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Experimentally Dissecting the Origins of Peroxiredoxin Catalysis

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Abstract

Aims: Peroxiredoxins (Prxs) are ubiquitous cysteine-based peroxidases involved in oxidant defense and signal transduction. Despite much study, the precise roles of conserved residues remain poorly defined. In this study, we carried out extensive functional and structural characterization of 10 variants of such residues in a model decameric bacterial Prx.

Results: Three active site proximal mutations of *Salmonella typhimurium* AhpC, T43V, R119A, and E49Q, lowered catalytic efficiency with hydrogen peroxide by 4–5 orders of magnitude, but did not affect reactivity toward their reductant, AhpF. pK_a values of the peroxidatic cysteine were also shifted up by 1–1.3 pH units for these and a decamer disruption mutant, T77I. Except for the decamer-stabilizing T77V, all mutations destabilized decamers in the reduced form. In the oxidized form, three mutants—T77V, T43A, and T43S—exhibited stabilized decamers and were more efficiently reduced by AhpF than wild-type AhpC. Crystal structures of most mutants were solved and many showed alterations in stability of the fully folded active site loop.

Innovation: This is the first study of Prx mutants to comprehensively assess the effects of mutations on catalytic activities, the active site cysteine pK_a , and the protein structure and oligomeric status.

Conclusion: The Arg119 side chain must be properly situated for efficient catalysis, but for other debilitating variants, the functional defects could be explained by structural perturbations and/or associated decamer destabilization rather than direct effects. This underscores the importance of our comprehensive approach. A remarkable new finding was the preference of the reductant for decamers. *Antioxid. Redox Signal.* 28, 521–536.

Keywords: thiol peroxidase, decamer, enzyme mechanism, regulation, peroxide signaling, AhpC

Introduction

PEROXIREDOXINS (Prxs, EC 1.11.1.15) are ubiquitous, cysteine-dependent peroxide reductases, which not only detoxify hydrogen peroxide (H_2O_2), lipid hydroperoxides, and peroxynitrite but also play pivotal roles in signal transduction involving H_2O_2 as a second messenger (36, 44, 47, 58). In this latter role, Prxs may be locally inactivated as floodgates to promote redox modification of other target proteins (27, 65, 67) or directly mediate oxidation of target proteins through a redox relay (19, 26, 36, 60). A number of

recent reviews provide a good overview of what is known about catalysis by Prxs and their physiological roles (7, 12, 36, 44, 58, 59).

Catalysis by Prxs relies on a conserved active site formed by a PxxxTxxC motif plus a conserved Arg residue (16). To initiate catalysis, the indispensable peroxidatic Cys residue, denoted by C_P , in its thiolate form performs a nucleophilic attack on the ROOH substrate, releasing ROH and becoming itself oxidized to a sulfenic acid (R-SOH, Fig. 1, step 1) (46). Details of the recycling events to restore the reduced C_P can vary, but generally proceeds *via* disulfide bond formation

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Innovation

Although peroxiredoxins (Prxs) are critical antioxidant enzymes and redox sensors that play important roles in protecting cells and regulating signal transduction, a molecular-level understanding of their catalytic function is still needed (44). In this study, putative roles for specific amino acids have been tested through complementary approaches, including assessment of catalytic activity, quaternary structure, local structure, and active site pK_a s. These studies have confirmed the complexity and inter-related nature of Prx properties and enabled deeper and more reliable insights into the factors influencing Prx activity than could have been obtained by the characterization of any single property.

with another resolving Cys, denoted by C_R (Fig. 1, step 2), then reduction by an electron donor such as thioredoxin (Fig. 1, step 3). To form a disulfide, the C_P -sulfenic acid must leave the fully folded (FF) active site pocket by locally unfolding, yielding a locally unfolded (LU) conformation of the C_P loop (Fig. 2A). Prxs are classified into six categories, some with varying C_R locations, defined as the Prx1, Prx5, Prx6, PrxQ, Tpx, and AhpC groups (15, 34).

FIG. 1. Catalytic cycle of AhpC, a bacterial Prx. Peroxidation (step 1) occurs when the thiolate form of peroxidatic cysteine (C_P-S^-) attacks the peroxide (e.g., H_2O_2), releasing the product (water in this case) and in the process becoming oxidized to sulfenic acid (C_P-SOH). Once the FF conformation undergoes a transition to an LU conformation, disulfide bond formation between the sulfurs of C_P and the resolving cysteine (C_R) occurs (step 2), followed by reduction by AhpF (or other thioredoxin-like reductants for other Prxs) and a reversal of the conformational change (LU $\rightarrow$ FF) to regenerate the substrate-ready active site (step 3). Some eukaryotic Prxs are sensitive toward oxidative inactivation (step 4) whereby a second molecule of peroxide reacts to generate the sulfonate ($C_P-SO_2^-$), but this reaction occurs with AhpC only in the presence of mM concentrations of peroxide. In Prx1-type dimers, as shown here for AhpC, the C_P - C_R disulfide is formed between subunits. Only one active site of the two per dimer is depicted as reacting. FF, fully folded; H_2O_2 , hydrogen peroxide; LU, locally unfolded; Prx, peroxiredoxin.

The most abundant and widely distributed Prx1 group includes *Salmonella typhimurium* AhpC (*StAhpC*) and four of the six human Prxs (PrxI, PrxII, PrxIII, and PrxIV) (34, 53). The Prx1 subgroup enzymes tend to be highly expressed in eukaryotes and are thought to be involved in the regulation of peroxide signaling (44, 67). Structurally, members of the Prx1 subgroup are noted for their intersubunit disulfide bonds formed between C_P and a C-terminally situated C_R in the partner subunit and for their tendency to form $(\alpha_2)_5$ decamers or $(\alpha_2)_6$ dodecamers (5, 14, 34, 44). Our work on *StAhpC* revealed that disulfide bond formation weakened the decamers and favored dissociation to dimers (Fig. 2C) (66), and this tendency is also seen for other Prx1-class enzymes (2, 4, 29, 55). Furthermore, we showed through making decamer-destabilizing (T77I) or -stabilizing (T77V) mutations of *StAhpC* that decamerization strongly promotes peroxidase activity by enhancing the stability of the FF substrate-ready form (41), agreeing with computational analyses describing activating influences of adjacent subunits in trypanothione peroxidase, another Prx1-class enzyme (69). A wealth of kinetic and structural experimental data have provided other mechanistic insights into Prx function (15, 17, 18, 28, 32, 33, 38–40, 42, 46, 48, 57, 61, 62, 64), as have emerging computational studies (54, 69, 70).

It is well accepted that the active site architecture of Prxs provides the specialized environment in which C_P and peroxide react not only through deprotonation of C_P to the thiolate form but also through interactions that are proposed to position and activate the peroxide substrate and lower the energy of the transition state (Fig. 2B) (16, 46, 54). A few studies have revealed defects in catalysis upon mutation of the conserved active site Arg and Thr side chains (13, 25, 32, 43, 54), but only one also tracked the structural consequences of such mutations (43), and none included comprehensive analyses of other functional and physical effects.

To quantitatively assess contributions to catalysis made by conserved residues in the active site and C-terminus, and regulating oligomeric state, we studied five newly created mutants of *StAhpC* at three key active site residues (R119A, E49Q, T43V, T43S, and T43A), two Trp mutants generated for our earlier rapid reaction kinetic studies (W81F and W169F) (40), a C_R mutant (C165S) used in earlier mechanistic investigations (9, 10), and the decamer-destabilizing (T77I) and -stabilizing (T77V) mutants (41) (Fig. 2C). For each mutant, we carried out C_P pK_a measurements, kinetic characterizations of reactions with peroxide and with the reductant, crystal structure determinations, and ultracentrifugation studies of the dimer/decamer equilibria. This work provides a new depth of understanding of the multifaceted effects that ensue when substitutions are made for residues with important roles in Prx catalysis.

