Published in final edited form as:

J Mol Biol. 2010 September 10; 402(1): 194–209. doi:10.1016/j.jmb.2010.07.022.

Structural evidence that peroxiredoxin catalytic power is based on transition state stabilization

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Abstract

Peroxiredoxins (Prxs) are important peroxidases associated with both antioxidant protection and redox signaling. They use a conserved Cys residue to reduce peroxide substrates. The Prxs have a remarkably high catalytic efficiency that makes them a dominant player in cell-wide peroxide reduction, but the origins of their high activity have been mysterious. We present here a novel structure of human PrxV at 1.45 Å resolution that has a dithiothreitol bound in the active site with its diol-moiety mimicking the two oxygens of a peroxide substrate. This suggests diols and similar di-oxygen compounds as a novel class of competitive inhibitors for the Prxs. Common features of this and other structures containing peroxide, peroxide-mimicking ligands or peroxide-mimicking water molecules reveal hydrogen bonding and steric factors that promote its high reactivity by creating an oxygen track along which the peroxide oxygens move as the reaction proceeds. Key insights include how the active site microenvironment activates both the peroxidatic cysteine side chain and the peroxide substrate, and is exquisitely well-suited to stabilize the transition state of the in-line S_N2 substitution reaction that is peroxidation.

Keywords

Peroxiredoxin; catalytic power; transition state stabilization; dithiothreitol; S_N2 displacement

INTRODUCTION

Peroxiredoxins (Prxs) are ubiquitous and dominant antioxidant enzymes found in organisms from all kingdoms. Based on sequence, Prxs separate into six subfamilies (Prx1, Prx6, Prx5, Tpx, BCP, AhpE), however all share a universal catalytic cycle and active site structure¹. Prxs reduce hydrogen peroxide, alkyl hydroperoxides and peroxynitrite using a Cys residue referred to as the peroxidatic Cys (C_p, S_p when referring to the sulfur atom of the C_p). In this role as a peroxidase, the Prxs are especially critical for many pathogenic bacteria that rely on the Prxs for protection from the human immune system. In addition to their role as

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Data bank accession numbers

The coordinates and structure factors for HsPrxV-DTT have been deposited in the Protein Data Bank as 3MNG.

an important antioxidant, in eukaryotes, some Prxs have additional roles in regulating hydrogen peroxide signaling pathways², although the details of how Prxs are involved are not yet known.

The importance of the Prx-catalyzed reduction of peroxides was met with skepticism during their initial characterization. Prxs do not contain a heme prosthetic group or reactive selenium hetero-atoms, but instead are dependent on a Cys residue (i.e. Cp) for peroxidase activity³, and this led people to doubt their ability to compete with other known peroxidases such as catalases and selenium-containing glutathione peroxidases⁴. Since then, kinetic data have shown that these thiol-based peroxidases have catalytic efficiencies (i.e. k_{cat}/K_m) on the order of $\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ^{5,6,7,8}, which is on par with those of catalase ($\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$), heme peroxidase ($\sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$), and selenium-containing glutathione peroxidases ($\sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$)⁹. Additional factors that make the Prxs important in antioxidant defense and redox signaling are their widespread distribution¹⁰, high expression levels (approaching 0.1 to 1% of soluble proteins in mammalian cells¹¹) and low K_m values¹². Taking these factors into account, competitive kinetic analyses have predicted that reduced Prxs would far out compete other peroxide-sensitive molecules and react with $\sim 90\%$ of mitochondrial hydrogen peroxide¹³ and almost 100% of cytoplasmic hydrogen peroxide¹¹.

For all Prxs, the first and chemically most interesting step of catalysis is peroxidation. This involves the Cp reacting with the peroxide substrate to form an enzyme-bound sulfenic acid (SpOH) and water (in the case of hydrogen peroxide). The conformation of Prxs that is competent for peroxidation is called the fully folded (FF) conformation. In this FF conformation, the conserved Cp is positioned in the first turn of helix $\alpha 2$ at the bottom of an active site pocket, surrounded by a trio of additional conserved residues: a Pro, a Thr (or occasionally Ser), and an Arg¹. The Pro and Thr are in a contiguous segment with the Cp, forming a PXXXTXXCp motif that has been referred to as the Cp-loop¹⁴. The conserved Arg comes from a position distant in sequence, and is positioned in the active site by stabilizing interactions from a residue one turn past the Cp in $\alpha 2$ that is commonly conserved as a Glu/Gln/His.

To carry out the peroxidation reaction, the Cp has long been presumed to be deprotonated because thiols are essentially unreactive towards peroxides in the absence of metals; thus proposals for how the enzymes work have focused on how the active site acts to lower the pK_a of the Cp from the typical Cys pK_a value of ~ 8.4 or higher¹⁵. Indeed, pK_a measurements of a few Prxs show values ranging from 5 to 6^{8,16,17}, implying that over 90% of the Cp will be deprotonated at pH 7. Although this begins to shed light on the catalytic power of Prxs, the presence of a Cp-thiolate can only explain a reactivity on the order of $\sim 20 \text{ M}^{-1} \text{ s}^{-1}$ ^{18,19}, leaving a factor of $\sim 10^6$ in the catalytic rate unaccounted for. Also noteworthy is that the Prx tertiary structure selectively directs this high reactivity towards peroxides⁸. This has presented the conundrum, what is it about these active sites that makes the Prxs so reactive and selective?

Here, we propose an answer to this question. We first present a novel structure of human PrxV with oxidized dithiothreitol (DTT) bound in the active site, with its diol-moiety near the Cp. We explore the details of DTT-binding as well as the binding of ligands in other Prx structures, including a recently determined H_2O_2 -bound structure²⁰ (Table 1). Then, by considering the peroxide reduction not as a redox reaction but as an $\text{S}_\text{N}2$ reaction, we show how the active site microenvironment activates not only the Cp to be a potent nucleophile, but also activates the peroxide substrate, and as a whole is remarkably well suited to stabilize the transition state of the reaction.

RESULTS

Structure determination of *HsPrxV* bound to DTT

Crystallization studies—For studying ligand binding to Prxs, we chose the system of human PrxV (*HsPrxV*). This system has yielded well-diffracting crystals containing an active site ligand (benzoate)^{21,22,23}, and provided key contributions to our understanding of Prxs. In our attempts to grow crystals of ligand-free wild type *HsPrxV* for soaking studies, DTT was added to the protein stock solution prior to crystallization to keep the *HsPrxV* protein in a reduced form. Optimization of the crystallization conditions with or without benzoate resulted in reliably grown, large, single crystals of *HsPrxV* isomorphous to the previously studied *HsPrxV*-benzoate complex (*HsPrxV*·BEZ)²¹. Crystals grown in the presence of benzoate contained bound benzoate in the active site as expected (data not shown) and those grown without benzoate surprisingly did not have an empty active site pocket but contained an oxidized DTT molecule (*HsPrxV*·DTT). Attempts to get ligand-free crystals using the reductant TCEP instead of DTT did not produce crystals unless benzoic acid was also added to the crystallization drop. Further cocrystallization trials of DTT-reduced protein mixed with sodium acetate, ethanol, isopropanol, tert-butanol, 1-pentanol, 1-hexanol and benzyl alcohol resulted in isomorphous crystals, however all had bound DTT.

