent leaving group pK_a of 8.4 (horizontal dotted line). Evidently, not all the catalytic efficiency arises from the stabilization of the negative charge on the transition state, but at least some of it can be ascribed to the cysteine of both Prx5[32] and GAPDH[78] sitting at the bottom of a conserved cationic cavity on the surface of the protein.

It is also evident that *tert*-butyl hydroperoxide and cumene hydroperoxide depart from the expected trend of Prx5, evidencing additional enhancement of the reactivity. This also may be due to the active site being able to accommodate hydrophobic anions as is apparent from the crystal structure of Prx5, that contains a considerable patch of hydrophobic residues flanking the catalytic cysteine[32] (see Figure 3).

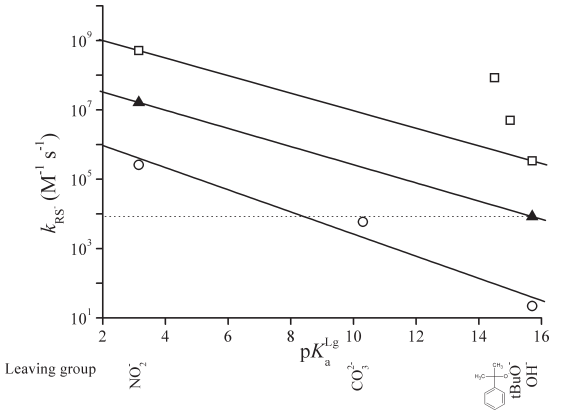


Figure 4. pH-independent rate constants for hydroperoxide reduction by thiols vs leaving group pK_a. Thiols, from bottom to top: glutathione (○), D-glyceraldehyde-3-phosphate dehydrogenase (▲), and peroxiredoxin 5 (□). Hydroperoxides, from left to right: peroxynitrous acid, peroxymonocarbonate, cumene hydroperoxide, *tert*-butyl hydroperoxide, and hydrogen peroxide. Data obtained from the literature.[20,75−77]

SPECIFICITY AND CATALYSIS OF PEROXIREDOXINS

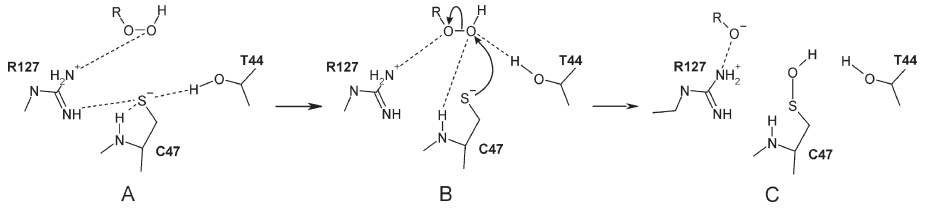
Reactivity and Substrate−Active Site Interaction. Hydrogen peroxide as a substrate poses an interesting problem to enzymologists. Enzymes, for both their reactivity and specificity, depend on the specific molecular recognition of the substrate and the exclusion of other potentially reactive species. In the case of large substrates, such recognition is easy to understand and can be exemplified by peptidases which bind the substrate via a number of specific weak interactions with amino acid residues often distant from the scissile bond. Hydrogen peroxide represents the opposite end in substrates: with only four atoms, it hardly has any recognizable molecular feature. Evolution has solved the problem of H_2O_2 binding in two different ways, one that implies metal binding in heme peroxidases and another that involves a reactive chalcogen such as selenolate in some glutathione peroxidases or a thiolate in peroxiredoxins and thiol peroxidases in general.

In the case of peroxide-reactive thiolates, a high reactivity has to be reconciled with the specificity of the catalyst. In this respect, peroxiredoxin 2 reacts extremely fast with H_2O_2 and ONOOH[2,3] but slowly with chloramines and other thiol reagents[3,4] as can be seen in Figure 1. As a general rule, the compromise of reactivity versus specificity can be achieved in enzymes by a "concealed" reactivity that is only apparent upon binding of the proper substrate in the active site. Once again, this is a problem with a substrate as small and water-like as hydrogen peroxide, but the network of polar interactions surrounding the thiolate may play a role in the catalytic mechanism.

A very recent report[79] has proposed the existence of an "oxygen track" along which the peroxide substrate is aligned by the protein and positioned for the nucleophilic attack of the thiolate. Their proposal is based on the analysis of crystal structures of Prx−ligand complexes, including the recently determined Prx-H_2O structure,[80] where the position of oxygen ligands binds to various peroxiredoxins and significantly supports the catalytic enhancement by means of the stabilization of a linear S−O−O transition state.

