Transition steps in peroxide reduction and a molecular switch for peroxide robustness of prokaryotic peroxiredoxins

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In addition to their antioxidant function, the eukaryotic peroxiredoxins (Prxs) facilitate peroxide-mediated signaling by undergoing controlled inactivation by peroxide-driven over-oxidation. In general, the bacterial enzyme lacks this controlled inactivation mechanism, making it more resistant to high H2O2 concentrations. During peroxide reduction, the active site alternates between reduced, fully folded (FF), and oxidized, locally unfolded (LU) conformations. Here we present novel insights into the divergence of bacterial and human Prxs in robustness and sensitivity to inactivation, respectively. Structural details provide new insights into sub-steps during the catalysis of peroxide reduction, enabling the transition from an FF to a LU conformation. Complementary to mutational and enzymatic results, these data unravel the essential role of the C-terminal tail of bacterial Prxs to act as a molecular switch, mediating the transition from an FF to a LU state. In addition, we propose that the C-terminal tail has influence on the propensity of the disulphide bond formation, indicating that as a consequence on the robustness and sensitivity to over-oxidation. Finally, a physical linkage between the catalytic site, the C-terminal tail and the oligomer interface is described.

Reactive oxygen species (ROS) are inevitable byproducts of normal aerobic metabolism, which at high levels can inflict damages on DNA, lipids and proteins. The intracellular concentration of peroxides is tightly maintained at very low levels by the oxidant-specific sensors that regulates the expression of oxidant-scavenger enzymes1,2. Peroxiredoxins (Prxs), a class of thiol-based peroxidases, present in all biological kingdoms from bacteria to human and consisting of six evolutionary subfamilies (Prx1, Prx5, Prx6, Tpx, PrxQ, and AhpE), are the dominant enzymes responsible for the reduction of over 90% of mitochondrial and cytoplasmic H2O23,4. These enzymes have recently grabbed attention not only due to their antioxidant function, but also for their additional significance in a broad range of cellular events including modulation of local intracellular H2O2 level required for signaling events5, and peroxide sensing for activation of transcription factors that control the expression of antioxidant enzymes6,7. Besides the importance of Prxs in keeping thioredoxins in a reduced state for maintaining cell viability under oxidative stress8, they act as peroxinitrite reductases9, chaperone holdases10, protein foldases11, and regulators of circadian rhythm12. Because of their fundamental cellular functions, Prxs are implicated in aging, cancer, cardiovascular diseases, diabetes and neurodegeneration, and are therefore appealing as therapeutic targets13,14.

The Prx subfamily enzyme Prx1, also typically called 2-Cys Prx, is among the most highly expressed, soluble proteins in the cell15, and is characterized by two conserved cysteine residues. One is represented by the peroxidatic cysteine (Cp), which becomes selectively oxidized by H2O2 to the Cp–SOH intermediate. This Cp further reacts with the so-called resolving cysteine (Cs). The Cs is located at the C-terminus of the other subunit of the basic functional dimer, forming an intermolecular disulphide with the Cp. Regeneration of the intermolecular disulphide bridge occurs via the Peroxiredoxin reductases (PrxR), enabling a continuous catalytic cycle16,17.

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As shown in Fig. 1, the detailed structural analysis of bacterial Prx1, called Alkyl hydroperoxide reductase subunit C (AhpC), reveals two distinct active site conformations linked with their catalytic cycle. In the reduced state, the active site is competent for productive substrate binding and is in a so-called fully folded (FF) conformation, where the peroxidatic cysteine, CP47 (according to the \textit{Escherichia coli} subunit AhpC numbering), is part of the \(\alpha_2\)-helix and is located at the bottom of the catalytic cavity, formed by the conserved residues, P40, T44 and R120. In parallel, the resolving cysteine (CR166), placed at the C-terminal tail of an adjacent subunit, is folded across the active site, placing CP and CR 14 Å away and in opposite orientation\(^1\) (Fig. 1). In the oxidized state, the CP and CR are disulfide bonded in the so-called locally unfolded (LU) conformation, where (i) CP of \(\alpha_2\)-helix is partially unwound, orienting CP towards CR, and (ii) the C-terminal tail unfolds from the active site region and becomes disordered\(^2\) (Fig. 1). Additionally, the redox-state dependent active site conformations are proposed to modulate the quaternary structure of Prxs, whereas the reduced FF active site favors the decamer formation and the oxidized LU active site weakens the decamer into a dimer\(^3\). Our recent studies on the \textit{Escherichia coli} AhpC (EcAhpC) explored the different functional role of its C-terminal tail, as influenced by the redox-state of the enzyme. In the oxidized state, the C-terminal tail of EcAhpC, which includes amino acids 172 to 187, is essential for regeneration by its specific Alkyl hydroperoxide reductase subunit F (AhpF) (Fig. 1). At the same time, the C-terminal tail is essential for the formation of a stable doughnut-shaped decamer under reduced conditions\(^4\)–\(^6\). Based on the elucidation of distinct redox-state-linked active-site conformations, it has been proposed that during the formation of the CP-SOH intermediate, the active site should move out of the FF conformation to facilitate the formation of the disulfide bond with CR\(^7\). Nevertheless, a detailed picture of the structural transition(s) between the FF and LU state during the catalysis is missing. In contrast, the structural studies on eukaryotic Prxs revealed that the FF active site conformation persists during and after oxidation of CP to CP-SOH (Fig. 1), which eventually promotes the over-oxidation of CP-SOH into CP-SO\(_2\)H/CP-SO\(_3\)H, and finally inactivates the enzyme\(^8\)–\(^10\). The presence of two conserved sequence features in human and other eukaryotic Prxs, the so-called GGLG motif and the extended C-terminal helix containing the YF motif, are thought to be essential for regulating H\(_2\)O\(_2\) mediated signal transduction\(^11\)–\(^13\). However, the greater importance of the C-terminal helix for sensitivity to over-oxidation has been confirmed\(^14\)–\(^16\). The C-terminal helix with its YF motif folds across the active site, thereby delaying the conformational change from FF to LU, and favoring over-oxidation\(^1\) (Fig. 1). It has been implicated that the disulfide formation or over-oxidation depends on the rate of FF to LU transitions. According to the proposed floodgate model, over-oxidation of Prxs facilitates the local accumulation of intracellular H\(_2\)O\(_2\) required for signaling events\(^17\). Finally, the enzyme sulfiredoxin (Srx) catalyzes the repair of over-oxidized Prxs, to restore their peroxidase activity\(^18\).