The *HsPrxV*·DTT structure—The structure of *HsPrxV*·DTT was refined at 1.45 Å resolution yielding a final R/R_{free} of 11.3% /13.6% (Table 2). The final model contains residues 1 through 161, and 1 DTT, 2 glycerol, 8 bromide and 288 water molecules, and was refined with individual anisotropic B-factors. The N-terminal His tag was not visible in the density and so was not modeled. The backbone for residues 2 through 161 is well ordered and has high-quality electron density (Figure 1). Clearly visible alternate side chain conformations were fit for Asn21, Glu27, Lys63, Ser74 and Lys93. *HsPrxV*·DTT adopts the standard Prx-fold and is in a fully folded conformation (Figure 2).

The structure of the *HsPrxV*·BEZ has been determined previously in the same crystal form and has been well described²¹. The *HsPrxV*·DTT structure determined here has no notable differences in the overall conformation or main chain B-factor values compared to *HsPrxV*·BEZ (rmsd of 0.10 Å for 161 Cα atoms, <ΔB_{main-chain}> = 2.8 Å²). Differences appear to be limited to the slightly higher resolution and the modeling of an additional 66 water molecules and 3 bromide ions, as well as a DTT molecule in the active site instead of the benzoate. Features of the *HsPrxV*·DTT structure already well described in previous publications^{21,22,23} will not be discussed here.

The bound DTT is well ordered and its conformation was unambiguously defined by unbiased electron density (Figure 1A). Within the active site, the DTT molecule binds in an unexpected orientation. Instead of the reactive sulfur atoms (typically thought of as the “business end” of the molecule) interacting with the C_p, the diol-hydroxyls are placed where one is in van der Waals contact with the C_p and where they are held in place by four hydrogen bonds to the protein (Figure 3). In this orientation, the six-membered ring of DTT mimics the benzene ring of benzoate, and the two diol-hydroxyls are placed similarly to the oxygens of the benzoate carboxylate moiety (Figure 1B inset). The DTT molecule is not only in the active site pocket, but also is involved in crystal packing interactions as its ring moiety packs against the backbone of a loop involving residues 64 through 66 of a symmetry mate. The importance for crystal formation of these packing interactions involving the DTT (or benzoate in *HsPrxV*·BEZ) is supported by two observations: first, no *HsPrxV* crystals form unless benzoate or DTT is present and second, the DTT-containing crystals do not appear for approximately four days, giving time for a sufficient amount of the oxidized DTT (disulfide form) to accumulate in the drops. Although the DTT (or benzoate) molecule appears to be necessary for crystal growth, it is the binding of the

oxygen atoms in the active site that is of most interest here because of the expectation that they, like the benzoate carboxylate¹, may mimic peroxide binding and thus give insight into the catalytic mechanism.

Survey of ligands that mimic peroxide binding in the Prx active site

This *HsPrxV*-DTT structure becomes the highest resolution Prx structure known and the seventeenth structure of a Prx with a ligand bound in the active site that may, in some way, mimic substrate binding (Table 1). These Prx-ligand structures include 73 ligand-bound chains and represent seven different Prxs from four of the six known subfamilies: two Prx6s, two Prx5s, one Tpx and two BCPs. The nine ligands represented are H₂O₂, glycerol, acetate, ethanediol, benzoate, DTT, sulfate, citrate and formate; of these, the *Aeropyrum pernix* Tpx (*ApTpx*)-H₂O₂ structure even appears to provide a true Michaelis complex (productively bound substrate) and we use it here as the reference structure for substrate binding. Additionally, the structure of a Prx from *Xanthomonas campestris* (*XcBCP*) has been determined with a molecule of DNS (naphthalene-2,6-disulfonic acid) binding near the active site pocket in a manner proposed to represent the alkyl moiety of organic peroxide substrates²⁴. As the DNS ligand does not occupy the peroxide-binding portion of the active site, it is not included in the following comparisons.

An overlay of all of the Prx-ligand structures onto the *ApTpx*-H₂O₂ structure reveals that the Prx FF active site geometry that has been considered to be conserved across all Prxs¹ actually comes in two varieties involving distinct conformations of the conserved Arg residue (Figure 4). Differences in backbone position and χ_1 -angles between the two groupings result in an ~1 Å difference in placement of the guanidinium group within its plane (Figure 4A). A more in-depth analysis of all FF Prx structures (Figure 4B) shows that the two distinct conformations for the Arg are not ligand-dependent, but are partitioned by subfamily with Prx1, Prx6 and AhpE subfamilies adopting position I, and all other Prxs adopting position II. The distinct position of the Arg in the Prx1, Prx6 and AhpE subfamilies is associated with the presence of a second Arg residue conserved in these subfamilies (residue 149 in *ApTpx*). The second Arg interacts with the backside of the Glu (residue 53 in *ApTpx*) that stabilizes the active site Arg (Figure 4). This Arg-Glu-Arg hydrogen bonding network leads the guanidinium group of the active site Arg to be in position I. When the second Arg is not present, the Glu (or other supporting residue) adopts a different conformation and the universally conserved active site Arg is situated in position II.

By comparing each of the Prx-ligand structures to the reference *ApTpx*-H₂O₂ structure, we can directly address how all the ligand oxygen positions compare with those of H₂O₂. For clarity, we distinguish the two oxygen atoms of the bound peroxide by labeling them O_A and O_B, with O_A being proximal to the C_P-thiolate and O_B being distal (Figure 5A). The overlays reveal three distinct binding modes for the ligands (Figure 5): (1) ligands binding with oxygen atoms in the positions of both O_A and O_B; (2) ligands binding with an oxygen atom in the position of O_A only; (3) ligands binding with an oxygen atom in the position of O_B only. Two of the ligands, ethanediol and glycerol (in structures 1X0R and 3A2W), are observed in more than one of these binding modes. In the following sections, we describe the ligands observed for each binding mode (Table 1 and Figure 5) and then summarize them along with the modes seen for water binding in the active site.

Ligands with oxygens occupying both the O_A and O_B positions—DTT, ethanediol, glycerol, citrate, acetate and benzoate all have examples showing binding with oxygen atoms at positions close to those of both the O_A and O_B atoms of H₂O₂. In all cases except for citrate, the two ligand oxygens of a diol or carboxylate straddle the positions of the O_A and O_B atoms. There are single examples of DTT and ethanediol (Figure 5B)

binding and two examples each of glycerol and citrate binding. Glycerol is found in the active sites of two chains of an *ApTpx* structure (Figure 5C). Citrate binding is observed in the two chains of the structure of *SsBCP1* (Figure 5D). As was noted above, a single carboxylate of the citrate molecule does not straddle the O_A and O_B positions; rather an oxygen atom from each of two carboxylates of citrate occupies the positions of O_A and O_B , respectively.

For acetate-binding, 12 examples exist (Figure 5E). Ten are from a single structure of *ApTpx* and the other two are from a structure of *MtTpx*. The ten acetate molecules in the *ApTpx* structure overlay in a tight grouping with an ~ 0.5 Å spread in position and the acetate molecules bound in *MtTpx* are similar to each other but shifted ~ 0.3 Å from the *ApTpx*-grouping in the direction of the active site Arg. The conserved Arg is in position I in *ApTpx* and in position II in *MtTpx*.

Lastly, the most abundantly represented ligand in this group is benzoate with 30 examples (Figure 5F): 16 are from 3 different structures of *AmPRDX6* and 14 are from 6 different structures of *HsPrxV*. In all cases, the carboxylate is roughly coplanar with the benzene ring as expected to allow for resonance stabilization. Within each group the structures overlay closely (~ 0.5 Å), but the benzoate molecules in *HsPrxV*·BEZ structures are twisted an approximate 45° compared to the benzoate molecules in *AmPRDX6*·BEZ structures where the benzoate carboxyl is more in-line with the S_P atom. The variability in the benzoate conformation appears related to the presence in *HsPrxV* of Ile119 and Phe120 lining the active site pocket on one side of the benzoate, and different backbone conformations for a loop on the other side of the benzoate (not shown). These features alter the shape of the active site pocket and force the benzene ring to rotate. Additionally, the two groups of benzoate-bound structures also have different positions of the active site Arg; in *AmPRDX6*, the Arg is in position I and in *HsPrxV*, the Arg is in position II.