Results

Strategy

To evaluate proposed roles of key residues and regions in *StAhpC*, we studied the structural and functional impact of 10 relatively conservative mutations introduced (i) proximal to the active site (R119A, E49Q, T43V, T43A, T43S, and W81F), (ii) in the C-terminal tail (C165S and W169F), and (iii) at the dimer–dimer interface (T77I and T77V) (Fig. 2C). Note that for C165S, the Ser substitution prevents disulfide

FIG. 2. Key structural features of AhpC. (A) The catalytic FF $\leftrightarrow$ LU conformational change is shown, whereby the active site and C-terminal regions unfold to facilitate the formation of a disulfide bridge between C_P of one chain (white) and the C_R in its partner chain (dark gray). Regions around C_P and C_R that undergo conformational rearrangements during catalysis are highlighted in cyan and gold, respectively. (B) The FF active site of AhpC (white) is overlaid with that of peroxide-bound ApTpx (pink, peroxide shown in green). Active site interactions with the catalytic C_P, Arg119, and Thr43 are highlighted with dashes. In the structure of WT AhpC, water mimics the position of proximal peroxide oxygen. (C) The sites of highly conserved residues are shown as sticks for a single AhpC monomer. C_P is also shown and noted with an asterisk. Note that as shown in panel (A), the C-terminal region of one chain packs against the active site of its dimeric partner. AhpC transitions between dimeric and decameric oligomers. Regions involved in the decamer-building interface are highlighted in pink. Regions involved in the homodimer interface are shown in teal. WT, wild-type.

bond formation, so the reductive recycling of this protein is mechanistically distinct from others, relying on direct reduction of C_P-sulfenic acid rather than the C_P-C_R disulfide bond. All proteins were expressed at similar levels from an *Escherichia coli* host strain lacking endogenous AhpC, were purified to homogeneity, and did not exhibit significant changes to global protein stability, as shown in Supplementary Results and Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/ars). Nearly all variants were amenable to crystallographic characterization. A synthesis of all findings in the Discussion section follows the Results section detailing (i) the C_P pK_a measurements, (ii) the kinetic characterizations of peroxide reduction, (iii) the crystal structure determinations revealing potential conformational changes and perturbations in the FF/LU equilibria, (iv) the ultracentrifugation studies of the dimer/decamer equilibria, and (v) the efficiency with which the StAhpC disulfide could be reduced by its natural reductant, StAhpF.

Effects of mutating key residues on pK_a modulation

pK_a determination involved assessment of StAhpC competition with horseradish peroxidase (HRP) for peroxide across a pH range, thus the observed pK_a of the C_P residue reflects the FF conformation. Relative to the pK_a of 6.0 for wild-type (WT) StAhpC, the C_P pK_a of R119A was raised by 1.3 pH units (evaluated by two complementary methods; Supplementary Results and Supplementary Fig. S2) and E49Q by 1.0 pH unit (Table 1 and Supplementary Fig. S3). Of the other active site variants assessed, only T43V exhibited a similarly large C_P pK_a shift of 1.2 pH units (Table 1). In contrast, a slight decrease in the pK_a was observed when Thr43 was replaced by Ser (T43S, Table 1); complete removal of the side chain hydroxyl group in the T43A mutant is quite well tolerated, yielding a C_P pK_a increased by just 0.2 pH units. This was surprising as computational studies of another Prx1-class enzyme showed that the active site Thr

TABLE 1. C46 pK_a AND RATE CONSTANTS WITH HYDROGEN PEROXIDE FOR AHpC VARIANTS

	pK _a *	k _{cat,app} [†] (s ⁻¹)	K _{m,app} ^{H2O2†} (μM)	k _{cat} /K _m ^{H2O2,†} (M ⁻¹ s ⁻¹)	Relative [‡] k _{cat} /K _m ^{H2O2}
WT	6.0 (0.1)	40 (1)	0.84 (0.09)	4.7 × 10 ⁷	1.0
R119A	7.3 (0.3)	11.7 (0.6)	1500 (300)	8.0 × 10 ³	0.00017
E49Q	7.1 (0.1)	0.73 (0.08)	740 (340)	9.9 × 10 ²	0.000021
T43V	7.2 (0.2)	2.8 (0.3)	1600 (500)	1.8 × 10 ³	0.000038
T43A	6.2 (0.4)	3.8 (0.1)	34 (3)	1.1 × 10 ⁵	0.0023
T43S	5.8 (0.2)	8.9 (0.1)	0.39 (0.03)	2.3 × 10 ⁷	0.48
W81F	6.5 (0.2)	19 (1)	2.7 (0.4)	7.2 × 10 ⁶	0.15
C165S	6.8 (0.2)	0.40 (0.03)	1.5 (0.4)	2.7 × 10 ⁵	0.0052
W169F	6.2 (0.2)	9.6 (0.4)	31 (4)	3.1 × 10 ⁵	0.0066
T77I	7.2 (0.1)	4.8 (0.3)	14 (4)	3.4 × 10 ⁵	0.0072
T77V	6.0 (0.1)	61 (3)	1.1 (0.2)	5.5 × 10 ⁷	1.2

Values in parentheses indicate standard error of the mean.

*The pH dependence for the AhpC reaction with H₂O₂ was determined as described in the Materials and Methods section by monitoring the ability of AhpC to compete with horseradish peroxidase across a range of pH values.

†To obtain pH-independent rate constants, k_{cat,app} and K_m^{H2O2,app} values were measured at pH 8.5 as described in the Materials and Methods section (except for W81F, which was unstable at pH 8.5 and therefore assessed at pH 8.0). Assays monitored the initial fluorescence decrease as 10 μM NTD_{S128W} becomes oxidized in the presence of each AhpC variant (0.05–1 μM) and various concentrations of peroxide.

‡Relative catalytic efficiencies compared with WT AhpC.

NTD, N-terminal domain; WT, wild-type.

had a strong effect on the pK_a of C_P (69), although perhaps in T43A, a water molecule mimics the Thr hydroxyl group.

W81F (conserved as either Trp or Phe across essentially all Prxs) exhibited a slightly larger increase in C_P pK_a at ~0.5 pH units. Surprisingly, mutation of the resolving cysteine within the C-terminus of AhpC, which is ~14 Å away from C_P in the FF structure (45), shifted the pK_a of C_P upward by ~0.8 pH units, whereas W169F exhibited a C_P pK_a just 0.2 pH units higher than WT AhpC (Table 1). Finally, the decamer-stabilizing T77V mutant exhibited no measurable pK_a change, whereas the decamer-disrupting T77I variant exhibited a pK_a increased by about 1.2 pH units. Thus, three substitutions of active site side chains (R119A, E49Q, and T43V) and one substitution known to disrupt the decamer and indirectly destabilize the FF active site (T77I) shifted the C_P pK_a upward by 1–1.3 pH units, C165S raised the pK_a by ~0.8 pH units, and other mutations had minimal effects on this property.

Peroxidase activities of AhpC variants

To assess steady-state kinetic parameters for H₂O₂ reduction (k_{cat,app} and K_{m,app} for H₂O₂), initial rates were obtained using spectral assays with a fixed concentration of reductant (10 μM of the engineered, fluorescent N-terminal domain [NTD] of AhpF) and varying concentrations of H₂O₂. These rates were purposefully assessed at pH 8.5 to fully stabilize the C_P thiolate, even in variants with elevated C_P pK_a values (the assay pH of 8.5 is more than 1.2 pH units above the highest pK_a measured for variant proteins [Table 1]).

In general, variants with substitutions introduced at conserved active site residues exhibited very low apparent k_{cat}/K_m^{H2O2} values. The most affected variants were T43V and E49Q at over 4 orders of magnitude lower catalytic efficiencies with H₂O₂. R119A is only slightly more reactive at 0.02% of the WT value. The T43A substitution also exhibited substantial impairment, but was 10 times more active than R119A (apparent k_{cat}/K_m ~0.2% of wild type). In contrast,

both T43S (which retains the hydroxyl group) and W81F (which retains an aromatic hydrophobic side chain) are much less affected at 48% and 15% of WT k_{cat}/K_m values, respectively.

Substitutions at the C-terminus of AhpC were also disruptive to catalysis. The catalytic efficiency of C165S dropped to about 0.5% that of WT AhpC, an effect that was almost exclusively caused by a drop in k_{cat,app}. C165S was also the only mutant that exhibited a greater propensity toward hyperoxidation during turnover than WT AhpC (Supplementary Fig. S4). In W169F, there was a similar drop in catalytic efficiency to about 0.7% that of WT AhpC, although in this case, both the apparent k_{cat} and K_m^{H2O2} values were affected (Table 1). As noted previously (41), T77V catalytic efficiency was slightly greater (by 20%) and T77I considerably lower (0.7%) when compared with WT *StAhpC*.