Substrate Recognition and Thiolate Activation. As a mechanism complementary to the proposal of Hall et al.[79] and congruent with both reactivity and specificity, we propose the sequence of events sketched in the first part of Scheme 2, where the approaching peroxide presents two atoms that are electronegative and are better hydrogen-bond acceptors than the thiolate to T44, R127, and probably also C47 amide (using residue numbers from human Prx5). The H_2O_2 approach causes the shift of the hydrogen bonds away from the thiolate, which in

Scheme 2. Diffusional Encounter of the Peroxide with the Peroxiredoxin Active Site (A), Shift of the Hydrogen Bonds from the Thiolate to the Substrate Increasing the Nucleophilicity of the Thiolate and Promoting the Attack on the Distal Oxygen (B), and Cleavage of the Peroxide Bond and Stabilization of the Leaving Anion by the Arginine Residue (C)



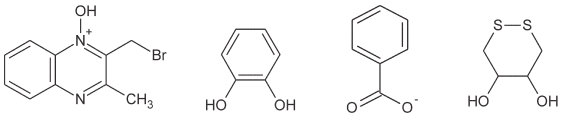


Figure 5. Inhibitors and active-site ligands of peroxiredoxins are expected to show optimal activity when they possess a pair of electronegative atoms in close vicinity.[79,84,85] The number in parentheses is the distance between the electronegative atoms.

turn enhances its nucleophilicity and favors the nucleophilic attack on the oxygen. Energetically, the shifting of the hydrogen bonds should be marginally favored as the newly formed hydrogen bonds are stronger than the ones broken. Nevertheless, energy distribution is different; thiolate nucleophilicity is no longer restrained by hydrogen bonds, and simultaneously the oxygens are stabilized by the newly formed polar interactions which provide a better leaving group. Thus, by binding near the thiolate, the peroxide springs the trap that starts the nucleophilic substitution reaction. From another perspective, the catalytic thiolate is only stabilized *in the absence of substrate binding*, thus providing specificity in its reactivity. Other oxidants such as chloramines[4] and alkylating reagents[3] are unable to unleash all the reactivity of the thiolate and then react as if it were an acidic thiol, or even more slowly due to steric hindrance (shown in Figure 1A for Prx2).

Our proposal is consistent with the fact that a threonine mutation should markedly decrease the activity of the enzyme. This has already been observed for tryparedoxin peroxidase of *Leishmania donovani*,[81] where the activity of the T49 V mutant is more than 2 orders of magnitude lower, and for peroxiredoxin Q of *Arabidopsis thaliana* in which the T107A modification lowers the activity by 60−90%.[82] Nevertheless, a threonine mutation should not decrease, or possibly even increase, the nucleophilicity of the thiolate measured with nonsubstrate reagents. Simple calculations using PROPKA[83] on reported Prx structures show that Thr contributes to decrease the pK_a of the peroxidatic cysteine by at most 1.6 units, not enough to significantly affect the availability of the thiolate. Another prediction of the model is that other electrophiles with two electronegative vicinal atoms, able to accept hydrogen bonds, could be either substrates or competitive inhibitors of peroxiredoxins and should be equally affected by the mutation on the conserved threonine.

The search for inhibitors of peroxiredoxins has yielded two examples of molecules that may support our hypothesis. In a recent report on the optimization of irreversible inhibitors of *Toxoplasma gondii* peroxiredoxin II,[84] the authors find that placing a *N*-oxide moiety close to the alkylating group doubles the efficiency of the inhibitor in the case of conoidin C7 (Figure 5a). Another example includes the search of protein ligands for binding peroxiredoxin 5,[85] which finds that the best ligand in the studied library for binding the active site of the enzyme is catechol (Figure 5b). Further, they calculate by docking that the ligand binds by establishing hydrogen bonds with T44 and R127 among other residues of the active site of Prx5. Finally, benzoate and oxidized dithiothreitol have been shown to crystallize in close vicinity to the active site of Prx5 with oxygens mimicking the putative position of the peroxide in the transition state.[32,79]

Thiolates Approaching Catalytic Perfection. The best characterized reaction between peroxynitrous acid and a peroxiredoxin is that with Prx5.[20,64] The reaction has a rate constant (k_{RS^-}) of 5.1×10^8 M^{-1} s^{-1}, which is 5×10^4 times faster than expected for a hypothetical thiol with the same pK_a according to Figure 1. Not only is this a big enhancement, but it is arguably one of the fastest possible reactions between peroxynitrous acid and a protein. The diffusion control limit for the reaction of a small molecule with a protein of 20 Å radius[32] can be calculated using the von Smoluchowski equation[86] as 1.7×10^{10} M^{-1} s^{-1} at 25 °C.