Ligands with oxygen occupying only the O_A position—Formate, ethanediol and glycerol all have examples showing binding with an oxygen atom occupying the position of the O_A atom of H_2O_2 only. Formate binding as O_A is seen in a structure of *XcBCP* (Figure 5G). Surprisingly, the formate does not bind in an equivalent location to the other carboxylates that straddle the positions of O_A and O_B in the active site; in fact, the single oxygen binding position for the formate, at 1.5 Å away from the position of O_A in H_2O_2 , is the most distinct of all the ligands. However, even with this shift, interaction geometries in the active site indicate that the formate molecule is still stabilized by similar hydrogen bonds as the other ligands.

For ethanediol binding in just the O_A site, four examples exist. All are from a structure of *ApTpx* (Figure 5H), and a comparison of the four molecules shows an ~ 0.8 Å spread in the oxygen positions. Glycerol binding in just the O_A site is seen in five chains of a structure of *ApTpx* (Figure 5I). While the position of the oxygen atom that mimics O_A is well conserved in three of the four chains, the rest of the glycerol molecule is varied while retaining a general trend of orientation towards the opening of the active site pocket.

Ligands with oxygen occupying only the O_B position—A sulfate ion is the sole ligand that has been observed to bind with an oxygen atom that occupies the position of the O_B atom of H_2O_2 only. This is seen in the two chains of the structure of *PtPrxD* (Figure 5G), and the sulfate positions overlay very closely (~ 0.1 Å shift).

Summary and comparison with water positions in unliganded FF active sites—A summary of all the relevant oxygen positions from the 73 examples of bound ligands (Figure 6A) emphasizes how well the binding of this whole set of molecules mimics the

peroxide binding. In compiling the series of ligand-bound structures and viewing their oxygen positions, we realized that even for unliganded active sites, water molecules can be considered as analogs of peroxide substrate. A survey of such unliganded FF structures revealed 55 chains from 12 structures with water molecules positioned in the active site. An overlay of these structures reveals that compared to the H_2O_2 molecule in $\text{ApTpx}\cdot\text{H}_2\text{O}_2$, the positions of observed water molecules are less clustered around the O_A and O_B positions themselves, but form a trough along the O_A - O_B axis that is in-line with the S_P atom and extends from a distance of 2.5 to 5.8 Å from the S_P (Figure 6B).

DISCUSSION

In this work, we have presented a novel structure of HsPrxV bound to DTT. Although not commonly thought of outside its role as a dithiol, DTT is a diol and in the $\text{HsPrxV}\cdot\text{DTT}$ complex, the two oxygen atoms of DTT bind in positions similar to the two oxygen atoms of a peroxide substrate in the Prx active site (Figure 5B). Extending the analysis of substrate binding to all known Prx-ligand structures (including a true Michaelis complex and water molecules) reveals a common theme of compounds binding in the active site along a narrow trough or track with two oxygen atoms separated by ~2 to 4 Å. The consistency of these structures implies that there is an active site energy landscape common to all Prxs. Using this information, we can now dissect the interactions in the Prx active site, and considering basic principles of enzyme catalysis, understand the origins of Prx catalytic power.

The peroxidation step as a simple $\text{S}_\text{N}2$ displacement

Enzymes are proteins that increase the rate of chemical reactions. As was first proposed by Pauling²⁵, enzymes are thought to accelerate reactions by binding most favorably to the transition state, as this will effectively lower the activation energy of the reaction^{26,27}. Powerful support for this concept is that compounds that resemble the transition state (i.e. transition state analogs) are often seen to bind many orders of magnitude more tightly than true substrates²⁸. Among enzymologists, a multitude of specific explanations and terms have been used to describe conceptually distinct contributions to the origins of catalytic power. However all of these explanations fit within the general framework of stabilizing the transition state, and in any given enzyme, many of these will concurrently contribute to its efficacy²⁶.

The net reaction that is catalyzed by the Prxs (together with their partner enzymes) is the addition of two hydrogen ions (H^+) and two electrons to H_2O_2 to produce two H_2O molecules:

Prxs catalyze this reaction by forming a catalytic covalent intermediate²⁸ ($\text{S}_\text{P}\text{OH}$). This changes the direct reduction of H_2O_2 to two H_2O molecules into a three-step mechanism,

with Rxn 3 representing the reductive recycling of the Prx by a system such as thioredoxin reductase/thioredoxin. The key peroxidation step of the reaction mechanism (Rxn 1) is chemically a simple S_N2 displacement reaction. As described in standard organic chemistry texts²⁹, an S_N2 reaction is a nucleophilic bimolecular substitution reaction where a nucleophile (electron rich) attacks an electrophile (electron poor) to form a nucleophile-electrophile bond and break the bond between the electrophile and the leaving group. In an S_N2 reaction, there are no stable intermediates and both the nucleophile and leaving group typically carry a negative charge. The incoming nucleophile attacks the electrophile 180° from the leaving group. In the transition state, the electrophilic atom at which the substitution reaction is taking place transitions from an sp^3 hybridized molecular orbital to an sp^2 hybridized molecular orbital, shifting the three substituents that remain bonded to the electrophile into one plane that is perpendicular to the line of the bond making and breaking. The partial bonds between the nucleophile-electrophile and electrophile-leaving group are formed by the overlap of lone pairs of the nucleophile and leaving group with the p orbitals of the electrophile, which explains the preferred in-line (i.e. 180°) attack geometry. The negative charge is distributed between the nucleophile and leaving group in the transition state, and in the course of the reaction, the stereochemistry on the electrophilic atom becomes inverted. In Prxs, the S_P atom is the nucleophile, the O_A atom is the electrophile and the O_B atom is the oxygen atom of the hydroxide (or alkoxide) leaving group (see Figure 7).

Structural origins for Prx catalytic power

Prxs share a common FF active site that includes a nearly universal PXXXTXXC_P peptide segment, as well as a conserved Arg that is distant in sequence¹. The conserved C_P, Pro, Thr and Arg are presumed to be required for catalysis and their constellation in the active site is spatially conserved¹, yet until now, specific roles for each of these residues have not been clearly proposed. The consensus has been that the Arg lowers the pK_a of the C_P and stabilizes the C_P-thiolate, that the Thr may also contribute to a lowered C_P pK_a value and play a role as a proton shuttle, and that the Pro shields the C_P from water and positions the subsequent peptide nitrogen to donate a hydrogen bond to contribute to activating the C_P^{1,14}. Now, with the known substrate bound structure (*ApTpx*·H₂O₂²⁰) and many ligand-bound structures, we can explore the geometry of the active site to gain further insight into the peroxidatic reaction and for the first time, propose specific roles for the conserved residues.

The dissection of active site geometries reveals two structural aspects of the Prx active site that help explain the previously unaccounted for rate enhancements of Prx-catalyzed peroxide reduction. First, we see that the Prx active site activates not only the C_P-thiolate but also the incoming peroxide substrate. Second, we see that the Prx active site is organized to maximally stabilize the transition state over substrate or product conformations. The details of these structural origins for Prx catalytic power and a specific proposal for the roles of conserved residues are discussed in the following two sections.

