Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4

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Prx4 (peroxiredoxin 4) is the only peroxiredoxin located in the ER (endoplasmic reticulum) and a proposed scavenger for H2O2. In the present study, we solved crystal structures of human Prx4 in three different redox forms and characterized the reaction features of Prx4 with H2O2. Prx4 exhibits a toroid-shaped decamer constructed of five catalytic dimers. Structural analysis revealed conformational changes around helix α2 and the C-terminal region with a YF (Tyr-Phe) motif from the partner subunit, which are required for interchain disulfide formation between Cys87 and Cys208, a critical step of the catalysis. The structural explanation for the restricting role of the YF motif on the active site dynamics is provided in detail. Prx4 has a high reactivity with H2O2, but is susceptible to overoxidation and consequent inactivation by H2O2. Either deletion of the YF motif or dissociation into dimers decreased the susceptibility of Prx4 to overoxidation by increasing the flexibility of Cys87.

Key words: crystal structure, overoxidation, peroxidase, peroxiredoxin 4 (Prx4).

EXPERIMENTAL

Mutagenesis, expression and purification

The coding sequences of mature human Prx4, Escherichia coli Trx and Trx reductase were cloned into pQE-30 (Qiagen), and all resulting proteins contain N-terminal (MRGSH6GS-) tags. Mutants of Prx4 were obtained by overlap-extension PCR, and all constructs were verified by DNA sequencing.

Proteins were expressed in M15[pREP4] (Qiagen) and purified with a nickel-chelating column (GE Healthcare). The protein eluates were dialysed against 20 mM Tris/HCl (pH 7.6) containing 0.1 M NaCl and 2 mM EDTA. Prx4 and its mutants were further purified on a Superdex 200 HR 10/30 column (GE Healthcare) for crystallization.

INTRODUCTION

In eukaryotes, disulfide bond formation driven by the Ero1/PDI (protein disulfide-isomerase) system in the ER (endoplasmic reticulum) generates H2O2, a highly reactive oxygen species [1,2]. It has been suggested that 105 disulfides are formed per second in a plasma cell, producing equimolar amounts of H2O2, which is potentially quite harmful to the cell [3]. Mechanisms, although poorly characterized to date, must exist to eliminate excess H2O2 in the ER. In mammalian cells, various peroxidases are distributed in the cytosol, nucleus and mitochondria, but only three, Prx (peroxiredoxin) 4 [4] and two GPx (glutathione peroxidase) homologues, GPx7 and GPx8 [5], have been reported to be located in the ER to date.

Prx4 belongs to a ubiquitous group of peroxiredoxins termed Prxs, containing a conserved peroxidatic cysteine which reacts with peroxides forming a Cys-SOH (cysteine sulfenic acid) [6]. In typical 2-Cys Prxs [7], the most abundant subclass including Prx4, the Cys-SOH can be resolved by forming an interchain disulfide bond with a second cysteine residue from the partner subunit within a dimer. This disulfide is usually recycled by Trx (thioredoxin). On the other hand, the -SOH can be overoxidized to -SO2H (sulfinic acid) or further to sulfonic acid, resulting in enzymic inactivation. The susceptibility to inactivation depends mostly on two motifs, GGLG (Gly-Gly-Leu-Gly) and YF (Tyr-Phe) which are conserved in eukaryotic, but lacking in prokaryotic, 2-Cys Prxs [8]. Prxs in the -SO2H form can be reduced and reactivated by sulfiredoxin in an ATP-dependent reaction [9]. In addition, oxidized Prxs have been reported to act as cell-cycle regulators [10] and molecular chaperones [11].

Prx4 was reported to protect cells from oxidative stress by metabolizing Ero1-generated H2O2 [12]. It is also capable of coupling H2O2 generation to productive disulfide bond formation even in the absence of Ero1 [13,14]. Prx4-knockout mice displayed elevated spermatogenic cell death via oxidative stress and testicular atrophy [15]. Increased Ero1 activity in cells was also reported to lead to overoxidation of Prx4 [12]. Since enzymes to reduce overoxidized Prxs, such as sulfiredoxin, have not yet been found in the ER, the overoxidation could result in irreversible inactivation of Prx4.