To put a simple energy equivalent to the magnitude of catalysis, an increase of 5×10^4 times in rate constant implies only 6.6 kcal/mol decrease in activation energy, equivalent to a few hydrogen bonds.[87] Reduction of H_2O_2 by Prx2, 4×10^6 times faster than cysteine, can be equated to a decrease in activation energy of about 9 kcal/mol. Such activation energy reduction can be constituted by the stabilization of the diffuse negative charge of the transition state and differential hydrogen bonding upon substrate binding to the active site.

Hydroperoxide Selectivity in Peroxiredoxins. Up to now, we can hypothesize that peroxiredoxins could use the shifting of the polar interactions away from the thiolate upon substrate positioning in the active site and the stabilization of the anionic leaving group of reaction[20] to augment the rate constant. Additionally, it can be seen in Figure 4 that in the case of Prx5 some peroxides such as cumene hydroperoxide and *tert*-butyl hydroperoxide depart from the trend and are more reactive than expected. In other peroxiredoxins, these divergences are even more dramatic, for instance Tsa1 and Tsa2 of *Saccharomyces cerevisiae*[67] and bovine peroxiredoxin 6[88] react faster with H_2O_2 than with ONOOH against the trend predicted by the basicity of the leaving group in Figure 4. Unfortunately, there are no published structures of reduced Tsa1, Tsa2, or Prx6 to shed light on this selectivity. However, the crystal structure of reduced Prx5 presents a benzoate ion cocrystallized in close association with the active site:[32] one side of the active-site pocket contains several hydrophobic residues, including Leu116, Ile119, and Phe120, whose side chains are located in the neighborhood of the benzoate aromatic ring (see Figure 3). It strongly suggests that the peroxides whose reduction is accelerated above the trend represented in Figure 4 are those with hydrophobic aliphatic and aromatic groups.

We can then propose that the existence of an anion-binding site in the neighborhood of the reactive cysteine of proteins furnishes a nonspecific acceleration for the reduction of hydroperoxides in general, by stabilization of the leaving group, and that this enhancement of the reaction can become specific in the case of larger peroxides having functional groups suitable for additional interactions with the surface of the protein.

According to Figure 4, the expected ratio $k_{RS^-}{}^{ONOOH}/k_{RS^-}{}^{H_2O_2}$ should be in the order of 10^3-10^4. Surveying the data in the literature, we found this ratio only for Prx5 and AhpE (see Table 3). The rest of the proteins have lower ratios, some even below 1. The data in Table 3 that compares the rate constants with those of cysteine reveal that whereas peroxiredoxins react faster with H_2O_2 by factors of 10^3-10^7, the acceleration in the case of ONOOH is much more modest, only reaching 10^3. This fact is often obscured by the similarity in magnitude of the rate constants.

Molecular symmetry of H_2O_2 may have a role in reactions that are close to the diffusion control limit, as every attack of the

Table 4. Reported Thiol Peroxidase Concentrations in Different Organisms[a]

Thiol peroxidase, organism	concentration (μM)[b]
cytosolic TXNPx, *Tc*[172]	2.75
mitochondrial TXNPx, *Tc*[172]	1.35
TXNPx, *Cf*[46]	535[c]
TXNPx, *Lm*[93]	70−280
mitochondrial Prx3, *Hs* [102,173]	60−125
erythrocyte Prx2, *Hs*[118,119]	240−410
Prx6, *Rn*[174]	0.24−13.6[d]
Prx5, *Rn*[174]	1.8−9[e]
Prx1, *Rn*[174]	1.7−17[f]
Tsa1 (aerobic), *Sc*[94]	48[g]
chloroplast 2-Cys Prx, *Hv*[175,176]	60[h]
chloroplast Prx Q, *At*[82]	30[i]

[a] Organisms: Tc, *Trypanosoma cruzi*; Cf, *Crithidia fasciculata*; Lm, *Leishmania major*; Hs, *Homo sapiens*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*. [b] Unless otherwise indicated, values account for cellular Prx content disregarding compartmentalization. Calculated according to the reported percentage or fraction of total soluble protein (tsp) assuming a mean protein concentration of 200 mg/mL and using the molecular weight of Prx. [c] 6% of tsp, 22.4 kDa. [d] 0.03−1.7 μg/mg tsp, 25 kDa. [e] 0.2−2 μg/mg tsp, 23 kDa. [f] 0.2−1 μg/mg tsp, 22 kDa. [g] 0.7% tsp, 29 kDa. [h] 0.6% tsp, 17 kDa. [i] 0.3% tsp, 22 kDa.