The Prx active site activates both the C_P and the peroxide substrate—Including the structure of *HsPrxV*·DTT determined here, we identified 17 Prx structures with a ligand bound in the active site, resulting in a total of 73 unique examples (Table 1). All of these ligands bind with an oxygen atom that mimics either the position of O_A , O_B or both the O_A and O_B atoms of the peroxide substrate. While geometry varies depending on the ligand, all of the bound ligands have similar hydrogen bonding geometries compared to the H₂O₂ molecule in *ApTpx*·H₂O₂ (Figure 5, Figure 6A), suggesting that all are acting as an analog of the substrate, transition state or product.

An overlay of Prx-ligand structures reveals that in most cases, the same ligand binds in a similar manner in different structures (Figure 5). The two exceptions are glycerol and ethanediol. For both of these, two binding modes are observed; one where ligand oxygen atoms bind as both O_A and O_B , and in the second case, only one oxygen atom of the ligand binds as O_A . This variation may be due to the conformational flexibility of these small ligands. Additionally, the ability of the some Prx active sites to interact with the alkyl groups of organic peroxide substrates may contribute competing forces involved in directing ligand binding.

The highly consistent positioning of ligand oxygens or water molecules in the active site (Figure 6) paints a clear and consistent picture of the key interactions common to Prxs. Using the peroxide-bound structure, Figure 7 summarizes the hydrogen bonding interactions that are present directly in the active site and some secondary hydrogen bonds that support the positions of the primary conserved residues. Within the active site, the two sets of interactions to consider in terms of the peroxidation reaction are first those involving only protein atoms, and second those involving the bound substrate molecule.

In terms of the interactions among protein atoms, the C_p -thiolate is stabilized and activated for nucleophilic attack on the peroxide substrate by hydrogen bonds with the conserved Arg and with a backbone amide hydrogen. The Arg is positioned by supporting hydrogen bonds to a side chain mostly conserved as a Glu/Gln/His and to one or two backbone carbonyls from the loop between $\beta 7$ and $\alpha 5$ that provide passing chain stabilization. The conserved Pro (residue C_p -7) provides a hydrophobic surface that helps to shield the activated C_p from unwanted reactions with solvent and positions the backbone of the following residue (C_p -6) to form two hydrogen bonds: the amide hydrogen is a donor to the C_p -thiolate and the carbonyl is an acceptor from the conserved Thr side chain hydroxyl (Figure 7). The presence of this Thr-to-carbonyl hydrogen bond specifies, as noted below, that the Thr must act as a hydrogen bond acceptor in its other interactions.

To consider how this environment contributes to catalysis, interactions with the peroxide molecule become important. In the H_2O_2 -bound structure, the H_2O_2 molecule is well-positioned for an S_N2 reaction, with the O_A atom ~ 3.5 Å away from the S_p with a $S_p \cdots O_A \cdots O_B$ angle of 172° , and is stabilized by four hydrogen bonds: the backbone amide hydrogens of the C_p and its preceding residue (C_p -1) and the NH of the conserved Arg side chain act as donors, and the conserved Thr acts as an acceptor for a hydrogen bond from the hydrogen on the O_A atom. Once the O_A - O_B bond is broken, the hydroxide (alkoxide) leaving group must be protonated; as no residue appears poised to be a proton donor and because the oxygen is highly accessible to bulk solvent, it seems reasonable that a water molecule will fulfill this role.

The two positions of the conserved Arg present in the Prx1, Prx6 and AhpE subfamilies (Arg I) versus the other Prx subfamilies (Arg II) do not significantly change the active site geometry. First, the variability in the placement of the guanidinium group seen in the entire Prx family shows that the two groupings spread enough to occupy overlapping space (Figure 4B). Second, when one considers that along with the shift in position, there is a slightly altered orientation of the guanidinium group (visible in Figure 4A), it becomes apparent that the hydrogen bonding acceptor atoms can be positioned in approximately the same place. This is most evident from the consistency in ligand positions in active sites containing an Arg in position I or II. Acetate bound to structures from both Arg classes are placed similarly (Figure 5E), and although bound benzoate binds in two slightly shifted positions (Figure 5F), this appears to be due to active site features unrelated to the Arg position (see Results). Thus the Arg I and II conformations would seem to represent alternate ways to stabilize the same geometry of reactants for efficient peroxidation.

The identification of these active site hydrogen bonds reveals three novel considerations for catalysis. First, the active site is not only activating the C_P to be a potent nucleophile, but is also positioning and thus activating the peroxide to be attacked. Secondly, we can now clearly propose specific roles for the three conserved residues around the C_P: the Pro shields the activated C_P-thiolate from unwanted reactions and positions the backbones of the two following residues for hydrogen bonding; the Thr positions and activates the protonated O_A atom of the incoming peroxide substrate; the Arg positions and activates both the C_P-thiolate and the O_A atom of the incoming peroxide substrate. Lastly, the conserved presence of the C_P in the first turn of a helix can now be seen to be crucial, as it places the backbone NH groups of residues C_P and C_P-1 where they can position and activate the incoming peroxide substrate for attack.

The Prx active site stabilizes the transition state—The geometries of the oxygen atoms of the ligand molecules, and especially the waters, define a “trough” (Figure 6) that shows what might be called an oxygen track along which there is an implied continuous well with favorable energy for the binding of oxygen atoms. This oxygen track is very well aligned along the S_P-O_A-O_B axis and in the case of the different ligands, the specific position occupied must reflect an optimal balancing of multiple hydrogen bonding interactions and van der Waals and other interactions of the ligand molecules. The results suggest that once aligned in the fairly narrow, in-line attack trough, oxygen atoms can be similarly stabilized at a number of positions along the axis, suggestive of their ability to slide along this axis during motions associated with the bond breakage and formation that takes place during peroxidation.

Based on the Michaelis complex seen in the *ApTpx*·H₂O₂ structure, we have generated a model for the product complex for the same enzyme and a simplistic model for the transition state in order to illustrate the reaction trajectory for the peroxidatic S_N2 reaction (Figure 8). Fortuitously, we noted that Figure 1B of the report of the *ApTpx*·H₂O₂ complex²⁰ showed difference electron density indicating that some S_POH product had formed during the 20 second peroxide soak used to generate the structure studied. By refining that model further (see Materials and Methods), we have shown the product occupancy is about 25% and that it provides an excellent model for the S_POH product structure that is ideally matched to the substrate complex (instead of, for instance being from a different protein). In this product complex, compared to the substrate complex, the only atom that has moved >0.2 Å is O_A, which moved 1.9 Å; S_P moved just 0.13 Å. Based on these two structures, we created the transition state model (Figure 8B) by simply positioning the O_A and S_P atoms half way between their positions in the substrate and product complexes. We realize that in the true reaction coordinate, O_A and S_P may move differently and that more atoms (especially O_B and the Arg and Thr side chains) may be shifting to optimize interactions as the reaction proceeds. In particular, we expect that the S_P will first move toward the O_A as the S_P-O_A bond forms before it ultimately moves to the further away position seen in the product structures.