In order to (i) gain deep insight into the delicate balance between the FF and LU active site during the catalytic cycle, (ii) to explore the underpinning role of the C-terminal tail in active site conformation, and (iii) to understand the features making the bacterial and human Prxs more robust and sensitive to inactivation, respectively, a combination of genetic engineering, enzyme kinetics and crystallography were used to establish the values

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**Figure 1. The catalytic cycle of 2-Cys Prxs.** The basic functional dimeric unit of 2-Cys Prxs and their active site region is shown. During peroxide reduction, the reduced CP assumes the FF conformation and reacts with H\(_2\)O\(_2\) to form a CP-SOH intermediate. This local unfolding of the active site enables the formation of a disulfide bond with the CR located in the C-terminal tail of adjacent subunit. AhpC is then reduced by AhpF to its FF conformation for future catalytic cycles, with AhpF being oxidized in this process. AhpF is regenerated with the utilization of NADH molecules for further catalytic cycles. In comparison, human Prx is stabilized in the FF active site conformation even after the formation of the intermediate CP-SOH form, which eventually promotes over-oxidation.
of kinetic parameters and sensitivity for inactivation using the well-established *E. coli* AhpC and its mutants. By generating and determining the structure of a chimeric *Ec* AhpC1-186-YFSKHN, which includes the extended C-terminal helix with the YF motif of human Prx2, we deduced for the first time the intermediate active-site conformations, lying between the FF and LU conformations. Furthermore, the studies reveal that the C-terminal tail acts as a molecular switch to mediate the structural transition between the FF and LU state. Finally, we propose the detailed conformational transition states that accompany the peroxide reduction and over-oxidation cycle, providing novel insight into the evolutionary divergence of the resistant and sensitive Prxs catalysts of bacteria and human, respectively.

**Results**

**Sensitivity to oxidative inactivation of a novel chimeric *Ec*AhpC1-186-YFSKHN protein.** The bacterial Prx is less sensitive to inactivation by hyper-oxidation, whereas the human Prx is sensitive towards inactivation with its C-terminal helix (YFSKHN-helix), including the conserved residues YF, playing a vital role. The question arises, whether the apparent sensitivity to over-oxidation exerted by the C-terminal helix of human Prx2 can be transformed into Prxs of prokaryotes. We used *E. coli* as a prototype to construct a chimeric Prx, *Ec*AhpC1-186-YFSKHN, which includes the extended C-terminal helix with the YF motif of human Prx2, we deduced for the first time the intermediate active-site conformations, lying between the FF and LU conformations. Furthermore, the studies reveal that the C-terminal tail acts as a molecular switch to mediate the structural transition between the FF and LU state. Finally, we propose the detailed conformational transition states that accompany the peroxide reduction and over-oxidation cycle, providing novel insight into the evolutionary divergence of the resistant and sensitive Prxs catalysts of bacteria and human, respectively.

As shown in Fig. 2A, WT *Ec*AhpC reacts with 1 mM H$_2$O$_2$ and actively consumes NAPDH. Increasing the H$_2$O$_2$ concentration to 10 and 20 mM caused an activity drop of ~12% and ~18%, respectively. The inhibitory effect of H$_2$O$_2$ to *Ec*AhpC increased significantly at a concentration of 30 mM, resulting in an enzyme activity reduction of ~44%. In comparison, an increase of 10 mM H$_2$O$_2$ decreased the chimeric *Ec*AhpC1-186-YFSKHN enzyme activity by already ~33% (Fig. 2B,C), followed by a further drastic decrease of ~50% in the presence of 20 mM H$_2$O$_2$, and finally to a ~64% drop with 30 mM H$_2$O$_2$ (Fig. 2C). The data indicate that WT *Ec*AhpC is resistant to inactivation up to 20 mM H$_2$O$_2$, as it shows little variation for peroxidase activity. In contrast, the peroxidase activity of chimeric *Ec*AhpC1-186-YFSKHN is affected at H$_2$O$_2$ concentrations above 1 mM, revealing that the transformation of the human C-terminal YFSKHN-helix inside the bacterial *Ec*AhpC increased its sensitivity to over-oxidation.
all the residues and the resolving cysteine (chains C, E, F, H and J), which is located about 10 Å away from the 4CP7 was determined to 3.1 Å resolution (Fig. 4A). Except for the C-terminus, clear electron density was observed for

Coming towards CP47 towards the CR166 was determined to be 1.44 μM and 0.09 s⁻¹, respectively. In case of EcAhpC1–186–YFSKHN a higher Km of 1.77 μM and slightly lower kcat value of 0.082 s⁻¹ was calculated, resulting in a lower catalytic efficiency of EcAhpC1–186–YFSKHN [kcat/Km (H2O2)], 4.8 × 10⁻⁴ M⁻¹ s⁻¹ when compared to the wild-type enzyme (6.2 × 10⁻³ M⁻¹ s⁻¹).

Crystallographic structure of oxidized and decameric-shaped EcAhpC1–186–YFSKHN. To understand the structural effect of the human YFSKHN-helix in general, in particular inside the chimera EcAhpC1–186–YFSKHN, as well as to identify the individual amino acids essential for sensitivity to oxidative inactivation of Prxs, the crystal structure of oxidized EcAhpC1–186–YFSKHN has been solved at 2.7 Å resolution. The asymmetric unit contains one decamer composed of five catalytic dimers (α2β2), with each subunit containing one catalytic active peroxidatic and one resolving cysteine (Fig. 3A,B). The subunit consists of a central seven-stranded β-sheet, flanked at one side by four and at the other side by two α-helices (Fig. 3C). Each subunit has a dimer and oligomeric interface. The dimer interface is mainly stabilized by salt bridge and hydrogen bond interactions, while the oligomer interface is mainly stabilized by hydrophobic interactions. Superposition of the recently resolved oxidized EcAhpC structure with the corresponding dimer of the oxidized EcAhpC1–186–YFSKHN one resulted in an r.m.s.d. value of 0.4 Å. The intermolecular disulphide bond observed between Cα47 and Cα166′ in the dimer interface (Fig. 3B,C) confirms the oxidized state of the EcAhpC1–186–YFSKHN structure. The peroxidatic cysteine (Cα47), located in the first turn of helix α2, adopts an LU conformation in the oxidized structure (Fig. 3B,C and Supplementary Fig. S1A). In most of the disulphide bonded active sites of the decameric and oxidized EcAhpC1–186–YFSKHN, the C-terminal arm beyond the resolving cysteine (Cα166) is disordered, indicating the high flexibility of the C-terminus.