thiolate on *any* oxygen results in the same transition state and products. For any other peroxide, the attack on the distal oxygen usually leads to the best leaving group and minimizes steric hindrance. Additionally, the small size and electronic characteristics of H_2O_2 could optimize the shifting of hydrogen bonds since both oxygens are equally good acceptors. In ONOOH, the electron withdrawing effect of the −NO moiety strongly diminishes the electron density of the proximal oxygen, thus compromising its ability as a hydrogen-bond acceptor.

Diffusion control limit also has to be considered. The reactions of thiolates with ONOOH have quite large rate constants even in the absence of catalysis (10^5−10^6 M^{-1} s^{-1}, Figure 1B) leaving limited room for improvement. However, rate constants for reactions of low-molecular weight thiols and noncatalytic protein thiols with H_2O_2 usually fall in a narrow range between 10 and 100 M^{-1} s^{-1} (see Figure 1C).[1]

Peroxide specialization and the apparent redundancy of peroxiredoxins still have unsolved questions that need to be approached from detailed kinetic and structural studies of the reaction with oxidants but also of the reaction with the possible reductants of the sulfenic/disulfide formed upon peroxiredoxin oxidation.

Extra-Chemical Factors Contributing to Preferential Reactivity. For a quantitative understanding of the relative efficiencies of protein thiols as targets for peroxide-mediated oxidation, the rate constants of peroxide reactions with thiol-containing proteins (P-SH) or with other relevant cellular targets (T), along with their concentrations should be taken into account. Then, we need to compare the products $k_{P\text{-}SH} \times [P\text{-}SH]$ vs $k_T \times [T]$ in a simple competition model. Moreover, it is important to consider the actual concentration of reactive thiol-containing protein, and the protein should be in the conditions required for the reaction to take place, i.e., active and reduced. In some cases, and mainly for those peroxides with limited membrane permeability, enzyme compartmentalization should also be taken into account.

Protein Concentration. Protein−thiol concentrations in the range of 45−180 nmol/mg protein (9−36 mM), have been reported for rat hepatocyte homogenates.[89,90] Protein−thiol concentrations have also been reported for selected compartments, for example, in the mitochondrial matrix, the concentration of exposed protein thiols is ∼60−90 mM.[91,92] For selected thiol-proteins specialized in peroxide reduction, protein content has been determined. For *Leishmania* major promastigote, GPx concentration was reported as 1.6−6.4 μM, much lower than that of peroxiredoxins (tryparedoxin peroxidase, TXNPx, 70−280 μM).[93] Indeed, peroxiredoxins, which are ubiquitous proteins expressed throughout life kingdoms in different subcellular compartments, are frequently very abundant (Table 4).

Several reports indicate that the concentrations of peroxiredoxins increase during conditions associated with oxidative stress.[94−97] In some cases, Prx expression also changes during development.[98] These enzymes were initially considered to be low-efficiency peroxidases, and their high abundance was rationalized as a way of compensating for their low catalytic activity.[46] Nowadays, many examples of peroxiredoxins with peroxidatic rate constants almost overlapping those of selenium-containing glutathione peroxidase (10^8 M^{-1} s^{-1}) or heme-dependent peroxidases like catalase (10^7 $M^{-1}s^{-1}$) have been described.[2,99,100] Thus, considering their concentrations and rate constants, peroxiredoxins should be regarded as preferential targets for hydrogen peroxide, peroxynitrous acid, and most probably for lipid hydroperoxides, leading to their reduction. However, in many cases, these simple calculations do not take into account factors such as enzyme oxidation, inactivation, and compartmentalization that can make effective concentrations different from those reported in the literature (Table 4).

A very recent article supports the view of thiol peroxidases as a kinetic bottleneck and a mandatory step in H_2O_2 reduction.[101] Using kinetic modeling and comparing with experimental data, the authors conclude that protein oxidation other than thiol peroxidases only becomes significant assuming the existence of "fast-reacting protein thiols" ($k = 5 \times 10^5$ M^{-1} s^{-1}) with a large concentration (200 μM). Considering a smaller rate constant ($k = 5 \times 10^2$ M^{-1} s^{-1}, still 2 orders of magnitude larger than that of nonspecialized thiols), the direct oxidation of protein-thiols other than Prx and GPx becomes nonsignificant. To the best of our knowledge, no such "fast-reacting protein thiols" have been described, implying that the indirect oxidation of protein thiols via oxidized Prx and GPx needs to be considered in order to account for the observed results.