Although the crystal structure of an N-terminal truncated human Prx4 (residues 47–234) in the reduced state has been deposited in the PDB (PDB code 2PN8), the relevant functional analysis is still lacking. In the present study, we have solved structures of the full-length human Prx4 decamer in three different redox forms and provide detailed structural insights into the reaction features of Prx4 with H2O2, in combination with biochemical studies. We show that Prx4 is able to function as an efficient peroxidase through conformational changes required for the interchain disulfide bond formation between the peroxidatic Cys87 and the resolving Cys208. Prx4 is also susceptible to inactivation caused by Cys87 overoxidation, and the inactivation is modulated via either limiting the conformational changes of the region around Cys87 by the YF motif or decameterization.

Abbreviations used: DTT, dithiothreitol; ER, endoplasmic reticulum; GPx, glutathione peroxidase; HRP, horseradish peroxidase; PDI, protein disulfide-isomerase; Prx, peroxiredoxin; Trx, thioredoxin.

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The co-ordinates and structural factors for Prx4 in three different redox forms and Prx4-T118E were deposited in the PDB with codes 3TKP, 3TKQ, 3TKS and 3TRK respectively.
Protein concentrations were determined spectrophotometrically at 280 nm with the absorption coefficients 36900 M$^{-1}$·cm$^{-1}$ for Prx4 and its point mutants, 28420 M$^{-1}$·cm$^{-1}$ for Prx4-ΔN, 35410 M$^{-1}$·cm$^{-1}$ for Prx4-ΔC, 13980 M$^{-1}$·cm$^{-1}$ for Trx, 17420 M$^{-1}$·cm$^{-1}$ for Trx reductase, and 1.02 × 10$^5$ M$^{-1}$·cm$^{-1}$ for HRP (horseradish peroxidase) at 403 nm.

**Crystallization and structural determination**

Proteins at 10 mg/ml were crystallized at 290 K by using hanging-drop vapour diffusion in 0.8 M NaH$_2$PO$_4$·K$_2$HPO$_4$ (pH 8.2) with or without 5 mM DTT (dithiothreitol). Crystals were soaked in a 1:1 mixture (v/v) of 50 % glycerol and reservoir solution for 1 h and then flash-frozen in liquid nitrogen. The soaking buffer containing 1 mM H$_2$O$_2$ was used for H$_2$O$_2$ treatment of the crystals obtained in the presence of DTT. Diffraction data were collected at 100 K on BL17A of Photon Factory, BL17U of Shanghai Synchrotron Radiation Facility or in-house, and processed using the HKL2000 software suite [16]. The structures were determined by molecular replacement using PHASER [17] with the truncated Prx4 (PDB code 2PN8) as the search model, and refined using Refmac [18] and Phenix [19]. Structural determination statistics are shown in Supplementary Table S1 at http://www.BiochemJ.org/bj/441/bj4410113add.htm. All structural Figures were generated with UCSF Chimera package [20] or PyMOL (http://www.pymol.org/).

**Electron densities averaging**

To solve the structure of Prx4 crystals obtained in the absence of DTT, we initially used the model for the reduced Prx4; however, it did not fit residues 82–90 in chains A, B, C and E for the continuous extra electron density around the region. To reduce the model bias, we recalculated the simulated annealing 2Fo-Fc omit map by omitting residues 81–91 from all five chains; unfortunately, the regions of residues 82–90 in the four chains were still not traced because of the weak electron density. However, we found that the extra electron densities among these four chains are quite similar, which allowed us to apply an averaging technique by AVE (Uppsala package) [21] to enhance the signal-to-noise ratio. The final electron density obtained with much improved appearance allowed us to unambiguously determine mixed conformations.

**Limited proteolysis assay**

Prx4 (50 μM), after incubation with 100 μM H$_2$O$_2$ for 5 min, was alkylated with 10 mM N-ethylmaleimide for 15 min to maintain thiol–disulfide status, and then digested with 0.1 mg/ml trypsin in 50 mM Tris/HCl buffer (pH 7.6) containing 0.1 M NaCl at 25°C for 2 h. The digestion was terminated by adding PMSF to a final concentration of 0.5 mM, and analysed by SDS/PAGE (12 % gels) and N-terminal sequencing.

**Activity assay**

The second-order rate constant for the reaction of Prx4 with H$_2$O$_2$ was determined by a competitive approach using HRP [22]. The peroxidase activity was measured by monitoring NADPH oxidation [23].

