Peroxisiredoxins (Prx) are thiol peroxidases that exhibit exceptionally high reactivity toward peroxides, but the chemical basis for this is not well understood. We present strong experimental evidence that two highly conserved arginine residues play a vital role in this activity of human Prx2 and Prx3. Point mutation of either ArgI or ArgII (in Prx3 Arg-123 and Arg-146, which are ~3–4 Å or ~6–7 Å away from the active site peroxidative cysteine (Cp), respectively) in each case resulted in a 5 orders of magnitude loss in reactivity. A further 2 orders of magnitude decrease in the second-order rate constant was observed for the double arginine mutants of both isoforms, suggesting a cooperative function for these residues. Detailed ab initio theoretical calculations carried out with the high level G4 procedure suggest strong catalytic effects of H-bond-donating functional groups to the Cp sulfur and the reactive and leaving oxygens of the peroxide in a cooperative manner. Using a guanidinium cation in the calculations to mimic the functional group of arginine, we were able to locate two transition structures that indicate rate enhancements consistent with our experimentally observed rate constants. Our results provide strong evidence for a vital role of ArgI in activating the peroxide that also involves H-bonding to ArgII. This mechanism could explain the exceptional reactivity of peroxiredoxins toward 

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investigation, Nakamura et al. (16) examined the archaean peroxiredoxin (ApTPx) in which a histidine residue (rather than an arginine) is in close proximity to C_p. (This His residue is absent at the active site of mammalian Prx.) Their theoretical study focused on the reaction steps subsequent to the reduction of H_2O_2, and they concluded that a hypervalent sulfurane intermediate is involved in the formation at that stage of a sulfenic acid (16). In contrast, the theoretical component of the present investigation focuses on the initial catalytic reduction of H_2O_2 by C_p.

In the current study, we have mutated ArgI and ArgII in human Prx2 and Prx3 and measured their rates of reaction with H_2O_2. We demonstrate that both arginines play important roles in the reactivities of Prx2 and Prx3, independently from one another as well as in a cooperative fashion. We also used high level ab initio calculations to mechanistically explain our findings and propose a novel model to elucidate the high reactivity of peroxiredoxins with peroxides.

**EXPERIMENTAL PROCEDURES**

Reagents, preparation of recombinant Prx and site-directed mutagenesis of arginine residues, biophysical characterization of the recombinant Prx, computational details, and additional computational results are described in the supplemental “Methods.”

**BLAST Search**—To analyze whether Arg-146 of human Prx3 is conserved among other human peroxiredoxin isoforms and whether it is conserved in other organisms, a BLAST search against NCBI protein reference sequences was performed using residues 121–170 of human Prx3 as a query sequence (BLASTP 2.2.24). All of the 250 retrieved reference proteins aligning with the query sequence contained a corresponding arginine at a similar position in the protein.

**Analysis of Oxidation State of Prx**—Reduced monomer and disulfide-bonded dimer were separated by nonreducing SDS-PAGE on 15% gels as in Ref. 6. Gels were Coomassie Blue-stained and scanned using a Fluor-S® Multimager (Bio-Rad). Bands were quantified using Quantity One® software from Bio-Rad.

**Kinetic Experiments**—Prior to each experiment, Prx was reduced with either 25 mM DTT (Prx3) in 50 mM phosphate buffer for 2 h or by tris(2-carboxyethyl)phosphine (Prx2) for 1 h at pH 7.4. Buffer solutions contained 1 mM diethylenetriaminepentaacetic acid to chelate trace metal contaminants. Reduced Prx were separated from excess DTT using Micro Bio-Spin 6 columns (Bio-Rad,) prewashed with 200 μl of 10 mg/ml bovine catalase and equilibrated with 50 mM phosphate buffer containing 100 μM diethylenetriaminepentaacetic acid. Prx concentrations were measured using Bio-Rad DC Protein Assay Reagent and bovine serum albumin as standard. For kinetic analyses, the initial concentration of reduced Prx was calculated based on the relative intensity of the bands representing oxidized and reduced forms determined by gel electrophoresis.