A similar scenario was described in a recent review on the role of Prx3 in mitochondria.[102] Using literature values of rate constants and mitochondrial concentrations, the authors conclude that 99.9% of H_2O_2 reduction occurs thanks to the action of thiol peroxidases. None of the other considered potential targets accounts for more than 0.0026% of H_2O_2 consumption.

Although the question of why peroxiredoxins are so abundant remains to be answered, there are some factors that should be considered: the distribution of the enzyme between reduced and oxidized forms, governed by enzyme turnover that is dependent on a separate enzyme system; the distribution of the enzyme between active and inactive forms, governed by inactivation−reactivation cycles; and the participation of these enzymes in alternative cellular functions, which do not always rely on the oxidation of the peroxidatic cysteine, such as chaperone,

phospholipase, and dethiolation activity, as redox sensors or other signaling functions.[103−112]

Enzyme Turnover. Thiol-dependent peroxidases catalyze two-substrate reactions by a ping-pong mechanism. Thus, their catalytic efficiency as peroxidases depends not only on the rate of their reaction with the peroxide but also on the rates of the rest of the catalytic cycle, including disulfide formation with the resolving cysteine in the case of 2-Cys Prxs and Cys GPxs, and reduction of the oxidized form of the enzyme by the reducing substrate that completes the catalytic cycle.

The rate constant of disulfide formation has only been determined for the atypical 2-Cys human Prx5 as 15 s^{-1} at pH 7.4 and 25 °C.[20] Thus, at least for human Prx5, considering H_2O_2 as oxidizing substrate and a rate constant for enzyme oxidation of 3×10^5 $M^{-1}s^{-1}$, reaction with this peroxide would be slower than disulfide formation, unless steady-state concentrations of the oxidant are higher than 50 μM. Indeed, H_2O_2 steady-state concentrations attained in cells during normal aerobic conditions are between $10^{-9}-10^{-7}$ M[113] reaching 15 μM in the vicinity of inflammation sites.[114,115] Therefore, intramolecular disulfide formation is most probably not rate-limiting Prx5 oxidation, although the situation may be different when analyzing other Prx and peroxides.

Two-cysteine Prxs (and also the functionally characterized thiol-containing GPx) are reduced by naturally occurring redoxins, including thioredoxins, glutaredoxins, tryparedoxins, or homologous domains in bacterial AhpF.[116] The rate constant of oxidized peroxiredoxin reduction by thioredoxin or related proteins has been determined by steady-state or presteady state kinetics and are usually in the 10^5-10^6 $M^{-1}s^{-1}$ range.[72] In the case of Cys GPx reduction by thioredoxins, the rate constants are in the 10^4-10^5 M^{-1} s^{-1} range.[116] Thus, considering reported thioredoxin concentrations in the micromolar range,[93] most of it maintained, reduced by thioredoxin reductase/NADPH systems, then the reductive part of the catalytic cycle could also be rate-limiting.

Let us consider the case of red blood cells, where Prx2 (2-Cys Prx) reacts with H_2O_2 with a rate constant of 1×10^8 $M^{-1}s^{-1}$,[2] and its reduction rate by the thioredoxin/TR/NADPH system has a measured value of 0.4 μM/s.[117] Considering an intraerythrocytic Prx2 concentration of 240−410 μM,[118,119] oxidation would be rate-limiting only with steady-state levels of the oxidant below 20 pM. Considering a physiological condition of fluxes of H_2O_2 formed from oxyhemoglobin autoxidation (4.6−7 nM/s)[2] and Prx2 as the only route for H_2O_2 consumption, steady-state concentrations of the oxidant would most probably be below pM levels. However, under experimental conditions where cells are exposed to bolus additions of H_2O_2 (μM), or fluxes of H_2O_2 (μM/s), higher intracellular concentrations can be achieved,[120] thus changing the rate-limiting step during the catalytic cycle.

In the case of most 1-Cys peroxiredoxins, natural reducing substrates are still a matter of debate, thus precluding kinetic considerations.