In summary, the apparent k_{cat}/K_m dropped by about 4 orders of magnitude or more for three active site variants (R119A, E49Q, and T43V), with both k_{cat} and K_m being severely impaired. Substantially, but not as severely, affected were T43A, C165S, W169F, and T77I, with catalytic efficiencies of about 0.2–1%. Minimal effects were observed for W81F and T43S, while T77V retained full activity.

Effects of amino acid substitutions on structure and FF/LU equilibria as seen in crystals of variants in their decameric forms

While functional analysis allows experimental assessment of the perturbing effects of select mutations, only a limited understanding of the origins of these effects is gained in the absence of accompanying structural information. Fortunately, using the approach of Perkins (45) (see the Materials and Methods section), crystals of the dithiol form of most of these *StAhpC* variants could be obtained in which four active sites per asymmetric unit are unperturbed by crystal contacts and allow for direct visualization of the FF/LU equilibrium and the fifth active site has an unfolded

FIG. 3. Structure of E49Q variant. (A) The interactions of WT Glu49 and Arg119 are shown, where Glu49 is seen to form hydrogen bonds to Arg119, as well as Arg142 and the Gly141 backbone. (B) The structure of E49Q is shown, which reveals that Gln49 adopts a different position, hydrogen bonding to the Gly141 backbone and to a water molecule. Arg119 is shifted away from C_P. We have interpreted the small electron density peak to the right of C_P-S_γ as a partially occupied water molecule (not shown here) that is bound when the S_γ (which based on its weak density is not well ordered) adopts other rotamers. 2Fo-Fc density contoured at 1.0 σ is shown as *blue mesh*.

C-terminus, allowing further insight into how C-terminal folding influences the stability of the FF conformation of the C_P loop (45). Such structures of the reduced forms were obtained for T43S, T43V, T43A, and E49Q, as well as for C165S and W169F. R119A never yielded diffraction-quality crystals despite multiple attempts to crystallize both oxidized and reduced proteins, and a structure for W81F was only obtained for its oxidized form (Supplementary Table S1).

Structures of disulfide forms of T77I and T77V were described previously (41) and those variants are not further analyzed in this section. For each of the new AhpC variant structures, the substituted amino acid is clearly visible in the electron density (Figs. 3–6).

Regarding conserved charged residues around the active site, while we do not have structural data for R119A, we do find that substitution of Gln for Glu49 (E49Q) results in large reorientation of the Arg119 side chain such that the Gln49 and Arg119 side chains move away from one another and away from Cys46 (Fig. 3). This new position for Gln49 clashes with the location in WT AhpC of the Ala167' methyl from the other subunit of the dimer, promoting unfolding of the C-terminus (not shown).

Considering the set of active site variants modified at T43, the Ser variant (T43S) has the fewest changes. The C_P loop adopts the FF conformation and Ser43 exhibits two alternative side chain conformations at roughly equal occupancy (Fig. 4C). In one, the hydroxyl group is positioned very much as the WT Thr43-OH, where it is near Cys46 and hydrogen bonds to the backbone carbonyl of Ala40. In the alternative conformation, the hydroxyl points away from Cys46 and hydrogen bonds to its own backbone NH (Fig. 4C, double-headed arrow). Phe76'' across the decamer-building interface, which normally packs against Thr43, also adopts two $\sim 50\%$ occupied conformations in T43S (not shown). In the mutant lacking both the hydroxyl and methyl groups, T43A, the active site loop structure is predominantly LU (Fig. 4B). T43V, like T43A, also adopts a predominantly LU

FIG. 4. Structure of Thr43 variants. The active site regions of WT, T43A, T43S, and T43V StAhpC are shown in *panels (A–D)*, respectively. 2Fo-Fc density for each structure is depicted as *blue mesh*, contoured at 1.0 σ . The T43S mutant shows two conformations both at ~ 0.5 occupancy (*double-headed black arrow*). For mutants T43A and T43V, the active site is seen to be predominantly LU (*gray arrow*).

FIG. 5. Structure of the W81F variant. In the WT FF structure (white sticks), W81 packs against C_p , and also hydrogen bonds to a well-ordered water molecule that is involved in anchoring the C-terminal Ile186' carboxylate of the partner chain (dark gray). The W81F mutation (pink sticks) adopts a similar position, but cannot form the hydrogen bond, and water is not present in the W81F structure. We infer that the loss of this interaction is destabilizing to the packing of the C-terminal tail. 2Fo-Fc density contoured at 1.0 ρ ms for Ile186' and the water seen in FF WT AhpC is shown as blue mesh. 2Fo-Fc density contoured at 1.0 ρ ms for the W81F mutation site is shown as pink mesh.

conformation in the crystal (occupancy near 100%), although with slightly different details (Fig. 4D).

For W81F, which possesses a smaller, but similarly hydrophobic, residue near the C_β of C_p , only crystals of the oxidized, but not reduced, protein were obtained. Fortunately, because this Trp does not move during FF $\leftrightarrow$ LU transitions (40, 47), we can infer from the LU structure how the mutation may impact the FF structure. As shown in Figure 5, Phe81 overlays rather well with the WT Trp81 side chain; by modeling this change in the FF WT structure, we see that the mutation would displace a well-ordered water molecule that bridges the Trp81 side chain NH with the C-terminal Ile186' residue (Fig. 5).

Both the C165S and W169F variants exhibited weak C-terminal density (Fig. 6B and C, respectively). In the case of C165S, $\sim 25\%$ of the C-terminal region is FF (as estimated during refinement, see the Materials and Methods section), and for W169F, still weaker density for the C-terminal region implies that there is even less of the FF form. In addition, the fraction of W169F that does adopt the FF conformation shows a slightly altered chain path beyond Glu163, including a perturbed location for Phe169 relative to the Trp in WT AhpC. This portion of the C-terminus packs against the back of the C_p -containing helix α_2 and, in the mutant, is associated with a displacement of its C_p -SY by ~ 0.55 Å in W169F relative to the WT enzyme (Fig. 6A, C).

To summarize, multiple types of alterations were observed in the active sites of the variants studied. E49Q has a mostly FF C_p loop, but Arg119 is displaced so it does not interact with C_p . In addition, T43V and T43A were seen to cause direct strong destabilization of the FF C_p loop, and C165S and W169F both led to partial C-terminal unfolding and indirect destabilization of the folded C_p loop. For T43S, perturbations were minimal, but Ser43 adopted approximately

FIG. 6. Structures of C-terminal mutants. (A) 2Fo-Fc electron density at 0.5 σ ms for the C-terminal tail of FF, WT StAhpC is shown at the top (white mesh), with the C_R , Trp169, and C-terminal Ile186 positions shown as sticks. Below, a close-up view of the C_R is region is shown, with 2Fo-Fc density contoured at 1.0 ρ ms. For reference, the well-stabilized α_5 region that contains Tyr156 is also shown. Similar depictions are shown for C165S and W169F mutants in panels (B, C), respectively, demonstrating that the C-terminal density is much weaker for the variants, indicating a shift from FF to LU. For the C165S and W169F close-up views, 2Fo-Fc density is also shown at 0.5 ρ ms to visualize the much weaker density in that region.

equal occupancy of what would appear to be productive and nonproductive conformations of the hydroxyl side chain.

Oligomeric properties of AhpC variants

We next assessed the influences of amino acid substitutions on oligomeric state. Using analytical sedimentation velocity experiments, we evaluated the dimer–decamer equilibrium of each mutant in both the reduced (dithiol) and oxidized (disulfide) states at a series of concentrations (1 and 10 μM , and 5–100 μM , respectively; Supplementary Fig. S5). We note that our previous crude estimate of 5 μM for the abundance of AhpC in *E. coli* (66), as well as the finding that AhpC concentrations can be induced by as much as 20-fold under conditions of oxidative stress that activate the transcription factor OxyR in *S. typhimurium* (6), suggests that this range of AhpC concentrations is physiologically reasonable.

The T77V and T77I variants studied earlier (41) represent extreme behaviors, with the decamer-stabilizing T77V forming substantial amounts of decamer in both the reduced and oxidized states at all concentrations studied and the decamer-destabilizing T77I showing no notable decamer formation even at the highest concentrations studied (Fig. 7A). For the other newly studied variants, we found for the reduced state that the decameric forms of all variants are destabilized (Fig. 7A). Not surprisingly, the two variants that retain the most activity, T43S and W81F (Table 1), are less affected than the others as seen by their higher decamer formation at 1 μM . Among the other variants, E49Q appears the most destabilized in reduced form, forming the smallest amount of decamer at the 10 μM concentration.