**Catalase Competition Assay**—As described (3), 8 μM Prx was reacted with equimolar H_2O_2 in the presence of bovine catalase (0.81–51 μM). Reactions were started by rapid addition of H_2O_2 (4.7 μM) at 20 °C, with vigorous stirring. Conversion of HRP to compound I was monitored spectrophotometrically by the loss of absorbance at 403 nm. The second-order rate constant for
the reaction of Prx with H₂O₂ was calculated based on the fractional inhibition and the reported rate constant ($k_{H11005} = 1.7 \times 10^7$ M⁻¹ s⁻¹) (17) of the reaction of HRP with H₂O₂.

**Measurement of Second-order Rate Constants of Arginine Mutants with H₂O₂**—Reactions were started by the addition of H₂O₂ to the mutant Prx during vigorous vortex mixing at 20 °C. Reactions were quenched either with 100 mM N-ethylmaleimide or 0.7 mg/ml catalase and analyzed by SDS-PAGE. Kinetic runs were carried out under pseudo first-order conditions with 5–10 μM Prx and 13–50 μM or 2.3–5 mM H₂O₂ for the single and double mutants, respectively. Pseudo first-order rate constants were obtained by correcting the pseudo first-order rate constants with the applied H₂O₂ concentrations. Kinetic curves were analyzed by SigmaPlot 11.

**Ab Initio Calculations**—Full computational details are presented in the supplemental data. Here, we note in addition that our discussion is based on enthalpies ($H$) rather than free energies ($G$) because our small model approach would not adequately take into account the preorganization provided by the enzyme (18). However, the latter quantities show the same qualitative trends (supplemental Table S2). We refer to the electrophilic oxygen of H₂O₂ (the one being attacked by HS⁻) as $O_a$ and to the oxygen of the leaving hydroxide group as $O_b$ (14). The theoretical results reported in this paper correspond to the CPCM-G4//B3-LYP/6–31+G(2df,p) level, which denotes the combination of a conductor-like polarizable continuum model (CPCM) solvation correction on top of a gas-phase G4//B3-LYP/6–31+G(2df,p) enthalpy.

**RESULTS**

**Two Highly Conserved Arginine Residues Play a Key Roles in the Peroxidase Activity of Prx2 and Prx3**—Single substitution of Arg-146 (ArgII) with various residues, Arg-123 (ArgI) with glycine, and a double substitution of both arginine residues with glycine, were introduced in Prx3. ArgI (Arg-127) and ArgII (Arg-150) in Prx2 were replaced with lysine, either singly or as a double mutant. WT recombinant proteins were constructed under the same conditions. Commercially available and recombinant WT and mutant proteins displayed similar CD spectra, suggesting that secondary structures were not affected by mutations (supplemental Fig. S1). Melting points of the recombinant WT and mutant proteins (supplemental Table S1) show that, although R146A and R146H of Prx3 behaved differently, other mutants melted at a similar temperature to the WT.

The reactivities of the WT recombinant proteins with H₂O₂ were investigated using competition kinetic assays against increasing amounts of bovine catalase. Prx2 and Prx3 are 2-Cys peroxiredoxins that form disulfide bonds between the Cₚ on one chain and the resolving cysteine on the other (Cys-172 and Cys-168 in Prx2 and Prx3, respectively). Oxidation can therefore be followed as dimer formation on a nonreducing gel. As shown in Fig. 2a, addition of H₂O₂ converted both Prx from reduced monomers to dimers. Catalase competed poorly against WT Prx2 and Prx3, with only marginal protection against dimerization at the highest concentration. This indicates a fast reaction between Prx and H₂O₂ and agrees with previous studies using purified human erythrocyte Prx2 (5) and commercially available Prx3 (3).
Prx also were competed against a constant amount of HRP for 
H₂O₂ as described (12). H₂O₂ reacts rapidly with the ferric 
form of HRP to give a stable intermediate (compound I) that has 
a lower Soret band absorbance at 403 nm. The reaction was 
quantified by measuring the decrease in this absorbance. The 
decrease was inhibited by increasing concentrations of the 
recombinant WT Prx (Fig. 2, c and d). Analysis of the competi-
tion data gave apparent second-order rate constants for re-
action with H₂O₂ that are similar to those reported for isolated 
and commercially available Prx (kₑff ~ 2 × 10⁷ M⁻¹ s⁻¹; Table 1) 
(3, 5).