Enzyme Inactivation. One interesting aspect of peroxiredoxins is their sensitivity to inactivation by overoxidation of the peroxidatic cysteine residue. The mechanism of overoxidation consists of the oxidation of the sulfenate form of the enzyme to sulfinic acid.[121] Thus, inactivation occurs during turnover, at least in 2-Cys Prxs.[122] In addition, overoxidation of peroxidatic cysteine has been observed by an excess of peroxynitrous acid, and a radical mechanism is involved.[2] Intriguingly, eukaryotic typical 2-Cys Prxs have evolved structural features that enhance susceptibility to oxidative inactivation compared to their prokaryotic counterparts.[122] The rate constant of H_2O_2-mediated peroxiredoxin sulfenate oxidation to sulfinic acid was determined as 57 M^{-1} s^{-1} for the 2-Cys human Prx1[122,123] and 40 ± 3 M^{-1} s^{-1} at pH 7.4 for the 1-Cys Prx *Mt* AhpE.[121] Thus, considering a rate constant of disulfide formation of 15 s^{-1} reported for human Prx5,[20] H_2O_2 steady-state concentration should be high (>mM) to cause a significant enzyme inactivation in a single enzymatic catalytic cycle. In any case, even at low oxidant concentrations, a minute fraction of the enzyme would be inactivated in each catalytic cycle and could become significant if accumulated over a long time. In several organisms including mammals, overoxidized 2-Cys Prxs can be slowly regenerated by sulfiredoxins in an ATP-dependent mechanism.[124,125] It has been proposed that this mechanism enables the function of H_2O_2 as a messenger in cell signaling.[126−128] However, it is still difficult to reconcile the concentrations of H_2O_2 physiologically produced during hormone-, growth factor- or cytokine-triggered signaling with those required for oxidative inactivation of these usually abundant enzymes.[116] Other peroxides such as peroxynitrous acid and lipid hydroperoxides can also lead to peroxidatic thiol overoxidation,[2,88,129,130] though their function in cell signaling is less documented. Alternatively, overoxidation of Prxs may lead to a gain of function, namely, chaperone activity.[106,108] Also a signaling role for inactive overoxidized Prxs has been proposed in plants.[131]

Enzyme Compartmentalization. Another concept that should be considered when analyzing extra-chemical factors contributing to the preferential reactivity of peroxiredoxins with peroxides is compartmentalization. Prxs are expressed in different cell compartments. Hydrogen peroxide is able to diffuse through membranes, but membranes can still impose a gradient in its concentration.[132] Peroxynitrous acid can diffuse through membranes, while for its conjugated base, peroxynitrite (pK_a = 6.8), diffusion seems to be limited to membranes possessing anion exchangers such as those of red blood cells.[133] Compartmentalization could also affect the reductive part of the catalytic cycle: there are cases where no solid evidence exists regarding the presence of the peroxiredoxin and its postulated reducing substrate in the same cellular compartment. For example, the role of mitochondrial tryparedoxin peroxidase in hydroperoxide and peroxynitrous acid reduction *in vivo* is well documented. Mitochondrial tryparedoxin peroxidases rapidly react with both H_2O_2, and peroxynitrous acid and can be reduced by thioredoxin 2 *in vitro*.[129] However, at least in *Trypanosoma cruzi* and *Trypanosoma brucei*, thioredoxin 2 has not been unambiguously detected inside mitochondria and most probably is located in the outer mitochondrial membrane facing the cytosol.[134] Thus, in those cases, the actual reducing substrate for peroxiredoxins is still under investigation.

THIOL REACTIVITY AND THE KINETIC CONTROL OF THIOL-MEDIATED REDOX SIGNALING

On thermodynamic grounds, thiol oxidation by hydroperoxides is always a favored reaction. Then, in principle, any biothiol could be capable of acting as a H_2O_2 sensor; moreover, the diversity of thiols and the multitude of possible formations of inter- and intramolecular disulfides present *a priori* a very complex scenario for thiol-mediated H_2O_2-dependent cell signaling. Thiol oxidations relevant in biology are then dictated by kinetics, steady-state concentrations, and compartmentalization. In order to unravel the different pathways of thiol-mediated

redox signaling, data are needed, not only on which thiols can be modified by hydroperoxides but also on the rates of these oxidations and how thiols compete with one another in the complexity and diversity of the intracellular medium.

In prokaryotes, transcription factors such as OxyR in *Escherichia coli*[135] and RosR in *Corynebacterium glutamicum*[136] appear to be fast-reacting and thus suffer the direct oxidation of their reactive cysteines by H_2O_2 resulting in activation of an antioxidant response, but that is not the norm in mammals and higher plants.