Interestingly, oxidized states had more diverse behavior, with the degree of and direction of the change in decamer stability differing widely across variants. Under the conditions used, WT AhpC exhibits a concentration-dependent shift from dimer to decamer between 10 and 20 μM proteins. Like wild type, all new mutants exhibited notable variation in the amount of decamer formed within the concentration range used. Two of the new mutants (T43S and T43A) had a more stabilized decamer relative to the WT protein, still exhibiting some decamer formation even at the lowest 5 μM concentration used. The other AhpC variants formed less stable decamers than wild type. Among these, two mutants with the most extreme destabilization were R119A and T43V. R119A was additionally unusual as it never exhibited any clear decamer in its oxidized form even at 100 μM (giving instead an intermediate s^* value, potentially signifying a rapid decamer–dimer equilibrium; Supplementary Fig. S5). Thus, the overall effect of substitutions made, except for T77V (which has decamer-stabilizing effects), is to enhance dissociation of decamers, in most cases, in both the reduced and oxidized forms. However, in two variants, T43A and T43S, decamers of the oxidized proteins are somewhat stabilized relative to WT AhpC.

Efficiency of reduction by AhpF

Noting large variations in stability of the oxidized decamer, we realized that this provided an opportunity to study how the oligomeric state of *StAhpC* influences the catalytic efficiency of reduction of the AhpC disulfide state by AhpF. We have previously shown that this step involves direct protein–protein contact with *StAhpF*, including formation of

FIG. 7. Oligomeric state of AhpC variants and their reactivity with AhpF. (A) Oligomeric state of AhpC variants. Each variant was evaluated in both oxidized and reduced states (except for the oxidized form of C165S) at multiple concentrations by analytical ultracentrifugation using sedimentation velocity experiments. The oligomeric state at each concentration ranged from fully dimeric (yellow, $s^* \sim 3.4$) to fully decameric (dark blue, $s^* \sim 7.5$) with many samples exhibiting a mixture of the two species; the colors represent the approximate relative values of dimer and decamer within the mixtures. At 100 μM , oxidized R119A exhibited a single peak at $s^* = 5.1$ (violet) that would suggest either the presence of an oligomeric species between dimer and decamer or a very rapid equilibrium between the two species (Supplementary Fig. S5). (B) AhpF-dependent peroxidase assays varying AhpC concentrations to assess reactivity of AhpC variants with AhpF. The absorbance at 340 nm was monitored as NADH becomes oxidized in the presence of WT or mutant AhpC (0.1–20 μM), 0.025 μM AhpF, 150 μM NADH, and either 50 μM (WT, T43S, W81F, C165S, W169F, T77I, T77V) or 1 mM (R119A, E49Q, T43V, T43A) H_2O_2 at pH 7.0 and 30°C. Under these conditions, the AhpC concentration was effectively below K_m and the linear fit of the initial rate versus the AhpC concentration approximates k_{cat}/K_m of AhpF for AhpC as substrate. Values shown are the averages of at least three independent measurements with standard errors; p -values were determined using Student's t -test. * p -value < 0.05, ** p -value < 0.005.

an interprotein disulfide bond involving the C_R residue of *StAhpC* (20). To study this reduction step, AhpF was held at very low concentration and AhpC was varied over a range of higher concentrations in the presence of saturating H_2O_2 (52). Under these conditions, reduction by *StAhpF* is rate limiting

(i.e., much slower than reoxidation of *StAhpC* by peroxide) and *StAhpC* is essentially all in its disulfide form.

As shown in Figure 7B, the worst electron acceptor for *StAhpF* is the C165S mutant. This is not surprising given that this variant has a major obligatory change in mechanism as the Cys129 thiolate of *StAhpF* must directly attack the Cys46 sulfenic acid of *StAhpC* rather than the sulfur of Cys165 within the Cys165-Cys46 disulfide bond. For all other *StAhpC* mutants, the presence of the native disulfide bond presents a more native-like substrate and catalytic efficiency is at least 35% of wild type (Fig. 7B).

Most of the variants (R119A, E49Q, T43V, and W169F) have reactivity toward *AhpF* that is not notably different from WT *StAhpC*, despite the large impairments they have in their peroxide reactivity. One mutant, W81F, exhibits a significantly lower reactivity with *StAhpF* than does wild-type *StAhpC* (Fig. 7B), and it also shows a significant destabilization of the decamer in its oxidized form (Fig. 7A). T77I, the previously generated decamer-destabilizing mutant, was also significantly lower in catalytic efficiency with *AhpF* (Fig. 7B). Importantly, variants that exhibited more stabilized decamers than wild type (T43S, T43A, and T77V) exhibited rather large approximately fourfold enhancements in the catalytic efficiency of electron transfer (Fig. 7B). Thus, while four mutants were unaffected in their turnover with *StAhpF* (R119A, E49Q, T43V, and W169F), three mutants were significantly less active (W81F, T77I, and C165S), and three significantly more active (T43A, T43S, and T77V) than WT *StAhpC* by this analysis.

Discussion

Prx catalysis depends on and can potentially be regulated through (i) proper positioning of critical side chains within the active site, (ii) appropriate balancing of the FF↔LU equilibrium, and (iii) redox-sensitive and dynamic changes in oligomeric state with respect to dimers and decamers for a Prx1 group enzyme. Indeed, we know that these physical properties are not static during catalysis as local unfolding around C_P and C_R is required for disulfide formation. Furthermore, we previously established that decamer dissociation is promoted by disulfide bond formation (66) and that simply stabilizing or destabilizing the reduced decamer (as in the T77V or T77I variants of *StAhpC*) leads to more or less effective reduction of peroxides (41).

In this study, we have carefully analyzed effects of a range of mutations within the active site, the C-terminal tail, and the dimer-dimer interface. In almost every case, we found that mutations which significantly altered one of these parameters also perturbed one or more of the other characteristics, highlighting the interdependence of various features in these enzymes. Important to note in our discussions is that by carrying out the peroxidase assays at high pH, we ensured that any changes in activity were not due to shifts in the C_P pK_a; pK_a shifts reported herein are considered indicators of disturbance of the active site conformation (not explanations for activity losses).

Regarding catalytic properties and oligomeric status, we note that enzymatic assays were conducted in two ways. One assay was designed to assess the reduction efficiency of full-length *AhpF*; under these conditions, the reaction with peroxide is rapid, reduction is rate limiting, and *AhpC* is predominantly in the oxidized form during the assay. In the

other assay, which was set up to sensitively evaluate the peroxide reactivity of mutants, reductive recycling is rapid and the enzyme is predominantly in its reduced form during the assay (the redox form for which decamers are more stable). In these assays, the *AhpC* concentration present was ≤1 μM (Supplementary Table S2), and from Figure 7A, we note that while WT *StAhpC* is predominantly decameric in reduced form, all reduced variants (except T77V) are entirely or primarily dimers at 1 μM. It is therefore not unexpected that the peroxide-dependent activity drops for all variants except for T77V, in part, due to their predominantly dimeric rather than decameric oligomeric state.

The case of T77I, which is an extreme case of decamer destabilization, proves that this effect on catalytic efficiency is about 140-fold *even in the absence of any changes in active site residues*. The values presented here were measured at a single NTD concentration and the apparent k_{cat}/K_m values (138-fold lower than wild type; Table 1) agree well with previously determined k_{cat}/K_m values observed by full bisubstrate turnover (T77I value 144-fold lower than that for wild type) (41).

Focusing on the peroxide-dependent reaction, it is notable that three other variants of *StAhpC*, C165S, W169F, and T43A, have $k_{\text{cat}}/K_{m,\text{app}}$ values for H₂O₂ in a similar range as T77I (0.7% of the WT value for T77I, and 0.5%, 0.7%, and 0.2% compared with WT value for C165S, W169F, and T43A, respectively). However, when considering the measured C_P pK_a and K_m values for these proteins, two types of defects are noted. For W169F and T43A, the $K_{m,\text{app}}$ values for H₂O₂ rise to ~30 μM, with a small fourfold drop in k_{cat} and no shift in the C_P pK_a (which measures the pK_a in the functional FF form of the C_P loop). From a simple interpretation of our previous rapid reaction kinetic data (40), K_m can be calculated as $(k_{-1} + k_2)/k_1$, where k_{-1} and k_1 represent the reverse and forward rates of substrate binding to the enzyme and k_2 is the rate of the chemical step of sulfenate formation on the enzyme and product (water) formation from substrate. As the increase in K_m for W169F and T43A is not expected to represent an increase in the rate of the chemical step, we infer that these two mutants have changes within their active sites that are moderately detrimental to substrate binding.