One notable feature seen in these images is the very clear inversion of the stereochemistry of the protein hydrogen bonds to O_A in the substrate versus the product (compare Figures 8A and 8C), consistent with an S_N2 reaction. Inspection of the geometries shown in Figure 8 suggests a couple of additional stereoelectronic factors that would seem to promote reactivity. The first is that based on the hydrogen-bonding environment, the *sp*³ orbitals of the thiolate and the O_A atom are held in a somewhat staggered conformation relative to each other. The other such factor is that the presence of the non-polar Pro residue on the upper backside of the thiolate may specifically promote its nucleophilicity by pushing the lone pair electrons into the reactive O_A-facing *sp*³ orbital. Also notable is how even with this simple analysis, it can be seen that neither the substrate nor product complexes have optimal

geometry for all hydrogen bonding interactions and that in the modeled transition state complex some of these interactions could have improved interaction geometry compared to the ground state. For example, if the S_P atom moves forward as the bond forms, that would allow the two hydrogen bonds it accepts to align better with its *sp*³ orbitals. Whereas the analysis we have done is rather simplistic, we suggest that the substrate-bound and product-bound structures presented here provide an ideal foundation for quantum mechanics/molecular dynamics simulations to better map the structural and energetic details of the peroxidation reaction. Indeed, we suggest that the simplicity of the chemistry involved, the abundant structural information available and the consistency of the active site geometry over the broad family make the Prxs an excellent model system for theoretical studies of enzyme catalysis.

Aside from the details that remain to be fleshed out, the structures analyzed here make it abundantly clear that the Prx active site is organized not just to activate the C_P-thiolate, but to stabilize the transition state of an in-line displacement reaction that is highly specific for a peroxide substrate having a hydrogen on its terminal oxygen (i.e. ROOH). This provides a compelling explanation for the catalytic power of Prxs and a rationale for the observation by Peskin *et al.* that Prx tertiary structure “facilitates reactions of the active site thiol with hydrogen peroxide while restricting its reaction with other thiol agents” such as chloramines, iodoacetamide and N-ethyl maleimide⁸.

Diols and carboxylates as classes of transition state analog inhibitors for Prxs

As the Prx active site is explicitly arranged to bind to the transition state of the peroxidatic reaction, transition state analogs should bind more tightly in the active site than true substrate²⁶. Combining the newly determined HsPrx·DTT complex with the 16 other liganded complexes seen for Prxs, we can now recognize carboxylate compounds and diols as two classes of molecules that can be considered transition state analogs for the peroxidation reaction. That they are transition state analogs rather than substrate or product analogs can be seen by considering the O_A■O_B separation distance. For the peroxide substrate, O_A■O_B separation is a covalent interaction at ~1.5 Å and in the product, this increases to become a noncovalent approach of ~3 Å. For carboxylates and diols, like the high resolution DTT structure reported here, the oxygen separations are in between these values at ~2.3 and 2.5 Å, respectively. These types of ligands thus are transition state analogs in that they sample intermediate positions along the linear oxygen track that is created in the Prx active site. To the best of our knowledge, this is the first proposal of any broad classes of small molecules that can serve as inhibitors for Prxs, and thus provides a jumping point for rational drug design efforts aimed at this widespread enzyme family.

MATERIALS AND METHODS

Protein purification and crystallization

The gene for expressing wild type cytosolic human PrxV (HsPrxV) was synthesized by GenScript with a hexahistidine tag. Protein was expressed in *Escherichia coli* strain B834 (pREP4) using a pQE-30 plasmid, purified as previously described²¹, and stored at 0.8 mM (14.6 mg/mL) in phosphate-buffered saline at pH 7.2. Crystals of HsPrxV bound to DTT (HsPrxV·DTT) were grown at 18 °C using the hanging-drop vapor-diffusion method. For crystallization, 2 or 3 µL of reservoir solution containing 1.6 M ammonium sulfate, 0.1 M sodium citrate pH 5.6 and 0.2 M sodium/potassium tartrate (optimization of Hampton Crystal Screen II #14 and previously published conditions²¹) was mixed with 1 µL of protein stock reduced with 10 mM DTT. Single chunky crystals grew to a typical final size of 0.4 × 0.3 × 0.3 mm³ in four days. Crystals were transferred to artificial mother liquor like the reservoir but with 1.8 M ammonium sulfate, 1 M sodium bromide and 15% (v/v)

glycerol, then flash-frozen in liquid nitrogen for data collection. Without the addition of sodium bromide, crystals after freezing diffracted at best to 7 Å.

Data collection

Data were collected on two isomorphous *HsPrxV*·DTT crystals using beamline 5.0.1 at the Advanced Light Source (ALS, Lawrence Berkeley National Laboratory). Data were processed and scaled using iMosflm v1.0.0³⁰ and SCALA³¹, and 5% of the data were flagged for use in R_{free} calculations. Final statistics showed the merged data set is usable to 1.45 Å (Table 2).

Structure determination and refinement

The *HsPrxV*·DTT crystals were isomorphous with those used to determine the published *HsPrxV*·BEZ complex (pdb code 1HD2²¹), and that structure with the bound benzoate molecule removed was the starting model for refinement. All refinements were done using Coot³² and REFMAC³³, and Molprobity³⁴ was used to monitor the model geometry. Rigid body refinement of the starting model yielded an R of 21.2% and further restrained refinement at 1.45 Å resolution resulted in an $R/R_{\text{free}} = 17.7\% / 19.3\%$. The resulting $2F_o - F_c$ and $F_o - F_c$ electron density maps showed clear density in the active site pocket corresponding to a small molecule. This region was left uninterpreted during iterative manual rebuilding and addition of more water molecules using standard criteria in Coot ($> 1 \rho_{\text{rms}}$ intensity in the $2F_o - F_c$ map, > 2.4 Å distance from nearest contact). Two glycerol molecules and eight bromide ions were also added to the model. Use of restrained anisotropic B-factor refinement and the addition of riding hydrogens significantly improved the model, decreasing both R and R_{free} by ~3%.

Before the final rounds of refinement, the active site pocket density was initially interpreted as a benzoate. The inadequacy of this interpretation was indicated by a poor refined geometry for the benzoate (e.g. internal ring angles ranging from 105° to 130°), a poor fit to $2F_o - F_c$ electron density maps, and strong residual $F_o - F_c$ electron density. A survey of the other chemical components in the crystallization solution led us to model the disulfide form of DTT into the density. Ideal geometry values for the oxidized DTT molecule were obtained from HIC-Up³⁵. Refinement of the model containing DTT resulted in no significant difference map peaks in the active site and the DTT molecule having both good geometry and a good fit to the $2F_o - F_c$ map (Figure 1B).

For the final rounds of refinement, B-factor and bond distance restraint weights were optimized leading to a final R/R_{free} of 11.3% / 13.6%. In the final structure, water molecules were renamed by electron density strength in the final $2F_o - F_c$ map using an in-house program, with water 1 having the strongest density. Refinement statistics for the model are in Table 2. Structural overlays of the active site regions were performed in Pymol³⁶ using only the backbone atoms of residues in the universal, contiguous PXXXTXXC_p segment for the alignment.

Additional refinement of ApTpx·H₂O₂

The *ApTpx*·H₂O₂ complex structure²⁰ was further refined in order to explicitly model the SpOH product in the active site and estimate its occupancy. Structure factors and the starting coordinate file were obtained from pdb code 3A2V. Initial refinement at 1.65 Å resolution of the deposited structure using REFMAC decreased R/R_{free} to 18.4% / 20.0%. Further refinement, including the use of restrained anisotropic B-factors and riding hydrogens, and addition of water molecules were performed as described above for the *HsPrxV*·DTT complex. The final R/R_{free} values were 13.0% / 18.3% and a total of 1951 water molecules were included in the model.