Evidence has accumulated indicating that upon activation of several cell-surface receptors (growth factors, cytokines, hormones, and inflammatory mediators), transient intracellular production of H_2O_2 initiates redox-signaling pathways. Hydrogen peroxide appears as a second messenger that can mediate signal transduction via the oxidation of specific cysteine residues. Transcription factors such as NFκB or Nrf2 are not directly oxidized, but translocated to the nucleus upon oxidation/modification of the cytosolic inhibitors bound to them (IκBα and Keap1, respectively), which are in turn redox-sensitive thiols.[137,138]

Several proteins involved in cell signaling contain thiols and have been shown to be up- or downregulated in response to hydroperoxide levels, but in general, there is a lack of knowledge about which cysteine residue is being oxidized, which one is the acting oxidant, and what the rate of oxidation is. It is surprising that in a PubMed search (Sep, 2010) of the 1021 articles containing the keywords "redox signaling" and "thiol", only 91 (8.9%) also contain the word "kinetics", and only four (0.39%) contain the words "rate constant".

Take as an example Keap1, which is modified by oxidation or reaction with an electrophile disrupting its interaction with Nrf2, decreasing proteasomal degradation, therefore accumulating free Nrf2 in the cytosol, and increasing Nrf2 translocation into the nucleus.[139] Keap 1 is a complex protein with over 20 cysteine residues (27 in the human variant) whose reactivity has only begun to be studied.[140,141] It is not even clear which cysteine residues are needed for Nrf2 sequestration, let alone what molecules are responsible for their modification *in vivo* and what the bases are for their preferential reactivity. The paucity of hard kinetic data is particularly troubling in an area involving thiol oxidation given the diversity and abundance of intracellular thiols that compete for the same oxidants and electrophiles.

Put in the perspective of previous sections, due to high rate constants, distribution and total concentration, thiol peroxidases (Prx and GPx) reduce the vast majority of ROOH, and particularly H_2O_2. This would create a kinetic bottleneck that separates ROOH from its redox-signaling targets, i.e., Prx and GPx would be mandatory intermediates in peroxide-mediated redox signaling (Figure 6) unless yet-undiscovered fast-reacting thiols are present.[101]

In fact, growth-factor-stimulated cells with overexpressed or partially depleted Prx respond differently to these ligands indicating that Prxs are critical components of cellular signaling cascades.[142,143]

Once Prx and GPx are oxidized, the thermodynamically favored routes of reduction end in NADPH oxidation (Figure 6), but there is also the possibility of kinetic competition by intermediate targets that can reroute oxidation to kinetically favored targets such as redox-sensitive transcription factors or divert it to other signaling mechanisms such as protein phosphorylation. Cross-talk between redox and phosphorylation

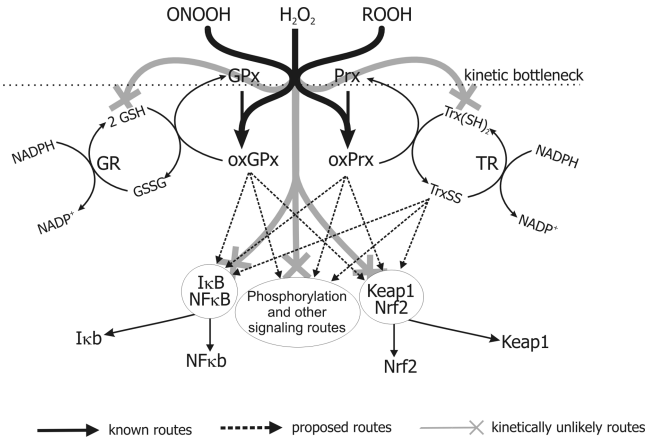


Figure 6. Pathways of thiol-mediated hydroperoxide signaling. Glutathione peroxidases (GPx) and peroxiredoxins (Prx) constitute kinetically favored targets of oxidation by hydroperoxide to an extent that they prevent practically all other thiol−peroxide reactions. GPx and Prx are reduced in their antioxidant routes by reactions ending in NADPH oxidation. The interaction between hydroperoxides and effector molecules such as the transcription factors NFκB and Nrf2 is achieved indirectly through protein−protein redox reactions involving GPx, Prx, Trx, and quite possibly other intermediates in kinetically favored reaction sequences.

signaling pathways has been postulated on the basis of the observed inactivation of protein tyrosine phosphatases via oxidation (PTP1B, PTP2a, Cdc25, and PTEN),[144−147] whereas kinases appeared activated under cellular oxidative stress (ASK1, Sty1, PKG-1a, PKA RI, and Src-PTK).[148−152] However, redox signaling via direct oxidation of PTP by H_2O_2 ($k = 20\ M^{-1}\ s^{-1}$ and 0.1 μM cytosolic concentration) as proposed[153] cannot kinetically compete with Prx (or GPx) and thus needs to occur by indirect oxidation involving other oxidized cysteines as intermediates.