In contrast, C165S, which also exhibited a decrease in k_{cat}/K_m to about 0.5%, exhibits alterations in k_{cat} (decreased 100-fold) and pK_a (rising from ~6.0 for wild type to ~6.8), but not K_m (at 1.5 ± 0.4 μM). This finding is similar to the increase of about 0.6 pH units observed for the C_R→Ser mutant of human Prx5 (64). For C165S, decamer destabilization and related lower stability of the FF C_P loop are major factors in lowering k_{cat}/K_m , but in this case, the apparent maladjustment in the FF active site affects k_{cat} rather than K_m . With results from these three mutants in mind, T77I is somewhat of a hybrid, with a similar decamer-destabilizing effect prevailing, but with the perturbation in the FF active site influencing C_P pK_a (to 7.2 ± 0.1), and k_{cat} (~10-fold lower) and K_m for H₂O₂ (~10-fold higher).

AhpC variants with only minor effects on k_{cat}/K_m , T43S and W81F, exhibit only small effects on C_P pK_a and $K_{m,\text{app}}$ and show at least some decamer still present at 1 μM of reduced forms (Fig. 7A). The connection between a better stabilized decamer and a more stabilized FF form of the C_P loop is not a coincidence (thermodynamically they influence each other to the same degree), and this result in a sense provides an independent confirmation of this linkage. The

high activity of T43S is expected given that not only the hydroxyl group is retained but also that Ser is found in this position in $\sim 5\%$ of known Prx sequences (44). The impact of the T43S mutation on catalysis, dropping k_{cat}/K_m by about 50% (Table 1), is plausibly explained by higher entropy (presence of two different side chain conformations) within this relatively stable FF active site. While the K_m for H_2O_2 is rather similar for WT and T43S AhpC (at 0.8 and 0.4 μM , respectively), the k_{cat} drops by 4.5-fold, consistent with a portion of the enzyme being in a less active or inactive state even when peroxide is bound. Notably, a similar mixture of the two conformations of a Ser at this position was seen in the *Saccharomyces cerevisiae* Tsa2 structure, for which Ser is the naturally occurring residue (37). They suggested this difference may explain the higher $\text{p}K_a$ of C_p in Tsa2 versus Tsa1, but our observation that the *StAhpC* T43S did not show an increased $\text{p}K_a$ implies that other factors may be behind the $\text{p}K_a$ shift seen in yeast Tsa2.

Also of interest for comparison is a very recent study, which concluded that the Thr versus Ser at this position in *S. cerevisiae* Tsa1 versus Tsa2 is a key switch, with the Ser promoting decamer stability (63). By measuring the oligomerization status at a single 50 μM protein concentration, it was seen that the reduced Ser mutant had similar decamer stability to WT Tsa1 and the oxidized (*i.e.*, disulfide) form had a more stable decamer. Interestingly, this observation matches the behavior we see for AhpC (Fig. 7A), with reduced T43S showing a slightly destabilized decamer and oxidized T43S a slightly stabilized decamer (Fig. 7A). This is consistent with their proposal that the Thr methyl group makes a stabilizing weakly polar interaction with the aromatic side chain across the decamer-building interface; however, our observation that the majority of *StAhpC* mutations influence decamer stability causes us to suggest caution in concluding that this particular residue is a key switch. In addition, because (from a thermodynamic point of view) the stability of the oxidized decamer is a property of that form itself and not the process of how it got there, we also find unsatisfying the explanation that the Thr methyl group is sterically disfavoring the decameric state during the transition from the FF to LU states.

The most severely affected mutants in both functional aspects requiring an intact FF C_p loop (C_p $\text{p}K_a$ and substrate binding) are R119A, E49Q, and T43V, with k_{cat}/K_m values lowered by about 4 or 5 orders of magnitude. In these cases, decamer destabilization may play a role, as does the related instability of the FF C_p loop, but K_m values are also up by ~ 3 orders of magnitude and C_p $\text{p}K_a$ values are strongly shifted upward by 1.0–1.3 pH units. Such dramatic effects argue that the FF active site, even when present, does not have key catalytic residues properly positioned. In particular, the Arg side chain, which arguably plays important roles both in lowering of C_p $\text{p}K_a$ and in binding and activating substrate, is in the wrong place. The E49Q structure emphasizes that rearrangement of Arg119 alone is quite detrimental for proper peroxide binding and activation. Not only is K_m affected in this mutant but also k_{cat} is down about 50-fold relative to WT *StAhpC*, indicating that even when high peroxide concentrations are present, which favor stabilization of the FF active site, this protein suffers from a lack of substrate activation.

Interestingly, the R119A mutant is almost entirely affected in K_m for H_2O_2 , which (as we argued above) is likely to represent an inability to bind the peroxide substrate with high

affinity. We speculate that the minimal impact on k_{cat} implies that with E49 present and peroxide at high enough concentration, the active site folds reasonably properly, and places water where the Arg would normally be, and these support catalysis quite well at the high pH of our assays. R119A was the only mutant studied here that did not crystallize, and we note that this would be expected if the oxidized protein does not form proper decamers (Fig. 7A). T43V has a very high K_m and so similarly takes a lot of peroxide to drive folding to the FF conformation, but with lower k_{cat} than R119A and high $\text{p}K_a$, we infer that in T43V, the FF C_p loop is more perturbed compared with R119A (due to collision of the unnatural Val methyl group).

We can compare our results for R119A and E49Q mutants with previous studies addressing the importance of the Arg-Glu-Arg interaction network of Prx1, Prx6, and AhpE-class proteins (16, 32). Glu is also conserved in PrxQ group enzymes, but is substituted by Gln, Ser, or His in Tpx and Prx5 group members (34, 44, 49). The second Arg is found only in the Prx1, Prx6, and AhpE groups. Early mutagenesis studies of these residues for Prx1 group members from *Leishmania donovani*, *Crithidia fasciculata*, and barley (13, 25, 30) used assays for which disulfide bond reduction was rate limiting and thus did not reveal the specific impact of the mutations on peroxide reactivity. More recent studies have accurately assessed peroxide reactivity of mutants lacking the conserved Arg [including studies of yeast Tsa1, human Prx2, Prx3, and Prx5, and *Mycobacterium tuberculosis* AhpE (32, 43, 54, 62)]; in all cases, the Prx proteins exhibited $\sim 10^5$ lower peroxide reactivity in striking agreement with results presented here for R119A.

In the two cases for which C_p $\text{p}K_a$ was assessed [R127Q of Prx5 and R116A of AhpE (43, 54)], it increased by ~ 1.5 or 0.7 pH units, respectively, again fitting well with the 1.3 pH unit increase in C_p $\text{p}K_a$ measured for *StAhpC* R119A. In the crystal structure of the AhpE R116A mutant (43), it was observed that the region normally occupied by the Arg side chain was filled with water (43). Mutagenesis of the second Arg of the Arg-Glu-Arg network (Arg150 and Arg146 in Prx2 and Prx3, respectively) also caused a very similar dramatic loss in peroxide reactivity (32), although based on earlier observations (16) and our results for E49Q, we suggest that these defects are caused by indirect effects on active site integrity (*e.g.*, the correct positioning of the Arg119 equivalent) rather than indicating a direct role for the second Arg in substrate binding and activity.

The E50A mutation of yeast Tsa1 (comparable with Glu49 in our study) decreased peroxide reactivity by only about 10-fold (62), implying that it causes less active site perturbation than we observed in this study with E49Q. It is plausible that in the active site environment with the Glu normally surrounded by hydrogen bond donors, an E $\rightarrow$ A mutation that allows water to fill in the space could be more conservative than an E $\rightarrow$ Q mutation that forces a dramatic local structural change.

Our results for the T43V variant are generally consistent with the observations of others even though our conclusions differ. For three other Prxs in which the Thr43 equivalent was replaced by Val [human Prx5 (54), *M. tuberculosis* AhpE (43), and *S. cerevisiae* Tsa1 (63)], peroxide reactivity was similarly lowered by at least 3 orders of magnitude. As no crystal structures of these mutants were determined, it was

natural for those studies to conclude that these results provide evidence for a crucial importance of the Thr hydroxyl in catalysis. However, given the high conservation of the FF active site geometry in Prxs, we suspect in all these cases that just as for *StAhpC*, the valine group effectively blocks formation of a properly folded FF active site, so one does not gain insight into the role of the Thr hydroxyl group in catalysis.