The occupancy of the SpOH product was estimated in the final rounds of refinement. The strongest difference density peak off of the C_P, at 4.8 ρ_{rms} , was seen in chain F. To initially model the conformation of SpOH, the SpOH of another Prx structure (pdb code 1PRX chain A) was used as a template and showed that the OH position matched the difference density peak very well. Parallel refinements were carried out with varying occupancies of the SpOH from 0.1 to 0.5 in increments of 0.1. A comparison of the refined B-factors for the C_P residue atoms at the different occupancies was used to judge the correctness of the occupancy of the SpOH product. As occupancy increased from 0.1 to 0.5, the B-factor value for the terminal oxygen of the SpOH refined to 17.4, 22.3, 26.0, 29.0 and 31.5 Å², respectively, while the B-factor value for the sulfur atom remained ~21 Å². As we expect the B-factor for the terminal oxygen to be slightly higher than that of the sulfur, we expect that the true occupancy is between 0.2 and 0.3. The refined structure with 25% occupancy of the SpOH product in chain F is used as the model for the product conformation.

Generation of a model for the transition state structure

A model for the transition state of the peroxidatic reaction was generated using the framework of the FF ApTpx·H₂O₂ active site structure. Changes were only made to the positions for the O_A and S_P atoms. The transition state was approximated by placing the atoms half way between their positions in the substrate bound ApTpx·H₂O₂ structure and the product ApTpx structure described above. This resulted in an ~0.05 Å shift in S_P and an ~0.9 Å shift in O_A. The O_A atom was kept in-line with S_P during positioning.

Molecular graphics were created using Pymol³⁶ and figures were prepared using GIMP.

Acknowledgments

This work was supported by a grant from the National Institutes of Health to L.B.P. and P.A.K. (R01 GM50389). The authors acknowledge the Proteins and Nucleic Acids Core facility of the Environmental Health Sciences Center at Oregon State University (NIEHS grant P30 ES00210). The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

ABBREVIATIONS

Prx	peroxiredoxin
C_P	peroxidatic cysteine
C_R	resolving cysteine
S_P	sulfur atom of the peroxidatic cysteine
SpOH	sulfenic acid product
FF	fully folded
O_A	peroxide oxygen atom proximal to the S _P
O_B	peroxide oxygen atom distal to the S _P
HsPrxV	human PrxV
HsPrxV·DTT	human PrxV bound to DTT
ApTpx·H₂O₂	<i>Aeropyrum pernix</i> Tpx bound to H ₂ O ₂
TCEP	tris(2-carboxyethyl)phosphine
DTT	dithiothreitol

References

1. Karplus, PA.; Hall, A. Structural survey of the peroxiredoxins. In: Flohé, L.; Harris, JR., editors. *Peroxiredoxin Systems*. Springer; New York: 2007. p. 41-60.
2. Hall A, Karplus PA, Poole LB. Typical 2-Cys peroxiredoxins--structures, mechanisms and functions. *Febs J*. 2009; 276:2469-77. [PubMed: 19476488]
3. Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG. Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. *Proc Natl Acad Sci U S A*. 1994; 91:7017-21. [PubMed: 8041738]
4. Flohe L, Budde H, Hofmann B. Peroxiredoxins in antioxidant defense and redox regulation. *Biofactors*. 2003; 19:3-10. [PubMed: 14757972]
5. Akerman SE, Muller S. Peroxiredoxin-linked detoxification of hydroperoxides in *Toxoplasma gondii*. *J Biol Chem*. 2005; 280:564-70. [PubMed: 15507457]
6. Budde H, Flohe L, Hecht HJ, Hofmann B, Stehr M, Wissing J, Lunsdorf H. Kinetics and redox-sensitive oligomerisation reveal negative subunit cooperativity in trypanothione peroxidase of *Trypanosoma brucei*. *Biol Chem*. 2003; 384:619-33. [PubMed: 12751791]
7. Parsonage D, Youngblood DS, Sarma GN, Wood ZA, Karplus PA, Poole LB. Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. *Biochemistry*. 2005; 44:10583-92. [PubMed: 16060667]
8. Peskin AV, Low FM, Paton LN, Maghzal GJ, Hampton MB, Winterbourn CC. The high reactivity of peroxiredoxin 2 with H₂O₂ is not reflected in its reaction with other oxidants and thiol reagents. *J Biol Chem*. 2007; 282:11885-92. [PubMed: 17329258]
9. Trujillo, M.; Ferrer-Sueta, G.; Thomson, L.; Flohe, L.; Radi, R. Kinetics of peroxiredoxins and their role in the decomposition of peroxynitrite. In: Flohé, L.; Harris, JR., editors. *Peroxiredoxin Systems*. Springer; New York: 2007. p. 41-60.
10. Leyens G, Donnay I, Knoop B. Cloning of bovine peroxiredoxins-gene expression in bovine tissues and amino acid sequence comparison with rat, mouse and primate peroxiredoxins. *Comp Biochem Physiol B Biochem Mol Biol*. 2003; 136:943-55. [PubMed: 14662316]
11. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol*. 2008; 4:278-86. [PubMed: 18421291]
12. Parsonage D, Karplus PA, Poole LB. Substrate specificity and redox potential of AhpC, a bacterial peroxiredoxin. *Proc Natl Acad Sci U S A*. 2008; 105:8209-14. [PubMed: 18165315]
13. Cox AG, Winterbourn CC, Hampton MB. Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling. *Biochem J*. 2010; 425:313-25. [PubMed: 20025614]
14. Wood ZA, Schroder E, Harris JR, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci*. 2003; 28:32-40. [PubMed: 12517450]
15. Poole, LB. The catalytic mechanism of peroxiredoxins. In: Flohé, L.; Harris, JR., editors. *Peroxiredoxin Systems*. Springer; New York: 2007. p. 61-81.
16. Nelson KJ, Parsonage D, Hall A, Karplus PA, Poole LB. Cysteine pK(a) values for the bacterial peroxiredoxin AhpC. *Biochemistry*. 2008; 47:12860-8. [PubMed: 18986167]
17. Ogusucu R, Rettori D, Munhoz DC, Netto LE, Augusto O. Reactions of yeast thioredoxin peroxidases I and II with hydrogen peroxide and peroxynitrite: rate constants by competitive kinetics. *Free Radic Biol Med*. 2007; 42:326-34. [PubMed: 17210445]
18. Makarov VL, Kochkina VM, Torchinsky YM. Reorientations of coenzyme in aspartate transaminase studied on single crystals of the enzyme by polarized-light spectrophotometry. *Biochim Biophys Acta*. 1981; 659:219-28. [PubMed: 7018583]
19. Winterbourn CC, Metodiewa D. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic Biol Med*. 1999; 27:322-8. [PubMed: 10468205]
20. Nakamura T, Kado Y, Yamaguchi T, Matsumura H, Ishikawa K, Inoue T. Crystal structure of peroxiredoxin from *Aeropyrum pernix* K1 complexed with its substrate, hydrogen peroxide. *J Biochem*. 2010; 147:109-15. [PubMed: 19819903]