With the arginine mutants, catalase was fully protective even 
at the lowest concentration, and the Prx mutants did not inhibit 
the formation of compound I by HRP. This indicates that all of 
the arginine mutants are several orders of magnitude less reac-
tive than the WT proteins. Reactions with these mutants are 
sufficiently slow to be followed over 15 min under pseudo first-
order conditions (excess of H₂O₂), using gel electrophoresis to 
quantify the relative amounts of the monomeric (reduced) ver-
sus dimeric (oxidized) forms (Fig. 3). Dimer formation is a two-
step process. The reaction of H₂O₂ with the Cₚ results in the 
formation of a sulfenic acid intermediate (Reaction 1), which 
reacts with the resolving cysteine of another protein molecule 
to give a disulfide-linked dimer (Reaction 2).

\[
\begin{align*}
\text{RS}^- + \text{H}_2\text{O}_2 & \rightarrow \text{RS}^-\text{OH} + \text{OH}^- \\
\text{RS}^-\text{OH} + \text{RS}^- & \rightarrow \text{RS}^-\text{SR} + \text{OH}^- 
\end{align*}
\]

REACTIONS 1 and 2

Reactions were quenched with catalase or by alkylating the 
reactive thiol groups of the proteins with N-ethylmaleimide.

| $kₑff$ | \(\text{M}^{-1} \text{s}^{-1}\) |
|-------|-----------------|
| Prx3  |                |
| WT    | \((1.9 \pm 0.1) \times 10^7\) |
| R146A | \((2 \pm 0.5) \times 10^7\) |
| R146H | \((3 \pm 1) \times 10^7\) |
| R146K | \((4 \pm 2) \times 10^7\) |
| R146G | \((2 \pm 0.9) \times 10^7\) |
| R123G | \((7 \pm 2) \times 10^7\) |
| R125G/R146G | \(2 \pm 0.5\) |
| Prx2  |                |
| WT    | \((1.6 \pm 0.1) \times 10^7\) |
| R150K | \((6 \pm 3) \times 10^7\) |
| R127K | \((3 \pm 2) \times 10^7\) |
| R127K/R150K | \(3 \pm 1\) |

Table 1: Second-order rate constants for the reactions of H₂O₂ with Prx2 and Prx3 WT and mutant proteins

Values are means ± S.D. from three to seven independent experiments using different H₂O₂ and Prx concentrations. Data are from experiments described in Figs. 2 and 3. (For details, see “Experimental Procedures.”)

The kinetic traces that were obtained by following the loss of the monomeric forms fit well to single exponential equations, indicating a first-order dependence on Prx concentration (Fig. 3). The second-order rate constants (Table 1) were obtained by correcting the pseudo first-order rate constants with the H₂O₂ concentrations. Second-order rate constants were similar at different H₂O₂ concentrations (in the range of 13–50 μM), suggesting overall second-order kinetics for the reaction. When ArgI was mutated, the second-order rate constants (for Prx3R123G and Prx2R127K) dropped by 5 orders of magnitude (to ~ 10⁷ M⁻¹ s⁻¹) compared with to WT. ArgI is therefore vital for the high reactivity of Prx.

Point mutation of ArgII (in Prx3R146A, Prx3R146H, Prx3R146K, Prx3R146G, and Prx2R150K) resulted in a similar decrease in the rate constant, demonstrating that ArgII is as important for the catalytic activity as ArgI. With both ArgI and ArgII mutated (Prx3R123G/R146G and Prx2R127K/R150K), the rate constants were a further 2 orders of magnitude smaller than for single mutants. This comprises a total of 7 orders of magnitude difference between double mutant and WT proteins (see Table 1), comparable with the difference in reactivity between free cysteine and Cₚ.