Consistent with our expectation that Val mutants cause unfolding of the C_P loop, the yeast Tsa1 Thr→Val mutant was shown to have a highly destabilized decamer in both reduced and disulfide states of the enzyme (63). One confusing finding is that the C_P pK_a of Prx5 T44V was reported to be shifted from 4.6 in the WT enzyme to 3.5 in the mutant (54); while we cannot readily reconcile this disparate finding, we note that the monobromobimane reactivity approach used would not constrain the pK_a measurement to be from the FF enzyme. As such a result has not been seen in any other Prx, this is an open question to be resolved by further studies.

In a new finding in the field of Prx research, our data across multiple mutants strongly support the conclusion that the physiological electron donor to AhpC, AhpF, is more reactive toward oxidized decamers than toward dimers. This was unexpected as we had previously speculated that redox sensitivity driving dissociation of decamers upon oxidation might have a positive effect on catalysis if dimers were better substrates for AhpF (41). We note that our findings are consistent with the demonstration by Dip *et al.* that C-terminally truncated *E. coli* AhpC (lacking 15 residues), which is less capable of forming decamers, also shows significant impairment in its interaction with the NTD of AhpF (8).

Whether or not other Prx1-class enzymes with different thioredoxin-like reducing partners share this linkage between reduction efficiency and oligomeric state remains to be tested, but an indication that this result might extend to other Prx1-group Prxs was obtained for the plant chloroplast 2-Cys PrxA interacting with *E. coli* Trx, although the decreased activity observed for the primarily dimeric mutant (F84R) was modest and within experimental error (24).

Importantly, this new finding implies that decamer dissociation, which can potentially be promoted by factors such as phosphorylation that modulate the dimer–dimer interfaces (68), lowers activity for both the oxidative and reducing phases of turnover. Dissociation to dimers does, however, serve one protective function as our earlier findings indicated that dimers are less prone toward hyperoxidative inactivation by their substrate (41). Moreover, as dimers offer more exposed surface area, this could promote the interaction of Prx1-like enzymes with other cellular proteins that could block oligomerization and thus hold the Prx in a less catalytically active state. As we have previously emphasized, molecules binding and stabilizing either the FF or LU conformations of Prxs in any oligomeric state will have an inhibitory effect (since catalysis requires dynamic changes between FF and LU), and thus a great potential exists for generating inhibitors targeted to regions that affect FF↔LU transitions, which may be far from the active site in less conserved regions and thereby less likely to cross-react with other Prxs (45, 46).

Materials and Methods

HRP type VI, NADH, iodoacetamide, trichloroacetic acid, calcium chloride, ammonium sulfate, sodium citrate, boric

acid, and sodium phosphate were purchased from Sigma. 5-Iodoacetamidofluorescein was purchased from Molecular Probes (Invitrogen). Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was from Worthington Biochemicals, and 1,4-dithiothreitol (DTT) was from Anatrache. Diethylenetriamine pentaacetic acid (DTPA) and 2,5-dihydroxybenzoic acid were purchased from Acros Organics. Guanidine hydrochloride (GuHCl) was from Lancaster Synthesis. Ethylenediamine tetraacetic acid (EDTA), β-mercaptoethanol (BME), ammonium bicarbonate, and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Research Organics. Sodium citrate, HCl, acetonitrile, and H₂O₂ were from Fisher, and PD-10 desalting columns were from GE Healthcare.

Protein mutagenesis, expression, and purification

AhpF and the AhpF NTD with one mutation, S128W (NTD_{S128W}), were expressed and purified as previously described (41, 51) and quantified using $\epsilon_{280} = 21,250 \text{ M}^{-1} \text{ cm}^{-1}$ for NTD_{S128W}. Untagged variants of AhpC were created by site-directed mutagenesis and expressed in TA4315 or JW0598, *E. coli* mutant strains lacking endogenous AhpC, and purified as previously described (40, 51) to >98% purity. For the C165S and W169F variants, reducing agent (5 mM DTT or 5 mM BME) was included throughout the purification process and in the storage buffer to prevent peroxide-mediated inactivation due to sulfinic acid formation.

The concentration of AhpC (in terms of monomers) was determined by absorbance at 280 nm. To determine the extinction coefficient for each variant, absorbance values at 280 nm were measured for four concentrations of each protein in dH₂O. One hundred microliters of each diluted sample was then removed from the cuvette and the protein concentration was measured with the BCA Protein Assay Reagent (Pierce-Thermo Scientific) using BSA as a standard. The concentration was calculated using the average molecular weight of the mutant protein without the N-terminal Met. The absorbance was plotted *versus* concentration and the extinction coefficient was calculated by direct fit to a linear equation. The measured extinction coefficients for the WT ($24,100 \pm 1200 \text{ M}^{-1} \text{ cm}^{-1}$), T43A and T43S variants agreed with the previously determined values of $24,300 \text{ M}^{-1} \text{ cm}^{-1}$ for WT AhpC (50). For the other mutants, the measured values used to calculate concentrations (in terms of monomers) were $27,000 \pm 600$ for T43V, $27,000 \pm 1000$ for E49Q, and $27,800 \pm 1200$ for R119A. Values of $19,400 \text{ M}^{-1} \text{ cm}^{-1}$ for W169F and $23,100 \text{ M}^{-1} \text{ cm}^{-1}$ for W81F were reported previously (40).

Measurement of AhpC stability

The intrinsic tryptophan fluorescence of AhpC decreases by ~70% as the protein becomes denatured (35). The change in reduced WT AhpC tryptophan fluorescence at increasing temperatures from 20°C to 80°C occurs at the same temperatures where a decrease in α -helical content is observed by circular dichroism spectroscopy (Supplementary Fig. S1), confirming that this assay reflects global protein stability and not local unfolding or alterations to the oligomeric structure. To measure the relative stability of AhpC mutants, WT and mutant AhpC proteins were reduced with 10 mM DTT for at least 30 min and then exchanged into 10 mM sodium

phosphate, and 1 mM EDTA, pH 7.0, using Bio-Gel P6 resin. Ten microliters of 0.013 mg/ml AhpC was mixed with an equal volume of GuHCl to provide final concentrations of 0–4 M GuHCl. Intrinsic tryptophan fluorescence was measured at $\lambda_{\text{ex}} = 280$ nm and $\lambda_{\text{em}} = 350$ nm using a Tecan Safire 2 monochromator-based microplate reader at 25°C. The photomultiplier tube voltage was set so that a fresh sample without GuHCl gave a fluorescence intensity that was 80% of the maximal fluorescence value. The concentration of GuHCl at which AhpC was 50% denatured was determined by fitting, using KaleidaGraph 4.0, to the following equation:

$$y = \frac{(F_F + m3 \times [\text{GuHCl}]) + (F_U + m4 \times x) \exp^{m2([\text{GuHCl}] - D_{50\%})}}{1 + \exp^{\frac{m2([\text{GuHCl}] - m1)}{R}}}$$

where [GuHCl] is the concentration of GuHCl, F_F is the fluorescence of the folded protein, F_U is the fluorescence of the unfolded protein, R is the gas constant, T is temperature, and $D_{50\%}$ is the GuHCl concentration at which the protein is 50% unfolded (23).

Determination of functional pK_a by pH dependence of H_2O_2 reactivity

The pH dependence of the AhpC reaction with H_2O_2 was determined as described previously by monitoring the ability of AhpC to compete with HRP in the presence of limiting amounts of H_2O_2 over a range of pH values (35); this approach was first applied to study Prx pK_a in 2007 (38). Briefly, when HRP reacts with H_2O_2 to form compound I, absorbance at 403 nm decreases with a second-order rate constant of $1.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at 25°C and pH 7. As increasing concentrations of reduced AhpC are included in the reaction mix containing HRP, followed by addition of a substoichiometric amount of H_2O_2 (relative to the HRP concentration), AhpC competes for peroxide, resulting in decreased oxidation of HRP and a smaller change in absorbance. By comparing the ratio of oxidized HRP at each concentration of AhpC with that in the absence of AhpC, a second-order rate constant for AhpC with H_2O_2 can be calculated (35, 38). Because the assay involves a single turnover and the C_p must be deprotonated to attack the peroxide substrate, the change in activity as a function of the pH of the reaction is taken to be a direct readout of the pK_a of the C_p .