21. Declercq JP, Evrard C, Clippe A, Stricht DV, Bernard A, Knoops B. Crystal structure of human peroxiredoxin 5, a novel type of mammalian peroxiredoxin at 1.5 Å resolution. *J Mol Biol.* 2001; 311:751–9. [PubMed: 11518528]
22. Evrard C, Capron A, Marchand C, Clippe A, Wattiez R, Soumillon P, Knoops B, Declercq JP. Crystal structure of a dimeric oxidized form of human peroxiredoxin 5. *J Mol Biol.* 2004; 337:1079–90. [PubMed: 15046979]
23. Smeets A, Marchand C, Linard D, Knoops B, Declercq JP. The crystal structures of oxidized forms of human peroxiredoxin 5 with an intramolecular disulfide bond confirm the proposed enzymatic mechanism for atypical 2-Cys peroxiredoxins. *Arch Biochem Biophys.* 2008; 477:98–104. [PubMed: 18489898]
24. Liao SJ, Yang CY, Chin KH, Wang AH, Chou SH. Insights into the alkyl peroxide reduction pathway of *Xanthomonas campestris* bacterioferritin comigratory protein from the trapped intermediate-ligand complex structures. *J Mol Biol.* 2009; 390:951–66. [PubMed: 19477183]
25. Pauling L. *Chem Eng News.* 1946; 24:1375.
26. Garcia-Viloca M, Gao J, Karplus M, Truhlar DG. How enzymes work: analysis by modern rate theory and computer simulations. *Science.* 2004; 303:186–95. [PubMed: 14716003]
27. Schowen, RL. *Transition States of Biochemical Processes.* Gandour, RD.; Schowen, RL., editors. Plenum; 1978. p. 77
28. Jencks, WP. *Catalysis in chemistry and enzymology.* McGraw-Hill Book Company; New York: 1969.
29. McMurry, J. *Organic Chemistry.* 6e edit. Kiselica, S., editor. Brooks/Cole-Thomson Learning; Belmont: 2004.
30. Leslie AGW. Recent changes to the MOSFLM package for processing film and image plate data. *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography.* 1992; 26
31. Evans PR. Recent advances in phasing. *Proceedings of the CCP4 study weekend.* 1997:97–102.
32. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:2126–32. [PubMed: 15572765]
33. Murshudov GN, Vagin AA, Dodson EJ. Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr.* 1997; 53:240–55. [PubMed: 15299926]
34. Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB 3rd, Snoeyink J, Richardson JS, Richardson DC. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res.* 2007; 35:W375–83. [PubMed: 17452350]
35. Kleywegt GJ. Crystallographic refinement of ligand complexes. *Acta Crystallogr D Biol Crystallogr.* 2007; 63:94–100. [PubMed: 17164531]
36. DeLano, WL. *The PyMOL Molecular Graphics System.* DeLano Scientific; San Carlos, CA: 2002.
37. Nakamura T, Yamamoto T, Inoue T, Matsumura H, Kobayashi A, Hagihara Y, Uegaki K, Ataka M, Kai Y, Ishikawa K. Crystal structure of thioredoxin peroxidase from aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1. *Proteins.* 2006; 62:822–6. [PubMed: 16342268]
38. Smeets A, Loumaye E, Clippe A, Rees JF, Knoops B, Declercq JP. The crystal structure of the C45S mutant of annelid *Arenicola marina* peroxiredoxin 6 supports its assignment to the mechanistically typical 2-Cys subfamily without any formation of toroid-shaped decamers. *Protein Sci.* 2008; 17:700–10. [PubMed: 18359859]
39. Evrard C, Smeets A, Knoops B, Declercq JP. Crystal Structure of the C47S Mutant of Human Peroxiredoxin 5. *J of Chem Cryst.* 2004; 34:553–558.
40. Echalié A, Trivelli X, Corbier C, Rouhier N, Walker O, Tsan P, Jacquot JP, Aubry A, Krimm I, Lancelin JM. Crystal structure and solution NMR dynamics of a D (type II) peroxiredoxin glutaredoxin and thioredoxin dependent: a new insight into the peroxiredoxin oligomerism. *Biochemistry.* 2005; 44:1755–67. [PubMed: 15697201]
41. Stehr M, Hecht HJ, Jäger T, Flohe L, Singh M. Structure of the inactive variant C60S of *Mycobacterium tuberculosis* thiol peroxidase. *Acta Crystallogr D Biol Crystallogr.* 2006; 62:563–7. [PubMed: 16627951]

42. D'Ambrosio K, Limauro D, Pedone E, Galdi I, Pedone C, Bartolucci S, De Simone G. Insights into the catalytic mechanism of the Bcp family: functional and structural analysis of Bcp1 from *Sulfolobus solfataricus*. *Proteins*. 2009; 76:995–1006. [PubMed: 19338062]
43. Li S, Peterson NA, Kim MY, Kim CY, Hung LW, Yu M, Lakin T, Segelke BW, Lott JS, Baker EN. Crystal Structure of AhpE from *Mycobacterium tuberculosis*, a 1-Cys peroxiredoxin. *J Mol Biol*. 2005; 346:1035–46. [PubMed: 15701515]
44. Diederichs K, Karplus PA. Improved R-factors for diffraction data analysis in macromolecular crystallography. *Nat Struct Biol*. 1997; 4:269–75. [PubMed: 9095194]
45. Weiss MS, Hilgenfeld R. On the use of the merging R factor as a quality indicator for X-ray data. *J Appl Cryst*. 1997; 30:203–205.

Figure 1.

Electron density for bound DTT in the active site. Shown is the electron density in the active site (A) before including DTT in the model and (B) after the final round of refinement of the model containing DTT (*HsPrxV*·DTT). A peptide segment including the C_P and the two residues prior to it is shown in sticks and colored by atom (oxygen = red, nitrogen = blue, sulfur = yellow), and electron density is shown as mesh: 2F_o-F_c density is contoured at 1.5 ρ_{rms} (pale blue) and 6.5 ρ_{rms} (dark blue), and F_o-F_c density is contoured at 4 ρ_{rms} (green). Panel B inset: An overlay of the final *HsPrx*·DTT structure (dark grey carbons) with the published *HsPrx*·BEZ structure²¹ (light grey carbons; PDB entry 1HD2) in the same orientation as the main panel.

Figure 2.

The *HsPrx*-DTT structure. The protein backbone ribbon is shown with α -helices (green), β -strands (purple), coils/turns (grey), and the C_P residue (yellow) highlighted. The bound DTT molecule (colored by atom, oxygen = red, sulfur = yellow) is shown as sticks, and the surface of the active site pocket into which it binds is shown. Secondary structures are labeled according to the common fold of all Prxs¹; the short helix between α_4 and α_5 is not universally conserved in the Prx fold and is labeled simply as " α ".

Figure 3.

Stereoview of the *HsPrx*-DTT active site with bound DTT. Shown are the Cp-loop and the first turn of $\alpha 2$ plus the conserved Arg colored by atom as in Figure 1. Hydrogen bonding (cyan dotted lines) important for DTT binding and the S_P-O_A interaction (orange dashed line) are indicated. The four residues conserved in all Prxs and the His that interacts with the active site Arg are labeled.

Figure 4.

Two distinct positions for the conserved Arg in the Prx active site. (A) The overlaid active sites from all ligand-bound Prx structures (Table 1) are shown with side chains (shown as sticks) for the C_p, the conserved active site Arg, the supporting Glu/Gln/His residue and for those that contain it, a second Arg (indicated by an asterisk) of the Arg-Glu-Arg hydrogen bonding network. The carbon atoms are colored according to the position of the Arg, light grey for position I and purple for position II; oxygen (red), nitrogen (blue) and sulfur (yellow) are colored by atom. The distinct positioning of the conserved active site Arg correlates with the presence of the second Arg residue. (B) Same as panel A but including all FF Prx structures. The overlay includes 179 chains from 32 pdb entries (3A2V, 3A2W, 1QMV, 2Z9S, 2PN8, 1UUL, 2I81, 2V2G, 2V32, 2V41, 1XCC, 2CV4, 1X0R, 3A5W, 1HD2, 1H4O, 1OC3, 1URM, 2VL2, 1TP9, 2VL3, 1NM3, 1XIY, 3HVV, 1Y25, 1PSQ, 2YZH, 2CX4, 1XXU, 3MNG, 3DRN, 3GKM).

Figure 5.