However, the structure in chain (H) could be resolved until residue 177, showing amino acids 167 to 177 oriented away from the active site and stacked against the neighboring symmetry molecule. Similarly, the C-termini of chains A, G and J are also stabilized by the packing interactions with the symmetry related molecules (Supplementary Fig. S1B,C). The average main chain B-factors analysis revealed three dynamic regions of the C-termini of chains A, G and J are also stabilized by the packing interactions with the symmetry related molecules. The sulfate ions observed in the interface region of the structure, emphasize their role in stabilizing the oxidized EcAhpC in a decameric form in solution.

The active site conformation in reduced EcAhpC1–186–YFSKHN. To shine a light on the redox modulated structural alterations in the active site, the crystallographic structure of reduced EcAhpC1–186–YFSKHN was determined to 3.1 Å resolution (Fig. 4A). Except for the C-terminus, clear electron density was observed for all the residues and the resolving cysteine (chains C, E, F, H and J), which is located about 10 Å away from the peroxidatic one (Fig. 4B). No disulphide bond between Cα47 and Cα166′ was observed in any of the active site interfaces of the decameric EcAhpC1–186–YFSKHN, confirming that the structure fairly represents the reduced form of EcAhpC1–186–YFSKHN. In the reduced state, the typical FF active site is arranged in a way that the Cα47 positions in the first turn of helix α2 and Cα166′, containing the C-terminal arm, is folded across the dimer interface to come in proximity to the first turn of helix α2 of another subunit. This conformational alteration brings the Cα47 and Cα166′ to face opposite directions and to become separated by about 10 Å.

Interestingly, two different active site conformations were observed in the reduced EcAhpC1–186–YFSKHN structure (Supplementary Fig. S2A), where the residues in the active site region are clearly defined in the electron density map except for the side chains of F45 and V46 (Fig. 4C,D). The first conformation (chains B–J) reveals that the peroxidatic Cα47 adopts helical conformation, bringing the Cα47 in a narrow solvent accessible pocket, which is surrounded by the highly conserved active site residues P40, T44 and R120 (Fig. 5A). In the second active site conformation (chain A), the first turn of α2-helix is partially unfolded to place Cα47 in the loop conformation (Fig. 5A). However, no significant influence of crystal packing on the conformation of Cα47 was observed in the reduced EcAhpC1–186–YFSKHN structure (Supplementary Fig. S2B,C). A comparison of these two reduced active sites with the oxidized LU state of EcAhpC1–186–YFSKHN reflects a significant difference in the loop region (T44–P48) (Fig. 5B), where the FF active site residues of the first active site of reduced EcAhpC1–186–YFSKHN are rotated, to expose Cα47 towards the Cα166′, and finally forming a disulphide bond in the oxidized structure.

### Table 1. Kinetic parameters of WT EcAhpC and its mutants.

|        | kcat ± SD (s⁻¹) | Km ± SD (μM) | kcat/Km (M⁻¹ s⁻¹) |
|--------|----------------|-------------|-------------------|
| WT EcAhpC | 0.090 ± 0.001 | 1.44 ± 0.1  | 6.2 × 10⁻⁴        |
| EcAhpC1–176 | 0.082 ± 0.004 | 9.45 ± 0.5  | 8.6 × 10⁻⁴        |
| EcAhpC1–166 | 0.089 ± 0.003 | 8.26 ± 0.4  | 1.0 × 10⁻⁴        |
| EcAhpC1–186–YFSKHN | 0.089 ± 0.004 | 9.74 ± 0.5  | 9.0 × 10⁻⁴        |
| EcAhpC1–186–YFSKHN | 0.082 ± 0.001 | 1.77 ± 0.1  | 4.6 × 10⁻⁴        |

Effect of the human YFSKHN-segment on catalysis. In order to address the question, whether the YFSKHN-helix affects the peroxidase activity, the catalytic turnover number (kcat), and the Michaelis-constant (Km) for substrate-binding were determined. The peroxide-dependent NAPDH-oxidation of EcAhpC and EcAhpC1–186–YFSKHN were measured with varying concentrations of H2O2 and Fig. 2D shows the NAPDH-oxidation at 30 μM H2O2 as a representative of all other measurements done. WT EcAhpC and EcAhpC1–186–YFSKHN exhibited typical substrate saturation kinetics of the Michaelis-Menten type (Fig. 2E,F). The determined kinetic constants and catalytic efficiency are reported in Table 1. The Km- and kcat-value of EcAhpC were determined to be 1.44 μM and 0.09 s⁻¹, respectively. In case of EcAhpC1–186–YFSKHN a higher Km of 1.77 μM and slightly lower kcat value of 0.082 s⁻¹ was calculated, resulting in a lower catalytic efficiency of EcAhpC1–186–YFSKHN [kcat/Km (H2O2)], 4.8 × 10⁻⁴ M⁻¹ s⁻¹ when compared to the wild-type enzyme (6.2 × 10⁻³ M⁻¹ s⁻¹).
While in the second active site of reduced EcAhpC1-186-YFSKHN, the unfolded CP47 adopts a lower magnitude of rotation, thereby preventing its full exposure towards CR166′ to form a disulphide (Fig. 5B).

FF to LU transition state like active site conformation. To gain insight into (i) the structural transition pathway between the FF and LU state, and (ii) the role of the C-terminal tail on the active site conformation, the two presented distinct active sites of reduced EcAhpC1-186-YFSKHN were compared with a typical reduced FF and oxidized LU conformation, represented by the crystallographic structure of the Salmonella typhimurium AhpC28 (PDB ID: 4MA9) and EcAhpC1-186-YFSKHN (see above), respectively. For clarity, we termed the first and second active sites of the reduced EcAhpC1-186-YFSKHN as FFlike and LUlike, respectively, to denote the folded and unfolded Cp47 in the active site environments (Fig. 6A).