The concentration of the HRP stock in 5 mM potassium phosphate buffer with 0.1 mM DTPA, pH 7.0, was determined at 403 nm ($\epsilon_{403} = 1.02 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$). Prerduced AhpC in the same buffer was mixed with HRP and an equal volume of a second buffer at various pH values between 4 and 9.5 to give a final concentration of 10 mM phosphate, 10 mM boric acid, 10 mM sodium citrate, 100 mM sodium chloride, 0.1 mM DTPA, 7.5 μM HRP, and various concentrations of AhpC (Supplementary Table S2) in a final volume of 150 μL . The starting HRP absorbance for each sample was determined at 403 nm in a Tecan Safire 2 microplate reader. The reaction was started by the addition of 10 μL of 45 μM H_2O_2 in deionized H_2O (final concentration of $\text{H}_2\text{O}_2 = 3 \mu\text{M}$). The extent of HRP oxidation by H_2O_2 was determined by monitoring the change in absorbance at 403 nm within 90 s of peroxide addition. The actual final pH was measured for each mixture. The percentage of inhibition of HRP oxidation (F /

$1-F$) at each AhpC concentration was plotted against AhpC concentration to obtain the second-order rate constant for AhpC (k_{AhpC}); k_{AhpC} was plotted *versus* pH and the pK_a was calculated as previously described (35).

For highly impaired Prx mutants, our data indicated that the competition kinetics with HRP overestimated the actual rate of reactivity with H_2O_2 (Supplementary Fig. S2). As a complementary approach, we measured the activity of R119A (one of the highly impaired mutants) as a function of pH by monitoring peroxide disappearance using the ferrous oxidation in xylenol orange (FOX) assay, as we did previously when studying the *E. coli* BCP protein (PrxQ group) (56). We were able to show that the change in activity across the pH range and the calculated pH were indistinguishable between the two assays within experimental error, confirming that the pK_a obtained by the HRP analysis was indeed reliable (Supplementary Fig. S2).

H_2O_2 reactivity of AhpC variants

To measure apparent k_{cat} and K_m values for AhpC with H_2O_2 , we utilized our previously established assay, which directly monitors the loss in fluorescence as the fluorescent variant of the NTD of AhpF (NTD_{S128W}), the direct electron donor to AhpC, becomes oxidized in the presence of low AhpC concentrations and varying peroxide concentrations (41). Briefly, NTD_{S128W} was prerduced with 10 mM DTT for 1 h and excess DTT was removed using a PD10 gel filtration column equilibrated with appropriate reaction buffer. Standard reaction buffer at pH 8.5 (50 mM Tris-HCl, with 0.5 mM EDTA and 100 mM ammonium sulfate) was used for WT AhpC and all variants (except W81F, which was assessed at pH 8 due to instability at pH 8.5) to maximize the amount of deprotonated C_p thiol present during the analyses, yielding pH-independent kinetic parameters.

Prerduced NTD_{S128W} (10 μM) and AhpC (0.05–1 μM ; Supplementary Table S2) in one syringe were mixed with various peroxide concentrations (in the same reaction buffer) in the second syringe of an Applied Photophysics SX.18 MV stopped-flow spectrophotometer at a constant temperature of 25°C. Fluorescence changes were monitored ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} > 320$ nm) using an emission filter. All rates reported are averages of at least three measurements in at least two independent assays. The initial rate of fluorescence decrease was determined by linear regression of data from the first 0.2–2 s of the reaction. The total change in fluorescence upon full oxidation in the presence of AhpC and excess peroxide was measured and used to convert the fluorescence change into the concentration of oxidized NTD_{S128W}. The values and standard errors for $k_{\text{cat,app}}$ and $K_m^{\text{H}_2\text{O}_2, \text{app}}$ at 10 μM NTD_{S128W} for each AhpC variant were determined by direct fit to the Michaelis–Menten equation in KaleidaGraph.

Evaluation of initial rates of AhpC reduction by AhpF

In a linked assay with excess NADH and H_2O_2 , low amounts of AhpF, and varying higher concentrations of AhpC, reduction of AhpC by AhpF has been shown to be rate limiting (52). This assay was previously used to demonstrate a k_{cat} and K_m of AhpF for AhpC of 237 s^{-1} and 19 μM , respectively (52), and was used here to measure the rate of AhpF reduction for each AhpC mutant. Steady-state assays employed various concentrations of each AhpC variant (0.1–20 μM ;

Supplementary Table S2), 0.025 μM AhpF, 150 μM NADH, and 50 μM H_2O_2 (or 1 mM for high apparent $K_m^{\text{H}_2\text{O}_2}$ mutants R119A, T43V, T43A, and E49Q) in 25 mM potassium phosphate with 1 mM EDTA and 100 mM ammonium sulfate at pH 7.0 and 30°C. The absorbance at 340 nm was monitored as NADH becomes oxidized using a Tecan Safire 2 microplate reader. The initial rate, which approximates the apparent k_{cat}/K_m of AhpF for AhpC, was assessed by a linear fit of the velocity data over the range of AhpC concentrations, which were kept below the previously determined K_m^{AhpC} value of 19 μM .

Analytical ultracentrifugation

Sedimentation data at 230 or 280 nm were collected every 4 min over ~ 4 h at rotor speeds of 40,000 or 42,000 rpm and a radial step size of 0.003 cm using an Optima XL-A analytical ultracentrifuge outfitted with absorbance optics (Beckman Instruments). Samples were evaluated in 25 mM potassium phosphate buffer, 1 mM EDTA, and 0.15 M NaCl, pH 7, at 20°C. To maintain the redox state of the protein throughout the run, oxidized protein was analyzed in the presence of 1 equivalent of H_2O_2 and prereduced protein was analyzed with 100 μM DTT present in both the sample and the reference cell. Proteins were evaluated in their oxidized state at 5, 10, 20, 50, and 100 μM and in their reduced state at 1 and 10 μM . Partial specific volumes were calculated for each mutant on the basis of amino acid compositions, and the buffer density of 1.00773 g/cm³ was determined using a DA-310 M precision density meter (Mettler Toledo) at 20°C. Data were analyzed using DCDT+ (version 2.4.3) from J. S. Philo.

Protein crystallography

Crystallization. The *StAhpC* variants were concentrated to ~ 10 mg/ml in 25 mM potassium phosphate and 1 mM EDTA, pH 7.0. Initial crystallization hits for some mutants were obtained through hanging drop optimizations of the 1.4 M MgSO_4 and 0.1 M MES, pH 6.5, reservoir used to grow WT crystals (45). As this crystal form grows from the LU disulfide form, we did not add reducing agent to the buffer. Crystals of T43V, E49Q, and W169F grew in 2 weeks at 26°C in drops of 4 μl protein and 1 μl reservoir. As previously reported (45), crystal growth was enhanced by seeding, using 0.5 μl of serially diluted crushed crystals added to fresh drops. This yielded single crystals of ~ 1 –2 mm³ after a few days. W81F grew small and poorly diffracting crystals in only a single drop of 2 μl protein and 1 μl of the WT condition, and many attempts to replicate these crystals were unsuccessful.

Additional sitting drop trials for the T43S, T43A, W81F, R119A, and C165S variants using a Phoenix Crystallization Robot (Art Robbins Instruments) and the commercially available Hampton Crystal I and II and Hampton Index I and II screens (Hampton Research) yielded hits for T43S and T43A. For T43S, the best among multiple leads led to optimized conditions of 4 μl protein and 2 μl of a reservoir of 1.2 M ammonium sulfate, 24% glycerol, and 0.1 M Tris, pH 7.3, at 26°C. For the T43A mutant, the best crystals grew at 4°C mixing 4 μl protein with 1 μl of a reservoir of 0.2 M ammonium acetate, 25% polyethylene glycol (PEG) 3350, and 0.1 M BIS-TRIS, pH 6.5.

As we reported earlier (45), AhpC variants lacking C165, in the presence of BME, can form a C_P-BME mixed disulfide adduct that stabilizes the LU conformation. Therefore, to crystallize C165S AhpC, we added 2 mM BME and 10 mM H_2O_2 in an attempt to form LU C_P-BME adducts; this dramatically improved crystal growth and generated well-diffracting crystals. Adding peroxide to the W81F and R119A samples did not improve their crystallization behavior and no further W81F crystals and R119A crystals were obtained.