Overlays of ligands that bind in the Prx active site. In all panels, a peptide segment containing the C_P-loop is shown as a ribbon in the same orientation as in Figure 3. Side chains are shown for the conserved Pro, Thr, C_P and Arg, and ligands are shown as sticks with coloring as in Figure 4. (A) H₂O₂-bound structure with O_A, O_B and S_P atoms identified. In the remaining panels, a bound H₂O₂ molecule (transparent pale green) is shown for reference. Panels B through I feature the following ligands: (B) DTT and ethanediol; (C) glycerol; (D) citrate; (E) acetate; (F) benzoate; (G) sulfate and formate; (H) ethanediol binding as only O_A; (I) glycerol binding as only O_A. The structures included in each panel are listed in Table 1.

Figure 6.

Summary of peroxide-mimics binding in the Prx active site. (A) The oxygen atoms (red spheres) mimicking O_A and O_B from all ligands shown in Figure 5. (B) Active site water molecules (red spheres) from unliganded FF structures. In both panels, the C_P-loop and conserved Arg are shown as sticks in the same orientation as Figure 3 and colored by atom type. As in Figure 5, a reference H₂O₂ molecule (pale green) is included for reference. In this figure the C_P-loop is extended by one residue to emphasize it is the first turn of helix α_2 by showing the first hydrogen bond of the helix (cyan). Waters shown are from pdb codes 1X0R, 1QMV, 2PN8, 1XXU, 1PSQ, 1XIY, 3A5W, 1CXX and 1N8J.

Figure 7.

Interactions in the Prx active site Michaelis complex. Shown is a schematic drawing of the peroxide-bound active site emphasizing the hydrogen bonding interactions present. The in-line geometry for S_p to attack O_A for the peroxidatic S_N2 reaction (bold dashed line) and key hydrogen bonding interactions (pale dashed, dotted, and solid lines) are indicated. The view is as in Figures 3, 5, 6 and 8. Backbone atoms in the C_p -loop are identified by their residue position relative to C_p . The negative charge on the C_p -thiolate is indicated.

Figure 8.

Reaction path for the peroxidatic S_N2 reaction. (A) The Michaelis complex structure as seen in *ApTpx*· H_2O_2 chain F (PDB entry 3A2V²⁰) shows how the active site aligns the peroxide substrate for an in-line attack from the C_P -thiolate (orange dashed line). (B) Simplistically-derived model for the transition state (see text) showing the partial S_P - O_A (orange dashed line) and O_A - O_B (red dashed line) bonds. The S_P - O_A and O_A - O_B distances are 2.6 and 2.4 Å, respectively. (C) The product complex as seen at ~25% occupancy in the rerefined *ApTpx*· H_2O_2 chain F (see text). The S_P O_A H and O_B hydroxide leaving group (red sphere) are shown. Stabilizing active site hydrogen bonds (cyan dotted lines) are shown in all panels.

Table 1

Published Prx structures with ligands bound in the active site

Structure ^a	Res. (Å)	Ligand Mimic	Oxygen ^b	Chain ^c	Mutation	PBD Code	Reference
Prx6							
<i>Ap</i> Tpx	1.65	H ₂ O ₂	N/A	all 10	C207S	3A2V	20
<i>Ap</i> Tpx	2.3	H ₂ O ₂	N/A	B,F,I	Cp50S	3A2W	20
<i>Ap</i> Tpx	2.3	glycerol	O _A , O _B	C,H	Cp50S	3A2W	20
<i>Ap</i> Tpx	2.3	glycerol	O _A	A,D,E,G,J	Cp50S	3A2W	20
<i>Ap</i> Tpx	1.9	acetate	O _A , O _B	all 10	Cp50S	3A2X	20
<i>Ap</i> Tpx	2.0	ethanediol	O _A , O _B	I	C207S	1X0R	37
<i>Ap</i> Tpx	2.0	ethanediol	O _A	A,B,C,D	C207S	1X0R	37
<i>Am</i> PRDX6	1.6	benzoate	O _A , O _B	A,B,C,D	Cp45S	2V2G	38
<i>Am</i> PRDX6	2.0	benzoate	O _A , O _B	A,B,C,D	Cp45S	2V32	38
<i>Am</i> PRDX6	2.4	benzoate	O _A , O _B	all 8	Cp45S	2V41	38
Prx5							
<i>Hs</i> PrxV	1.45	DTT	O _A , O _B	A	-	3MNG	this study
<i>Hs</i> PrxV	1.5	benzoate	O _A , O _B	A	-	1HD2	21
<i>Hs</i> PrxV	1.7	benzoate	O _A , O _B	A	Cp47S	1URM	39
<i>Hs</i> PrxV	1.92	benzoate	O _A , O _B	A,B	-	2VL2	38
<i>Hs</i> PrxV	1.95	benzoate	O _A , O _B	all 8	-	1H4O	21
<i>Hs</i> PrxV	2.0	benzoate	O _A , O _B	A,B	-	1OC3	39
<i>P</i> PrxD	1.62	sulfate	O _B	A,B	-	1TP9	40
Tpx							
<i>M</i> Tpx	2.1	acetate	O _A , O _B	A,B	Cp60S	1Y25	41
BCP							
<i>Ss</i> BCP1	2.15	citrate	O _B	A,B	Cp45S,Cp50S	3DRN	42
<i>Xc</i> BCP	1.53	formate	O _A	A	Cp48S,Cp84S	3GKM	43

Structure ^a	Res. (Å)	Ligand Mimic	Oxygen ^b	Chain ^c	Mutation	PDB Code	Reference
XcBCP	1.47	DNS ^d	-	A	C ₁ 48S	3GKN	43

^a Structures are divided by subfamily. Organism abbreviations are as follows: *Am* = *Arenicola marina*; *Ap* = *Aeropyrum pernix*; *Hs* = *Homo sapiens*; *Mt* = *Mycobacterium tuberculosis*; *Pt* = *Populus trichocarpa*; *Ss* = *Sulfolobus solfataricus*; *Xc* = *Xanthomonas campestris*.

^b The oxygen atom of the peroxide substrate that is mimicked by an oxygen atom of the substrate analog is listed.

^c The chains containing the ligand in the described binding mode are listed.

^d The DNS molecule (naphthalene-2,6-disulfonic acid) binds near the active site pocket, but has no atoms that mimic the oxygen atoms of the peroxide substrate.

Table 2

Data collection and refinement statistics

HsPrxV·DTT	
PDB code	
<i>A. Data Collection^a</i>	
Space group	P4 ₁ 2 ₁ 2
Unit cell axes (Å)	a=b=67.3 , c=123.9
Resolution limits (Å)	59-1.45 (1.53-1.45)
Unique observations	51438 (7365)
Multiplicity	36.7 (14.0)
Average I/σ	30.9 (6.9)
Completeness (%)	100.0 (100.0)
R _{meas} ^b (%)	9.5 (40.9)
R _{pim} ^c (%)	1.5 (10.9)
<i>B. Refinement</i>	
R _{cryst} (%) / R _{free} (%) ^d	11.3 / 13.6
Molecules in AU	1
No. protein residues	161
No. water/Br/DTT	288/8/1
rmsd bond lengths (Å)	0.012
rmsd bond angles (°)	2.7
< B > protein ((Å) ²)	12.5
< B > DTT ((Å) ²)	24.6
< B > water ((Å) ²)	35.2
Ramachandran plot ^e	
Favored (%)	96.9
Outliers (%)	0.0

^aNumbers in parentheses correspond to values in the highest resolution bin.

^bR_{meas} is the multiplicity-weighted merging R-factor⁴⁴.

^cR_{pim} is the precision-indicating multiplicity-weighted merging R-factor⁴⁵.

^dFive percent of the data were set aside for R_{free} calculations.

^eRamachandran plot generated using Molprobity³⁴.