Ab Initio Calculations—High level calculations using the composite Gaussian-4 (G4) procedure were performed to assess possible H-bonding interactions at the active site that would lower the activation energy and increase the rate of the reaction of Cₚ with H₂O₂. For reasons of computational efficiency, the Cₚ and arginine residues were modeled by HS and guanidinium cation, respectively. All of the uncatalyzed and catalyzed reductions of H₂O₂ by HS are found to proceed via an S₂O2 mechanism. Fig. 4 and supplemental Fig. S2 display the reactant complexes, transition structures (TS), and product complexes located along the uncatalyzed and catalyzed reaction pathways. Table 2 gives the calculated reaction barriers ($\Delta H^\ddagger$) at 298 K.

In our analysis, the catalytic efficiency or rate enhancement of the catalyst is taken as the difference in barrier between the uncatalyzed process in aqueous solution and the enzymatic reaction (i.e. it is given by $\Delta \Delta H^\ddagger = \Delta H^\ddagger(\text{Uncat,aq}) - \Delta H^\ddagger(\text{Cat,enz})$), where the enzyme-like environment in the catalyzed reaction is simulated by a homogeneous polarizable continuum model with the dielectric constant $\epsilon = 4.0$. We note that, according to the Arrhenius equation, a change of 5.7 kJ mol⁻¹ in the barrier corresponds to a change of 1 order of magnitude in the reaction rate at 298 K.

Formation of the reactant complex (Fig. 4, Uncat-RC) is thermodynamically unfavorable in aqueous solution ($\Delta H_{\text{aq}} = +5.2$ kJ mol⁻¹). Thus, the enthalpic barrier in aqueous solution is taken relative to the free reactants ($\Delta H_{\text{Uncat,aq}} = 67.2$ kJ mol⁻¹). In all other cases, the barrier is taken as the energy of the TS relative to that of the reactant complex.

Probing the Effect of Hydrogen Bonding—The partial protonation or deprotonation that accompanies H-bonding can play an important role in facilitating enzymatic reactions (18). To consider the effect on the reaction profile of H-bonding at the oxygen centers of the H₂O₂, HF was chosen as a model H-bond
FIGURE 3. Time course for conversion of reduced (monomer) to oxidized (dimer) of the ArgI, ArgII, and ArgI/ArgII mutants for reaction with H₂O₂. The arginine residues of Prx3 (a–c) and Prx2 (d–f) were converted to Gly in Prx3 or Lys in Prx2. Data points on the kinetic traces represent the loss of reduced Prx as a % of total Prx (dimer + monomer; obtained from the depicted gels). Second-order rate constants obtained from the single exponential fits are shown in Table 1. Reactions were quenched by catalase (a) or N-ethylmaleimide (b–f). For experimental details, see “Experimental Procedures.”
Mechanism of Peroxiredoxin Peroxidase Activity

Barrier heights associated with the reactions in Fig. 4 and supplemental Fig. S2.

Values are given as kJ mol\(^{-1}\), calculated at the CPCM-G4//B3-LYP/6−31+G(2df,p) level in a simulated protein-like phase.

| Reaction | \(\Delta H^\ddagger\) |
|----------|----------------------|
| Uncat\(^a\) | 67.2\(^d\) |
| Uncat | 70.6 |
| [FH−O\(_a\)] | 56.8 |
| [O\(_a\)−HF] | 55.7 |
| [FH−S] | 65.1 |
| [FH−O\(_a\),O\(_b\)−HF] | 52.1 |
| [FH−S,O\(_a\),O\(_b\)−HF] | 56.6 |
| [FH−S,O\(_a\),O\(_b\)−HF] | 47.8 |
| [Gua+−S,O\(_a\),O\(_b\)] | 49.7 |
| [H\(_a\)C = NH−S,O\(_a\),O\(_b\)−Gua+] | 44.8 |
| [H\(_a\)NH−S,O\(_a\),O\(_b\)−Gua+] | 40.4 |
| [HOH−S,O\(_a\),O\(_b\)−Gua+] | 38.3 |
| [FH−S,O\(_a\),O\(_b\)−Gua+] | 32.2 |
| [Gua+−S,O\(_a\)] | 26.4 |
| [Gua+−S,O\(_a\),O\(_b\)−HOH] | 48.2 |
| [Gua+−S,O\(_a\),O\(_b\)−HF] | 40.1 |
| [Gua+−S,O\(_a\),O\(_b\)−HF] | 27.8 |