As previously described for WT AhpC (45), the crystals grown in the LU disulfide form were soaked with DTT to generate the thiol/thiolate form, designated here with a subscript DTT (*i.e.*, WT_{DTT}). For harvesting, crystals were equilibrated for 3 min in a fresh ~ 10 μl drop of their respective mother liquor with DTT added to a concentration of ~ 0.1 M. Subsequently, $\sim 20\%$ glycerol was added to the drop as a cryoprotectant, and after another 3 min, the crystals were scooped and flash-frozen by plunging into liquid nitrogen. The few small W81F crystals grown did not survive the DTT treatment.

Data collection. Frozen crystals were sent to the Advanced Light Source (ALS) Lawrence Berkeley National Laboratory, and data were collected on beam lines 5.0.1, 5.0.2, 5.0.3, and 8.2.2. The resolution to which the crystals diffracted varied widely, and ~ 30 –60 crystals were screened for each variant to identify those that were best diffracting. For W169F, the highest resolution dataset for a DTT-soaked crystal was obtained using our in-house R-AxisIV X-ray detector and Cu-K α radiation. All data were processed with Mosflm 7.1.1 (3). The T43A crystal, which grew with PEG as a precipitant, was in space group P1, whereas the T43S, T43V, W81F, E49Q, C165S, and W169F variants crystallized from sulfate solutions in the space group C222₁ as seen for previous *StAhpC* structures (Supplementary Table S1) (45).

The resolution cutoff criterion used here was CC_{1/2} in the 0.2–0.3 range based on evidences that including such weak data tends to improve models and does not make them worse (21, 22). The resolutions at which $\langle I/\sigma \rangle \sim 2$ also reported for reference, and for W169F_{DTT}, a paired refinement test (22) confirmed that including the additional data (between 2.7 and 2.3 Å resolution) did improve the model (Supplementary Table S1). Specifically, after the final W169F_{DTT} structure was slightly perturbed (atoms given 0.2 Å random shifts and all *B*-values set to 30 Å²) and subjected to automated rerefinement at 2.7 and 2.3 Å resolution, the 2.3 Å refined model had at 2.7 Å resolution an *R*-value 0.7% higher (*i.e.*, less overfit) and an *R*_{free} value 0.3% lower. *R*_{free} flags for five percent of the data were imported from the WT_{DTT} dataset (PDB code 4ma9) (45).

Refinement. Initial refinements for the C222₁ variants utilized the WT_{DTT} model (PDB code 4ma9) (45), with active site residues 40–50 and 161–186 removed for the five chains (half decamer) in the asymmetric unit. This model was also used as a molecular replacement search model for the P1 T43A structure using Phaser-MR, which yielded a solution with two decamers in the asymmetric unit. As was seen previously (45), the DTT soaks reduced the disulfides. An initial rigid-body refinement was done with Refmac 5.8.0073 (31), then manual model building and adjustments were

performed by Coot (11); subsequent refinements were performed with Phoenix 1.9-1692 (1).

Each chain was modeled as FF, LU, or mixed FF/LU conformations as determined by the initial omit map density. It was found that T43S_{DTT} was predominantly FF, E49Q_{DTT} adopted a perturbed FF, C165S_{DTT} and W169F_{DTT} were a mix of FF/LU_{SH}, T43V_{DTT} and T43A_{DTT} were predominantly LU_{SH}, and W81F (not treated with DTT) was LU_{SS}, although the resolution was not good enough to model the disulfides (Supplementary Table S1). For chains that exhibited active sites with substantial electron density for both conformations, the ratio of FF to LU_{SH} occupancy was refined. Refinements with riding hydrogens were tested and yielded better R_{free} values for T43S_{DTT} and E49Q_{DTT}.

In late stages of refinement, adding one TLS group per monomer improved R_{free} by 1–3% in all cases. Positions modeled as ions in the WT_{DTT} structure (45) and with suitable electron density and environment were similarly modeled. Generally, Cl[−] was placed at the N-terminus of each protein chain, and K⁺ was placed between the Thr72 and Thr72' carbonyl oxygen at each decamer-building interface. Finally, as was noted for the WT_{DTT} structure (45), a series of peaks with strong difference density were commonly seen at the decamer-building interfaces near the Arg105 and Arg105' side chains. With the slightly higher resolution provided by the T43S_{DTT} structure, we speculate that these peaks are disordered solvent molecules and ions that adopt symmetric alternate conformations; we have modeled a Cl[−] ion in the higher resolution structures and left the site unmodeled in the lower resolution structures.

A few notable features were observed for this set of structures. First, for structures in the C222₁ crystals, chain A maintains an LU C-terminus due to crystal packing interactions. For the WT_{DTT} structure, the lack of chain A C-terminal packing resulted in the BA' active site to be ~50/50 FF↔LU_{SH} (45). We expected this to be similar for mutants that were mostly FF, but in fact, we saw that T43S_{DTT} had an ~100% LU_{SH} BA' active site, which we think may be due to the crystals for this mutant being grown in the presence of 20% glycerol; density for disordered solvent, possibly a glycerol, suggests that this molecule may bind and stabilize the LU_{SH} active site.

Second, as was seen for the C165A_{DTT} structure (45), the C165S_{DTT} structure exhibited weak FF C-terminal density and we have therefore modeled the residues beyond Val164 as having 0.75 occupancy based on trials with several occupancies. Because of the weak density for the C-terminal region, we utilized the WT_{DTT} model for reference restraints in Phoenix, which resulted in improved geometry and R_{free} .

In addition, residual electron density was observed near the chain C_P, and mass spectroscopy analysis of C_R mutant crystals treated with millimolar levels of peroxide has shown that some SO₂[−] or SO₃[−] can be formed (45), so we have modeled this chain as the SO₃[−] form and allowed the occupancy of the oxygen to refine, yielding close to ~0.5 for all three oxygens. Extra electron density is also seen near the C_P S_γ for all chains in the E49Q_{DTT} structure that we have modeled as a partially occupied (~0.5) C_P-SOH; however, the density is quite weak and does not form a favorable bond angle and may actually be from a solvent molecule that occupies the site when the enzyme is LU. The final statistics for data collection and refinement are reported in Supplementary Table S1.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

AhpC = alkyl hydroperoxide reductase C-22
(or catalytic) component
AhpE = alkyl hydroperoxide reductase E
AhpF = the natural, flavin-containing reductant
of AhpC enzyme
ALS = Advanced Light Source
BCP = bacterioferritin comigratory protein
BIS-TRIS = Bis(2-hydroxyethyl)amino-
tris(hydroxymethyl)methane
BME = β -mercaptoethanol
C_P = active site peroxidatic cysteine
C_R = resolving cysteine
D_{50%} = concentration of GuHCl that causes
50% unfolding of the protein
DTPA = diethylenetriamine pentaacetic acid
DTNB = 5,5'-dithiobis(2-nitrobenzoic acid)
DTT = 1,4-dithiothreitol
EDTA = ethylenediamine tetraacetic acid
FF = fully folded
FOX = ferrous oxidation in Xylenol orange
GuHCl = guanidine hydrochloride
H₂O₂ = hydrogen peroxide
HRP = horseradish peroxidase

$k_{\text{cat,app}}$ = apparent maximal turnover rate under
a specific set of conditions
 $K_{\text{m,app}}$ = apparent Michaelis constant for one specific
set of conditions
LU = locally unfolded
MD = molecular dynamics
MES = 2-(N-morpholino)ethanesulfonic acid
NADH = nicotinamide adenine dinucleotide, reduced
NTD = N-terminal domain
NTD_{S128W} = the N-terminal domain of AhpF in which
Ser128 has been replaced by Trp
PEG = polyethylene glycol
Prx = peroxiredoxin
 ρ_{rms} = root-mean-square electron density
ROH = alkyl alcohol
ROOH = alkyl hydroperoxide
R-SOH = alkyl sulfenic acid
St = *Salmonella typhimurium*
TPCK = tosyl phenylalanyl chloromethyl ketone
Tpx = thiol peroxidase
Tsa1, Tsa2 = thiol-specific antioxidant 1 and 2 (Prx) of
Saccharomyces cerevisiae
TLS = translation/libration/screw (used for rigid-
body motion of large groups of atoms)
WT = wild-type