Firstly, the reduced EcAhpC1-186-YFSKHN and the S. typhimurium AhpC structure (PDB ID: 4MA9) share an overall similarity indicated by an r.m.s.d. of 0.5 Å for the superposition of the catalytic dimers. When zoomed into the catalytic relevant α2-helix, the FF and FFlike active sites differ significantly (Fig. 6A). While the residues

Figure 3. Structural features of oxidized EcAhpC1-186-YFSKHN. (A) Crystal structure of oxidized EcAhpC1-186-YFSKHN in decameric form (α2)5. Each subunit in the basic functional dimeric unit is shown in light and bright colors denoted as A and A′. The intermolecular disulphide bond between the peroxidatic (Cp) and resolving (Cr′) cysteine is shown in ball representation. (B) The 2Fo-Fc map contour at 1 σ level around the Cp and Cr in disulphide bond conformation. (C) Each subunit is composed of two interface regions, namely the dimer and oligomer interface. The secondary structural features are highlighted according to their position in the structure. (D) The average main chain B-factor of ten chains showed three highly dynamic regions in the structure.
F45 and V46 are placed in the starting position of helix $\alpha_2$ inside the FF state, the $\alpha_2$-helix becomes unwound in the FF-like conformation and the main chain $C_\alpha$ atoms F45 and V46 shifted by about 0.9 Å. Similarly, in the FF-like conformation the main chain $C_\alpha$ atoms of C47 and P48 moved about 0.6 Å and 0.5 Å, respectively, compared to their corresponding positions in the FF state (Fig. 6A). Furthermore, no obvious difference in the main chain conformations of the loop amino acids P40, T44 and R120 were observed between the two conformations.

The comparison of the FF (PDB ID: 4MA9) and LU-like active site (see above) shows, significant structural variation in the first turn of helix $\alpha_2$ and the loop region (Fig. 6B,C). Due to the partial unfolding of the $\alpha_2$-helix, the main chain $C_\alpha$ atoms of V44, F45, V46, C47 and P48 of the FF active site moved by 0.4, 1.8, 1.9, 5.1 and 1.1 Å, respectively, to achieve the LU-like state positions. As shown in Fig. 6B,C, the same residues moved during the transition from the FF to the LU state but with a higher magnitude of 1.4, 3.8, 3.8, 5.9 and 1.7 Å, respectively. Because of the magnitude of displacement, the data indicate that the FF-like and LU-like state, adopt intermediate conformations, lying between the catalytic FF and LU conformation. In addition, significant differences in the backbone torsion angles were determined between the FF, LU-like and LU active sites (Supplementary Table S1).
Figure 5. Comparison of FF and LU states from EcAhpC1-186-YFSKHN crystal structures. (A) Superimposition of two different active sites, the FF-like (brown) and LU-like state (salmon) which were observed in the reduced EcAhpC1-186-YFSKHN structure. (B) Comparison of FF-like (brown) and LU-like state (salmon) in the reduced EcAhpC1-186-YFSKHN structure and the LU state of the oxidized EcAhpC1-186-YFSKHN (green) structure.

Taken together, it can be proposed that the FF-like and LU-like active sites mimic the initial and the intermediate phase of the structural deformation that occurs during the redox-modulated FF to LU conformational switch.

Oligomeric interface region. The oligomeric behavior of Prxs is proposed to be redox modulated. Under reduced conditions, the FF active site buttresses the oligomer interface, and favors decameric ring formation. Whereas under oxidized conditions, the LU active site mediates the restructuring of the oligomer interface region, resulting into lower order oligomers. A structural view to the oligomeric interface in Fig. 7 highlights differences of the distinct active site loop conformations observed between FF, FF-like, LU-like and LU, where the C_p-containing loop residues of the LU conformation are pulled away from the oligomer interface. This facilitates the destabilization of the oligomer interface through structural rearrangements of F43 and F45, which participate in the interface region. The rest of the interface region residues remain unchanged in the crystal structures.

Effect of the C-terminal tail on substrate-binding and catalytic efficiency. By genetically engineering and enzymatically characterizing the EcAhpC mutants AhpC1–172, AhpC1–186, AhpC1–186-YFSKHN and AhpC586A,788A, the questions were addressed, whether the folding of the C-terminal tail across the active site region as well as the dynamic region of helix α5 with residues S86 and T88 (Fig. 8A), influences the enzyme kinetics.

Like WT EcAhpC, all EcAhpC mutants exhibited Michaelis-Menten substrate saturation kinetics with a $k_{cat}$ of 0.089 s$^{-1}$ for EcAhpC1–172 and EcAhpC586A,788A, a value similar to EcAhpC (Table 1, Fig. 8B–E). In comparison, the $k_{cat}$ of 0.082 s$^{-1}$ of the C-terminal truncated EcAhpC1–172 was slightly lower but similar to the one determined for the chimeric EcAhpC1–186-YFSKHN. In contrast to EcAhpC1–186-YFSKHN and WT EcAhpC, all three EcAhpC mutants showed a significant decrease in H$_2$O$_2$-binding, with $Km$ values of 8.26 μM, 9.74 μM, and 9.45 μM for EcAhpC1–186, EcAhpC586A,788A and the C-terminal truncated EcAhpC1–172, respectively. The increased $Km$ values suggest that the active site binding pocket is impaired in all EcAhpC mutants. The respective values of catalytic efficiency of the enzyme [$k_{cat}/Km$ (H$_2$O$_2$)] determined for these mutants are $1.0 \times 10^4$, $9.0 \times 10^3$, and $8.6 \times 10^3$ for EcAhpC1–172, EcAhpC586A,788A and EcAhpC1–172, respectively (Table 1).

The role of the C-terminus of bacterial AhpC in H$_2$O$_2$ robustness. Revealing that the transformation of the human C-terminal YFSKHN-helix inside the bacterial EcAhpC increased its sensitivity to over-oxidation, leads now to the question of whether the C-terminus of bacterial 2-Cys Prxs keeps the enzyme more robust and enabling the prokaryotes to survive under higher peroxide concentrations. To test this, the ability of the two C-terminal variants, EcAhpC1–172 and EcAhpC1–186, to inactivation was studied. As shown in Fig. 9A,B, EcAhpC1–172 and EcAhpC1–186 showed NADPH consumption at 1 mM H$_2$O$_2$ concentration. Increase of H$_2$O$_2$ (10 mM) dropped the peroxidase activity of EcAhpC1–172 and EcAhpC1–186 by ~32% and ~34%, respectively (Figs 2C and 9A,B). The inhibitory effect of H$_2$O$_2$ in EcAhpC1–172 and EcAhpC1–186 increased significantly at a concentration of 30 mM, resulting in an enzyme reduction of ~64% and ~62%, respectively. These values are comparable to the ones of the chimeric EcAhpC1–186-YFSKHN, which were determined to be ~33% at a concentration of 10 mM H$_2$O$_2$, followed by a further drastic decrease of 50% in the presence of 20 mM H$_2$O$_2$, and finally to ~64% drop with 30 mM H$_2$O$_2$ (Fig. 2B). The results demonstrate that both EcAhpC1–172 and EcAhpC1–186 are sensitized to inactivation in a magnitude similar to that of EcAhpC1–186-YFSKHN, and finally, that the C-terminus, in particular residue I187, is essential for the robustness at high H$_2$O$_2$ concentrations (Supplementary Fig. S3). The sensitivity of EcAhpC1–172 and EcAhpC1–186 is surprising, since the destabilized active site, indicated by the increased $Km$ value, is supposed to expose the C_p for disulphide formation with C_p. We propose, that the EcAhpC1–172,
Figure 6. **FF to LU transition state like conformation.** (A) Comparison of the FF\(_{\text{like}}\) active site of EcAhpC\(_{1-186}\)-YFSKHN (brown) with the typical FF conformation in the reduced StAhpC (light blue), where the C-terminal tail is folded across the active site (blue), which is disordered in reduced EcAhpC\(_{1-186}\)-YFSKHN. (B) The oxidized- (green) and reduced (salmon) active sites observed in the EcAhpC\(_{1-186}\)-YFSKHN structures are compared with the reduced StAhpC (light blue) FF active site. The intermediate FF\(_{\text{like}}\) (brown) and LU\(_{\text{like}}\) (salmon) conformations, represent the transition state from the FF to the LU state. (C) Another view to close look at the comparison of FF, LU, FF\(_{\text{like}}\) and LU\(_{\text{like}}\) active sites.