\(^a\) Unless otherwise specified, the barriers are taken relative to the reactant complex.

\(^b\) In enzyme-like medium (\(\epsilon = 4.0\)), unless otherwise noted.

\(^c\) In aqueous solution (\(\epsilon = 78.4\)).

\(^d\) Relative to the free reactants (see text).

 donors (reactions [FH−O\(_a\)], [O\(_a\)−HF], and [FH−O\(_a\),O\(_b\)−HF] in supplemental Fig. S2). H-bonding of HF to O\(_a\) in the transition structure will render the oxygen center a better electrophile, whereas H-bonding to O\(_b\) will facilitate departure of the hydroxide moiety. Indeed, we observe catalytic enhancements of \(\Delta \Delta H^\ddagger = 10.4, 11.5, \) and 15.1 kJ mol\(^{-1}\) for the [FH−O\(_a\)], [O\(_a\)−HF], and [FH−O\(_a\),O\(_b\)−HF] reactions, respectively.

An additional H-bonding interaction at the S center in [O\(_a\)−HF] (reaction [FH−S,O\(_a\),O\(_b\)−HF], supplemental Fig. S2) increases the catalytic enhancement from \(\Delta \Delta H^\ddagger = 11.5\) to 19.4 kJ mol\(^{-1}\). However, interaction at the sulfur atom in [FH−O\(_a\)], Uncat, and [FH−O\(_a\),O\(_b\)−HF] results in little or no reduction of the barrier heights (namely, by 0.2–2.4 kJ mol\(^{-1}\)). Finally, we note that in all cases, the hydrogen bonds at the various O or S centers stabilize the transition structures more than they stabilize the reactant complexes (see supplemental data).

Single-arginine Model—To model the effects of Arg1, we incorporated a single guanidinium cation moiety into our calculations instead of HF. We located two reaction pathways, shown as reactions [Gua+−S,O\(_a\),O\(_b\)] and [Gua+−S,O\(_a\)] in Fig. 4. The transition structure in [Gua+−S,O\(_a\)] is qualitatively similar to the insightful structure proposed by Hall et al. (14) for a related system. The two transition structures are fairly close in energy, in the enzyme-like environment [Gua+−S,O\(_a\),O\(_b\)]-TS lies lower in energy by 3.4 kJ mol\(^{-1}\). In [Gua+−S,O\(_a\),O\(_b\)]-TS, the guanidinium cation interacts with all three S, O\(_a\), and O\(_b\) centers, as evident from hydrogen bond distances of 2.14 (H\(_a\)−S), 2.18 (H\(_a\)−O\(_b\)), 2.03 (H\(_b\)−O\(_a\)), and 1.80 Å (H\(_b\)−O\(_a\)), where H\(_a\) and H\(_b\) are the hydrogens closer to the S and O\(_a\) atoms, respectively. On the other hand, in [Gua+−S,O\(_a\)]-TS the guanidinium cation interacts mainly with S and O\(_a\) (the H\(_a\)−S and H\(_b\)−O\(_a\) distances are 2.12 and 1.61 Å, respectively) (see also supplemental Table S4).