Concentrating the first-hit oxidation in a limited set of thiol proteins (Prx and GPx) constitutes a good starting point for thiol-mediated redox signaling as it provides the specificity that is missing in the hydroperoxide, via protein−protein interaction, and also places the oxidized residue in the complex, yet regulated, thiol map of the cell.

Thiol−disulfide exchanges are in many cases reactions involving little energy, which may convey the false idea of rapid equilibration, but the attempts of measuring an intracellular "redox potential" based on thiol−disulfide couples have consistently encountered several nonequilibrium or "steady state" redox potentials[154] coexisting in the same subcellular compartment. This constitutes evidence that the cellular thiol pool is kinetically compartmentalized by way of specific catalysis linking the exchange between particular redox couples. A very recent study in *S. cerevisiae*[155] indicates that not all reducible cysteines react with all possible reductants and that there are mutually exclusive protein-thiol pools reducible by glutaredoxin 1, thioredoxin 1, and tris (2-carboxyethyl)phosphine (a standard thiol-reducing agent).

Temporary inactivation of Prx via overoxidation has been evoked as a mechanism of redox signaling (the so-called "floodgate" mechanism[122]) since under Prx inactivation, the intracellular H_2O_2 levels would increase, allowing direct oxidation of other critical cysteine residues in redox-sensitive signaling proteins.[127] Prx overoxidation, albeit detected in cells stimulated

with TNFα during 48 h, does not affect the level of reduced Prx unless cells are subjected to acute oxidant exposure such as 150 μM *tert*-butyl hydroperoxide [156]

A mechanism of signal transduction based on Prx and GPx as the kinetically favored targets has been suggested before,[111157−159] and experimental evidence in eukaryotes is accumulating discovering new interactions of Prx with other redox-sensitive proteins as signaling intermediates.[106,152,160−163]

In addition, the initial oxidation of Prx is transformed to thioredoxin oxidation in the antioxidant pathway of most Prxs (Figure 6), and it has been shown that the redox state of thioredoxin can also modulate cellular signaling.[151,164]

CONCLUDING REMARKS

The function of numerous proteins depends on the reactivity of specific cysteine residues toward specific targets. In particular, protein-thiols are relevant in toxicology, participating in drug activation, metabolism and toxicity, and oxidant detoxification. Protein-thiol reactivity is complex, but in general, the reaction involves the nucleophilic attack of the thiolate on an electrophile. A thiol with low pK_a has the kinetic advantage of higher availability of the thiolate at neutral pH but usually the disadvantage of a lower nucleophilicity. Several protein structural factors (charge and dipole interactions, hydrogen bonding, and solvent exclusion) contribute to increasing the reactivity of the thiol in very specific reactions, but these factors do not provide an indiscriminate augmentation in general reactivity.

The outstanding rate of hydroperoxide reduction by the catalytic cysteine of peroxiredoxins, close to the diffusion-control limit, is explained by features in the structure of the catalytic site. Protein structure affords stabilization of the anionic transition state via a relatively static positive charge distribution and a dynamic set of polar interactions that are established once the peroxide substrate is bound.

This particularly high thiol reactivity toward peroxides of Prx places them not only as efficient antioxidant enzymes but also as cellular redox sensors. The detailed participation of Prx in redox signaling is still not clear. In order to elucidate potential signaling pathways, further studies need to be done on redox kinetics, structural modifications that regulate activity, subcellular localization, and interactions with other proteins.

ASSOCIATED CONTENT

Supporting Information. pH profiles of the reaction of peroxynitrous acid with thiolates of different pK_as and trends of the apparent rate constant plotted vs thiol pK_a. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

Prx, peroxiredoxin; ε, dielectric constant; SN_2, bimolecular nucleophilic substitution; β_{Nuc}, nucleophilic constant; β_{Lg}, nucleophilic constant of the leaving group; GST, glutathione *S*-transferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PTP 1B, protein tyrosine phosphatase 1B; AhpE, alkyl hydroperoxide reductase E; AhpC, alkyl hydroperoxide reductase C; AhpF, alkyl hydroperoxide reductase F; Tsa, thiol specific antioxidant; TXNPx, tryparedoxin peroxidase; GPx, glutathione peroxidase; Trx, thioredoxin; GR, glutathione reductase.

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