Figure 7. **The oligomer interface region.** The decamer building interface region between two subunits are shown. The significant structural difference in the interface region is observed only in the residues that form the C\(_p\)-loop. Compared with “FF” (green) C\(_p\)-loop structure, the “FF\(_{\text{like}}\)” (blue), “LU\(_{\text{like}}\)” (magenta) and “LU” (cyan) is shifted away (solid arrow) from the interface region and this facilitates the destabilization of the oligomeric interface through F43 and F45 (dotted arrow). The complementary interface region formed by the neighboring subunit is shown with light colors shade.
and EcAhpCI187G mutants might affect the propensity of the disulphide formation (Supplementary Fig. S3) which would indicate that the C-terminus plays a role in aiding the disulphide bond formation.

**Discussion**

Evolutionary adaptation to differing environmental conditions was fundamental to the formation of the different kingdoms of life. Eukaryotic 2-Cys Prxs show a high sensitivity to peroxide inactivation by over-oxidation of the peroxidatic cysteine, C_p, to sulphinic acid (C_p-SO2H). Due to this adaptation, the enzyme keeps resting levels
of H₂O₂ low, while permitting higher levels during signal transduction⁵,¹⁸. The eukaryotic GGLG motif and its extended C-terminal helix, including the YF motif (residues YFSKHN), are responsible for regulating H₂O₂ mediated signal transduction¹⁸. The chimeric EcAhpC¹-186-YFSKHN described here demonstrates, that transformation of the human C-terminal YFSKHN-helix into the related bacterial AhpC confers sensitivity to over-oxidation on the chimeric enzyme. As for human Prx2 (Fig. 1), the engineered YFSKHN-helix inside the chimeric EcAhpC¹-186-YFSKHN falls across the active site of the chimeric enzyme, thereby delaying the FF to LU transition during the intermediate Cₚ-SOH state. In such case, the Cₚ-SOH form can further react with peroxide and leads to enzyme inactivation (VII). (B) The cartoon representation shows that the Cₚ and the first turn of the α₂-helix is stabilized by the weak interactions with the I₁₈⁶ and C-terminal tail, which is hold by residues S₈⁶ and T₈⁸. Disruption of weak interactions of the C-terminal tail, like between I₁₈⁶ and the α₂-helix or between I₁₈⁶ with S₈⁶A and T₈⁸A, leads to unfolding of the C-terminal tail from the active site region.

Figure 10. The proposed transition state(s) during the catalytic cycle of Prxs. (A) (I) The reduced Cₚ adopts FF conformation and is essential to activate and stabilize the Cₚ-thiolate for peroxidation. (II) The oxidative modification of Cₚ to Cₚ-SOH leads to localized structural alterations around the conserved active site pocket as seen from the "FFlike" conformation of the reduced EcAhpC¹-186-YFSKHN. (III) The "LUlike" conformation in the reduce EcAhpC¹-186-YFSKHN structure, reveals a severe clash between the active site residues with the C-terminal tail. The entire C-terminal arm (aa 166–187) is needed to undergo alterations and to enable the disulphide formation between Cₚ and Cₚ (solid arrow). (IV) The disulphide bonded active site is stabilized in the LU conformation, wherein the C-terminal tail is disordered (denoted by asterisk). (V) The C-terminal tail of AhpC binds to its reducing partner, the N-terminal domain (NTD) of AhpF, to regenerate for another catalytic cycle. (VI) In human Prxs, the extra C-terminal YFSKHN-helix is folded across the active site, thereby delaying the FF to LU transition during the intermediate Cₚ-SOH state. In such case, the Cₚ-SOH form can further react with peroxide and leads to enzyme inactivation (VII). (B) The cartoon representation shows that the Cₚ and the first turn of the α₂-helix is stabilized by the weak interactions with the I₁₈⁶ and C-terminal tail, which is hold by residues S₈⁶ and T₈⁸. Disruption of weak interactions of the C-terminal tail, like between I₁₈⁶ and the α₂-helix or between I₁₈⁶ with S₈⁶A and T₈⁸A, leads to unfolding of the C-terminal tail from the active site region.
The critical step, which decides about inactivation or continues resolving and recycling of AhpC, is combined with the newly described LU\textsubscript{like} conformation in the reduced EcAhpC\textsubscript{1-186}-YFSKHN structure (Figs 6B and 10A-II). This structurally trapped conformation reveals a severe clash between the active site residues C\textsubscript{p}47 and F45 with the C-terminal tail, causing the C-terminal tail residues L183\textsuperscript{′}, V184 and I187, to move away in order to accommodate the preceding structural alteration in the FF active site due to oxidative modification of the peroxidatic cysteine, C\textsubscript{p}. This dynamic behavior of C-terminal tail residues is supported by the FF\textsubscript{like} structure (Fig. 6A), demonstrating that alterations in the C\textsubscript{p} and the first turn of the \(\alpha\)2-helix caused by oxidation can disrupt interactions of the C-terminal tail, like between L183\textsuperscript{′}, I187\textsuperscript{′} and the C\textsubscript{p}-loop residues or between I187 with S86A and T88A, the C-terminal tail unfolds partially and moves away from the active site (Fig. 10B). Therefore, alterations of the C-terminal tail confer instability on the FF active site, which in turn increases the rapid shift from the FF to the LU state. These structural rearrangements explain the significant increase of the binding constants observed for the EcAhpC\textsubscript{I187G} and EcAhpC\textsubscript{S86A,T88A} mutants (Table 1). Therefore, we propose that the side chain interactions of I187 with the C\textsubscript{p}-loop as well as with amino acids S86A and T88A affect binding of peroxide in the catalytic site.