For the [Gua+−S,O\(_a\)] reaction, we obtain \(\Delta H^\ddagger (\text{cat,enz}) = 48.2\) kJ mol\(^{-1}\), thus \(\Delta \Delta H^\ddagger = 19.0\) kJ mol\(^{-1}\), which corresponds to a rate enhancement of ~3 orders of magnitude. Reaction [Gua+−S,O\(_a\),O\(_b\)] has a slightly lower barrier (\(\Delta H^\ddagger (\text{cat,enz}) = 44.8\) kJ mol\(^{-1}\)), which leads to \(\Delta \Delta H^\ddagger = 22.4\) kJ mol\(^{-1}\) and corresponds to a rate enhancement of ~4 orders of magnitude.

Single-arginine Model Plus an Extra H-bond Donor Moiety—The computational experiments (found under Probing the Effect of Hydrogen Bonding) suggest that a strong hydrogen-bonding interaction at the S center in [Gua+−S,O\(_a\),O\(_b\)]-TS or at the O\(_a\) center in [Gua+−S,O\(_a\)]-TS will further enhance the catalytic activity of the guanidinium catalyst. Indeed, if we consider the simultaneous interaction of a guanidinium at O\(_a\) and
O_b and an HF molecule at S (reaction [FH−S,O_a,O_b−Gua], Fig. 4), we find a barrier of just 26.4 kJ mol⁻¹ (Table 2). The catalytic efficiency is thus increased in this way to ΔH‡ = 40.8 kJ mol⁻¹, which corresponds to a rate enhancement of ~7 orders of magnitude relative to the uncatalyzed reaction in water. Consideration of weaker hydrogen-bond donors at S (namely H₂O, NH₃ or H₂C=NH, Table 2) results in smaller catalytic enhancements.

When the transition structure involves a simultaneous interaction of a guanidinium at S and O_a and an HF molecule at O_b (reaction [Gua−S,O_a,O_b−HF]), we find a barrier of 27.8 kJ mol⁻¹. The catalytic efficiency is thus increased in this way to ΔH‡ = 39.4 kJ mol⁻¹. A weaker hydrogen bond donor at O_b (e.g. [Gua−S,O_a,O_b−HOH]) reduces ΔH‡ to 27.1 kJ mol⁻¹.

**DISCUSSION**

H₂O₂ is a strong oxidant. However, its reactions with most thiols have a high activation barrier and therefore, although thermodynamically favored, are relatively slow. The structures of Prx overcome this barrier. This study investigated the roles of two highly conserved arginine residues (Fig. 1) in the exceptional reactivity of Prx with H₂O₂. Kinetic data obtained with various mutants of human Prx2 and Prx3 provided strong kinetic evidence that both arginine residues are vital for the peroxydase activities of these Prx. ArgI has been proposed to contribute significantly to the catalytic reaction. Indeed, we show mutations of ArgI in Prx2 and Prx3 result in a 5 orders of magnitude drop in reactivity with H₂O₂. Surprisingly, comparable losses in reactivity were also observed in ArgII mutants of both Prx. Importantly, simultaneous mutation of both arginine residues in Prx2 and Prx3 further decreased the rate constant to a total of 7 orders of magnitude less than the WT value.

High level quantum chemistry calculations provide mechanistic insight into the roles of these arginine residues in the reduction of H₂O₂ by C₆. The calculations indicate that partial protonation through H-bonding at the S, O_a, and O_b centers significantly lowers the activation barrier in a cooperative manner. A model using a single guanidinium cation moiety identifies two reaction pathways for catalysis ([Gua−S,O_a,O_b] and [Gua−S,O_b], Fig. 4), with reductions of 22.4 and 19.0 kJ mol⁻¹ in the barrier compared with the uncatalyzed reaction in water.

The presence of an additional H-bond donor such as water lowers the barrier further. We located two relevant transition structures ([Gua−S,O_a,O_b−HOH]-TS and [HOH−S,O_a,O_b−Gua]−TS, Fig. 4) that lead to barrier reductions and are consistent with the position of ArgI at the Prx active site. The reductions in the barriers are 27.1 and 35.0 kJ mol⁻¹, respectively, compared with that for the uncatalyzed reaction. This translates to an increase of ~5−6 orders of magnitude in the second-order rate constant of the reaction. This is consistent with the experimentally observed difference in reactivity between the ArgI mutants and the WT proteins.