The comparison of the presented LU\textsubscript{like} and oxidized structure of EcAhpC\textsubscript{1-186}-YFSKHN also provides novel insights into the resolution of the C\textsubscript{p}-SOH form. As shown in Fig. 10A-III, the transfer from a LU\textsubscript{like} to a LU state requires the entire C-terminal arm, including residues 166 to 187, to undergo a structural rearrangement that brings the resolving cysteine, C\textsubscript{p}166\textsuperscript{′}, into proximity to finally resolve the C\textsubscript{p}-SOH form. Unfolding of the C-terminal tail might aid disulphide formation by favorably orienting both C\textsubscript{p} and C\textsubscript{p}′ disulphide bonded active site becomes stabilized (Fig. 10A-IV), which weakens the oligomer building interface\textsuperscript{19}. In this oxidized state, the C-terminal tail is highly disordered and not visible in the EcAhpC\textsubscript{I187G} -YFSKHN structure (Fig. 3C), but essential for binding with its reducing partner, the N-terminal domain (NTD) of AhpF (Fig. 10A-V). In this essential regenerative step of the catalytic cycle, the C-termius of EcAhpC wraps around the NTD and slows the dissociation rate for an efficient electron transfer process\textsuperscript{22}. Once the disulphide bond is reduced by the NTD of AhpF, the active site is present in equilibrium between the FF and LU state. In this way, the folding of the C-terminal tail of AhpC back into the active site region and the formation of a stable decamer interface maintain the conformational integrity of the FF active site (Fig. 10A-I). Such a FF active-site pocket is essential to activate and stabilize the C\textsubscript{p}-thiolate for peroxidation\textsuperscript{16}.

Establishing the delicate balance between FF and LU conformation and identifying the critical C-terminal tail (amino acids 172 to 187 according to the EcAhpC) and residues (S86, T88) are important as they provide insights not only into the catalytic cycle of Prxs, but also the divergence of the bacterial and human 2-Cys Prxs in robustness and sensitivity to inactivation, respectively. In the sensitive human and more generally eukaryotic Prxs, the two important segments identified here, namely the C-terminal tail and residues S86 and T88, of the less sensitive bacterial Prxs are structurally replaced by the conserved eukaryotic GGLG motif and the YF motif inside the extra C-terminal helix (YFSKHN -helix). Although both the eukaryotic as well as the bacterial enzyme reveal similar catalytic efficiency, the structurally distinct motifs/segments make the eukaryotic enzyme susceptible to over-oxidation\textsuperscript{21}. As revealed in Fig. 10A-VI, the eukaryotic C-terminal YFSKHN -helix is complemented by the GGLG motif, forming a stable structural segment across the active site to securely pack the peroxidatic cysteine, C\textsubscript{p} in the FF conformation, even after the oxidative modification of the C\textsubscript{p} to the sulfenic acid form\textsuperscript{24,25}. Based on the proposed model of bacterial AhpC, the C-terminal tail is resistant to unfolding from the active site due to the oxidation induced structural perturbation, thereby delaying the unfolding step needed to switch from the FF active site to the LU conformation. Moreover, the resolution of C\textsubscript{p}-SOH depends on the availability of the resolving cysteine, C\textsubscript{p}′, which is also mediated by the unfolding of the C-terminal tail. This perspective is reflected in the rate of disulphide formation during catalysis, which in case of the bacterial AhpC is significantly higher (75 s\textsuperscript{−1})\textsuperscript{32}, when compared to the low rate of 1.7 s\textsuperscript{−1} determined for human Prx2\textsuperscript{23}. Taken together, natural selection for the stable C-terminal tail over a stable FF active site reveals the essential role of the C-terminal tail to act as a molecular switch that mediates the structural transition between the FF and LU state during the catalytic cycle.

The oxidized EcAhpC\textsubscript{1-186}-YFSKHN structure in Fig. 3C,D reveals that the active site loop-helix-motif and the C-terminal tail region adopt structural alteration, bringing C\textsubscript{p} and C\textsubscript{p}′ to the disulphide bonded LU state. This structural feature is important, since the redox-linked active site conformations are proposed to regulate oligomerization. This regulative mechanism proposes that the disulphide bonded LU state destabilizes the decamer while the C\textsubscript{p} and C\textsubscript{p}′ reduced FF conformation favors decamer formation\textsuperscript{29}. With the studies presented here, we delineate the linkage between the active site conformation and the C-terminal tail residues. Our earlier studies demonstrated that the EcAhpC\textsubscript{1-172} -EcAhpC\textsubscript{1-182} and EcAhpC\textsubscript{I187G} mutants prevented decamer formation in solution\textsuperscript{11}, leading to the conclusion that the destabilized active site, caused by these mutants, might confer instability to the oligomer building interface (Fig. 7). Taken together, we propose a physical linkage can be established between the three major regions, namely the C\textsubscript{p} containing active site, the C-terminal tail and the oligomer interface. In the oxidized state, this physical linkage enables the folded C-terminal tail and stable oligomer interface to facilitate formation of the FF active site and vice versa. However, in the oxidized state, these three structural regions are destabilized\textsuperscript{19,30}.

In summary, a combination of complementary approaches of genetic engineering, protein chemistry, enzymatic assays, and structural biology has provided new insights into the unique aspect of divergence between the bacterial and human 2-Cys Prxs in robustness and sensitivity to inactivation, respectively. Structural details of the oxidized and reduced chimeric EcAhpC\textsubscript{1-186}-YFSKHN provide novel insights into sub-steps during the catalysis of peroxide reduction, enabling the transition from a fully folded to a locally unfolded conformation. Together with mutational and enzymatic studies these data unravel the fundamental role of the C-terminal tail as a molecular switch that mediates the structural transition between the FF and LU state during the catalytic cycle. Finally, a physical linkage between the CP-containing active site, the C-terminal tail and the oligomer interface was established.
Materials and Methods

Cloning and overexpression. The chimeric protein EcAhpC1-186-YFSKHN, composed of the N-terminal 186 amino acids of *E. coli* AhpC (187 residues) and the C-terminal residues 19-YFSKHN(19) of human Prx2, was designed based on a protein sequence alignment. EcAhpC1-186-YFSKHN was amplified by polymerase chain reaction (PCR) using forward primer 5′-CAT GCC ATG GCA ATG TCC TGT ATT AAC ACC AAA ATT AAA CCT-3′ with an *NcoI* restriction site (bold) and reverse primer 5′-GCC AGC TCT TAG TTG TGC TTA CTA AAG TAT TTA CCA ACC AGG TCC AG-3′ with a *SacI* restriction site (bold), respectively, and using the pET9-d1-His6 plasmid34, which contains the gene encoding EcAhpC20. The double mutant, S86 and T88 to alanine (EcAhpC{	extsubscript{S86A,T88A}}) was amplified using site-directed mutagenesis with HiFi KAPA DNA polymerase (KAPA Biosystems, USA) and the forward primer 5′-AGC AGC GCT GAA GCT ATC GCT AAA ATC AAA TAT GCG-3′ as well as the reverse primer 5′-GAT AGC GCC GCT GTG CCA TGC TTT TTT-3′. The coding sequences of EcAhpC{	extsubscript{1-186}}, YFSKHN and EcAhpC{	extsubscript{S86A,T88A}} were verified by DNA sequencing.

Wild type *E. coli* thioredoxin (Trx) and thioredoxin reductase (TrxR) was amplified by PCR using forward primers 5′-GCC ATG GCC AGT AGC GAT AAA ATT ATT CAC CTG-3′ and 5′-TAT GCC ATG GCG ACG AAA CAC AGT-3′ with an *NcoI* restriction site (bold) and reverse primers 5′-TAG AGC TCG TTA CGC CAG GTT AGC GTC GAG GAA-3′ and 5′-GCC AGC TCG TTA TTT TGC GTC AGC TAA AC-3′ with a *SacI* restriction site (bold), respectively. *E. coli* genomic DNA was used as a template.

Liquid cultures were shaken in Luria Broth medium (LB-medium) containing kanamycin (30 μg/ml) at 310 K until an optical density OD{	extsubscript{600}} of 0.6–0.7 was reached and isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration of 1 mM), was added for production of recombinant proteins. The incubation took 3 h at 310 K, except for EcAhpC{	extsubscript{S86A,T88A}}, which was grown overnight at 291 K after induction. Recombinant *E. coli* AhpC, the C-terminal truncated EcAhpC1–172, and mutant EcAhpC{	extsubscript{I187G}} were produced by the method described earlier21.

Purification of recombinant proteins. BL21 (DE3) *E. coli* cells containing recombinant EcAhpC, EcAhpC{	extsubscript{1-172}}, EcAhpC{	extsubscript{I187G}}, EcAhpC{	extsubscript{S86A,T88A}} and EcAhpC{	extsubscript{1-186}}, YFSKHN were purified according to Dip et al.21. Recombinant *E. coli* Trx (EcTrx) and -TrxR were purified with a slightly modified protocol, in which the purified Ni{	extsuperscript{2+}}-NTA fractions were directly loaded on the Superdex 75 HR 10/30- (GE Healthcare) and Superdex 200 column (GE Healthcare), respectively. The purity and homogeneity of the protein samples were analyzed by a 17% SDS-gel35. The gels were stained with Coomassie Brilliant Blue G250. The gels of the generated and purified EcAhpC{	extsubscript{1-186}}, YFSKHN, EcAhpC{	extsubscript{S86A,T88A}}, EcTrx, and EcTrxR are shown in Supplementary Fig. S5. Protein concentrations were determined using a BioSpec-nano Spectrophotometer (Shimadzu, USA).

Enzymatic characterization using a peroxidase assay. Peroxide-dependent activity of the various forms of purified recombinant *E. coli* AhpC proteins was measured by coupling its activity with NADPH-oxidation (ε{	extsubscript{280}} = 6220 M⁻¹ cm⁻¹) catalyzed by EcTrxR and EcTrx. The peroxidase activity was carried out at 25°C by monitoring the decrease in NADPH-absorbance at 340 nm for 120 sec using a stopped-flow spectrophotometer SX20 (Applied Photophysics, UK). The reaction mixture containing 100 μM NADPH, 50 mM HEPES buffer pH 7.0, 100 mM of ammonium sulfate, 0.5 mM EDTA, 0.25 μM of EcTrxR, 4 μM of EcTrx, and 4 μM of EcAhpC were mixed with varying concentrations of hydrogen peroxide (250 nM–100 μM) to initiate NADPH-oxidation. The rate of NADPH-oxidation was calculated by a least square fit to the linear portion of the curve. NADPH consumption measured in the absence of peroxiredoxin was taken as a control. The background rate was subtracted from the experimental rate to determine the activity due to EcAhpC. All rates reported here are the average of three independent experiments.

Inactivation assay. Peroxide-dependent overoxidation of EcAhpC, -AhpC1–172, -AhpC{	extsubscript{I187G}} and the chimeric EcAhpC{	extsubscript{1-186}}, YFSKHN were measured similar to the above mentioned condition, using hydrogen peroxide concentrations of 1–30 mM. NADPH-oxidation was monitored at 340 nm at 25°C for 210 sec. Background NADPH-oxidation observed for EcTrx and EcTrxR in the absence of Prx, which is significant at higher H₂O₂ concentrations, was subtracted to estimate the activity due to Prx. The rate of reaction for each hydrogen peroxide concentration was calculated by fitting the linear portion of the curve. The rate observed for the lowest concentration of hydrogen peroxide is taken as 100% activity, to calculate the remaining enzyme activity in percentage at each concentration of hydrogen peroxide.

Peroxide reduction assay using SDS-PAGE. This assay is based on the observations that the reduced and oxidized 2-Cys Prxs run as a monomer and dimer, respectively, in a non-reducing SDS-PAGE33. Prior to each experiment, WT EcAhpC, EcAhpC{	extsubscript{1-172}} and EcAhpC{	extsubscript{I187G}} were reduced with 20 mM dithiothreitol (DTT) in 50 mM phosphate buffer, pH 7.4 containing 1 mM diethylenetriaminepentaacetic acid for 1 h. Reduced proteins were separated from excess of DTT using a PD-10 desalting column (GE Healthcare). The different forms of EcAhpC (30 μM) were incubated with varying concentrations of H₂O₂ for 5 min. The reaction was stopped by adding 50 mM N-ethyl maleimide in a sample buffer (4% SDS, 10% glycerol and 62.5 mM Tris–HCl, pH 6.8) and analyzed by a non-reducing 17% SDS-gel.