H-bonding of a guanidinium nitrogen to O_a and either the leaving group (O_b) or the active site thiol has a profound effect on the catalytic activity. However, to account for the reactivity of the Prx compared with free cysteine, the barrier needs to be lowered further. This is achieved by introduction of a second, stronger H-bond donor (modeled by an HF), interacting at S or O_b ([FH−S,O_a,O_b−Gua]−TS and [Gua−S,O_a,O_b−HF]−TS, Fig. 4). The barriers then become 26.4 and 27.8 kJ mol⁻¹, corresponding to a total of 7 orders of magnitude increase in rate constant, as observed experimentally for the double mutant proteins. Thus, the computational results as well as substantiating the proposal (14) that ArgI is crucial in the catalytic mechanism also indicate a critical role for a second protonating agent.

Computational modeling suggests that reduction of H₂O₂ by cysteine proceeds via an S₅,2 mechanism with a single transition structure (Scheme 1).

On the basis of our experimental observations and theoretical computations, we propose that ArgI is involved in lowering the electron density of the reacting oxygen (O_a) in H₂O₂ (Fig. 1c) via H-bonding, making it a better electrophile. Simultaneously, ArgI could also be engaged in an H-bonding interaction with C₆, which would not only contribute to lowering its pK_a but would also anchor O_b to C₆ (further increasing reactivity in line with theoretical calculations). This role is consistent with structural data on the position of ArgI in the Prx active site and similar to that proposed by Hall et al. (9, 14).

Computational data indicate that additional interaction of either O_b or C₆ with a strong H-donor in the Prx structure is needed to explain the high reactivity with H₂O₂. The dramatic decrease in reaction rate by ArgII mutation also needs to be taken into account. The crystal structures of Prx2 and Prx3 suggest that ArgII would be ideally located to donate an H-bond to O_b. The dimensions of the active site (Fig. 1c) enable H₂O₂ to sit within bonding distance of both ArgI and ArgII. This would lower the energy of the transition structure, thus facilitating the reaction. An H-bonding network between the two arginine residues and H₂O₂ would also pull the two peroxide oxygens apart, thereby activating the oxidant by weakening the O_b−O_b bond. This would favor the OH⁺ transfer to C₆ as well as facilitate the departure of OH⁻.

This role for ArgII in the reactivity of Prx differs from that proposed by Hall et al. (9, 14). They suggest that ArgII assists ArgI to adopt the correct conformation via an Arg-Glu-Arg H-bonding network to exert its catalytic effect. Our observation that point mutation of either arginine residue results in a similar drop in reactivity, for both Prx2 and Prx3, is consistent with this suggestion. However, the further 2 orders of magnitude
drop in reactivity observed for double arginine mutants suggests a cooperative function for both residues.

We acknowledge that the orientation of H$_2$O$_2$ in the transition structure of our proposed mechanism is different from that of the H$_2$O$_2$ crystallized in the active site of the archael peroxiredoxin ApTPx (21). Hall et al. (14) used the ApTPx-H$_2$O$_2$ structure as the basis of their proposed model and also have shown that it is consistent with the orientation of different substrate mimics that have co-crystallized at the active site of other Prx. This model therefore favors involvement of residues other than ArgII in the H-bonding network. Further mechanistic and structural investigations are therefore required to reconcile these observations with our kinetic data.

In conclusion, our kinetic and computational studies substantiate structural studies and provide strong evidence for a vital role of ArgI in the high reactivity of Prx2 and Prx3 with H$_2$O$_2$. Our data also indicate that ArgII is critical in the activity of these proteins, and we propose that it could act through H-bonding to H$_2$O$_2$.

Acknowledgments—We thank the NCI National Facility and Intersect Australia, Ltd. for the provision of generous grants of computer time. We are grateful to Andrew Cox who helped with some of the initial analyses and Tong Zhu and Grant Pierce (BioInteraction Center at the University of Canterbury) for help with CD and melting point experiments.

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