Crystallization of EcAhpC{	extsubscript{1-186}}, YFSKHN. Chimeric EcAhpC{	extsubscript{1-186}}, YFSKHN was concentrated to 8 mg/ml in buffer containing 50 mM Tris–HCl pH 7.5, 200 mM NaCl, using a Millipore spin concentrator with a molecular-mass cutoff of 10 kDa. An initial crystallization attempt was carried out using the recent protocol of EcAhpC (1.8 M ammonium sulfate, 100 mM MES (2- (N-morpholino) ethanesulfonic acid), pH 6.5 and 5% Dioxane)20, and the hanging-drop vapour diffusion method in 24-well VDX plates with sealant at 291 K. Diffraction quality crystals were obtained in the optimized condition of 1.6 M ammonium sulfate, 100 mM MES pH 6.5, 5% Dioxane and a protein concentration of 4.5 mg/ml, yielding rod shaped crystals of 0.3 mm × 0.2
mm × 0.1 mm. Reduced EcAhpC1-186-YFSKHN crystals were grown by soaking the oxidized crystals with 1 mM Tris(2-carboxyethyl) phosphine (TCEP) for 1–3 min. There was no cracking or disintegration of crystals observed during the soaking.

Data collection and structure determination. Crystals of EcAhpC1-186-YFSKHN were quick-soaked in a cryoprotectant solution containing 25% glycerol in the mother liquid and flash-cooled in liquid nitrogen at 100 K. A single wavelength dataset for both the oxidized and reduced EcAhpC1-186-YFSKHN were collected at 140 K, beamline 13B1 of the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan) using a ADSC Quantum 315 CCD detector. The diffraction data were indexed, integrated and scaled using Mosflm 36 and HKL2000 suite37. Data collection and processing statistics for oxidized and reduced EcAhpC1-186-YFSKHN are summarized in Table 2. Oxidized EcAhpC1-186-YFSKHN crystals belong to the monoclinic space group P2₁ with the unit cell parameters

| Ox. EcAhpC1-186-YFSKHN | Red. EcAhpC1-186-YFSKHN |
|-------------------------|-------------------------|
| Wavelength (Å)          | 1.000                   | 1.000                   |
| Crystal-to-detector distance (mm) | 360                     | 420                     |
| Rotation range per image (°) | 1                      | 1                       |
| Total rotation range (°) | 140                     | 140                     |
| Exposure time per image (s) | 5                      | 5                       |
| Space group             | P2₁                     | P2₁                     |
| Unit cell parameters (Å,°) |                |                          |
| a=                      | 99.47                   | 100.49                  |
| b=                      | 134.73                  | 135.65                  |
| c=                      | 107.53                  | 106.49                  |
| α=                      | 90                      | 90                      |
| β=                      | 111.06                  | 111.22                  |
| γ=                      | 90                      | 90                      |
| Molecules in asymmetric unit | 10                    | 10                      |
| Solvent content (%)      | 60.72                   | 60.96                   |
| Resolution limits (Å)    | 50.0–2.70 (2.85–2.70)  | 50.0–3.10 (3.21–3.10)   |
| No. of reflections       | 202994                  | 138462                  |
| Unique reflections       | 69616                   | 44808                   |
| Multiplicity             | 2.9 (2.9)               | 3.1 (2.9)               |
| Completeness (%)         | 96.1 (91.3)             | 92.9 (92.0)             |
| Rmerge (%)               | 12.8 (58.4)             | 11.7 (58.6)             |
| <I/σ(I)>                | 7.2 (1.8)               | 8.9 (1.5)               |
| CC½                      | 98.4 (66.7)             | 91.5 (66.3)             |

| Refinement statistics    |                          |                          |
| R-factor (%)             | 24.63                   | 25.03                   |
| R-free (%)               | 28.29                   | 28.36                   |
| Number of waters         | 320                     | 19                      |
| Number of sulphates      | 23                      | 14                      |
| Number of glycerol       | 22                      | –                       |
| MolProbity statistics    |                          |                          |
| Ramachandran favoured (%)| 97.3                    | 98.2                    |
| Ramachandran outliers (%)| 0                      | 0                       |
| Clashscore               | 0.61                    | 0.39                    |
| R.M.S. deviations        |                          |                          |
| Bond lengths (Å)         | 0.006                   | 0.007                   |
| Bond angles (˚)          | 0.994                   | 0.946                   |
| Overall B values         |                          |                          |
| From Wilson plot (Å²)    | 48.20                   | 89.60                   |
| Mean B value (Å²)        | 30.31                   | 81.83                   |

Table 2. Data collection, processing and refinement statistics of EcAhpC1-186-YFSKHN. Values in parentheses refer to the corresponding values of the highest resolution shell. Rmerge = ΣΣΣΣ|Ih - Ih|/ΣΣΣΣIh, where Ih is the mean intensity for reflection h. ‘R-factor = ΣΣΣΣ|Fo| - |Fc||ΣΣΣΣ|Fo|, where Fo and Fc are measured and calculated structure factors, respectively. R-free = ΣΣΣΣ|Fo| - |Fc||ΣΣΣΣ|Fo|, calculated from 5% of the reflections selected randomly and omitted during refinement.
using the crystallographic structure of oxidized EcAhpC (PDB ID: 4O5R)\textsuperscript{10} as model for molecular replacement by the program PHASER\textsuperscript{38}. The reduced form of EcAhpC1-186-YFSKHN was solved using the reduced structure of Salmonella typhimurium AhpC (PDB ID: 1N8F)\textsuperscript{39}. Refinement\textsuperscript{38} was done until convergence and the geometry of the final model was validated with MolProbity\textsuperscript{40}. The figures were generated using PyMOL\textsuperscript{34} and structural comparison analysis was carried out by SUPERPOSE\textsuperscript{42} as included in CCP4 suite\textsuperscript{44}.

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Author Contributions
N.K., M.F.S., F.E., B.E. and G.G. designed the experiments. N.K. and M.F.S. performed the experiments. N.K., and M.F.S. and G.G. analyzed the data. N.K., M.F.S., F.E., B.E. and G.G. wrote the paper.

Additional Information
Accession codes: The atomic coordinates of the models and their corresponding structure factors of oxidized and reduced EcAhpC1-186-YFSKHN have been deposited in the Protein Data Bank (www.pdb.org) with the entry codes 5B8A and 5B8B, respectively.

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