**Western blotting**

Prx4 or mutants at 2.5 μM was incubated in 50 mM Hepes (pH 7.0) with 1 mM EDTA and different concentrations of H$_2$O$_2$ for 5 min at 25°C, followed by addition of reducing SDS/PAGE loading buffer to remove excess H$_2$O$_2$. Aliquots of 5 μl from each reaction were subjected to Western blotting with a Prx-SO$_2$/3H-specific antibody (generously provided by Hyun Ae Woo and Sue Goo Rhee, Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea) [24] and rabbit anti-Prx4 serum (Animal Facility, Institute of Genetics and Developmental Biology, Chinese Academy of Science, Beijing, China).

**RESULTS**

**Overall structure of reduced Prx4**

In the presence of DTT, Prx4 was crystallized into space group C2 with five chains in an asymmetric unit. The structure was solved and refined to 2.5 Å (1 Å = 0.1 nm) with 84 % residues traced clearly into electron-density maps (see Supplementary Table S1). The overall structure resembles a toroid-shaped decamer from two asymmetric units (Figure 1A). The toroid has a maximal diameter of ∼120 Å and an inside diameter of ∼60 Å. The ten subunits in the decamer were nominated as chains A–J separately, where chains F–J are symmetrical to chains A–E respectively by the crystallographic 2-fold axis. Then chains A/I, B/H, C/G, D/F and E/J form five dimers accordingly, each of which acts as the basic catalytic unit.

Each subunit exhibits a typical extended Trx fold ($\beta_6\alpha_6\beta_6\alpha_6\beta_6\beta_6\alpha_6$) (Figure 1B). Two subunits are related by a molecular dyad within a dimer, and the C-terminal arm with the conserved YF motif (Tyr$^{258}$ and Phe$^{260}$) in one subunit folds over the other (Figures 1B and 1C). The $\beta$-sheets of each subunit combine to form a ten-stranded $\beta$-sheet, linked between two $\beta$-strands (Figure 1B). Two $\alpha$-helices from adjacent dimers serve as a scaffold to build up the decamer, with a distance of only 4.4 Å between the hydroxyl groups of Thr$^{118}$ (Figure 1B and see Supplementary Figure S1 at http://www.BiochemJ.org/bj/441/bj4410113add.htm). Replacement of Thr$^{118}$ with a glutamic acid residue caused dissociation of the decamer to dimers in solution (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/441/bj4410113add.htm).

The peroxidatic Cys$^{65}$ is located in the first turn of helix $\alpha_2$ and surrounded by three conserved residues: Pro$^{60}$, Thr$^{64}$ and Arg$^{103}$. The resolving Cys$^{266}$, located on a flexible loop between $\alpha_5$ and $\alpha_6$, is ∼13.0 Å away from Cys$^{65}$ of the partner chain (Figures 1B and 1C). As the distance is obviously unfavourable for disulfide formation, conformational changes are required for the resolving process.

This overall structure is similar to that of the N-terminal truncated Prx4 in the reduced state (PDB code 2PN8), and the two can be superimposed well with a RMSD (root mean square deviation) of 0.273 over 188 residues within one subunit.

**Unique flexible N-terminal region of Prx4**

Different from other typical 2-Cys Prxs, Prx4 has a unique N-terminal extension of approximately 40 residues, which was lacking in 2PN8. However, in the full-length Prx4 structure, the N-terminal 1–37 residues were invisible (which was not due to degradation; results not shown) and residues 38–46 adopt an unwound loop. Thus this region was proposed to be flexible and exposed to the solvent. Proteolysis studies (Figure 2A) showed that, after digestion with trypsin, the molecular mass of Prx4 shifted from 28 kDa (lane 1) to 25 kDa (lane 2) under reducing conditions, and N-terminal sequencing of the 25 kDa fragment revealed a cleavage site at the C-terminus of Arg$^{65}$, confirming the flexibility of this N-terminal region. On non-reducing SDS/PAGE,
Figure 1 Crystal structure of Prx4 obtained in the presence of DTT

(A) Overview of the Prx4 decamer from two asymmetric units shown in ribbon and backbone bond representations respectively. The five chains within one asymmetric unit are coloured differently, and the corresponding crystallography-symmetric chains are in the same colour. Chains are indicated with capital letters. (B) Ribbon diagram of one dimer with two subunits in magenta and green respectively. Cys87 and Cys208 are shown as spheres. (C) Close-up view of the active site and the surrounding environment with 2Fo − Fc electron-density map contoured at 1.5σ.

Figure 2 Disulfide bonds between subunits in Prx4

(A) SDS/PAGE (12% gel) of Prx4 treated with or without H2O2 after trypsin digestion. Molecular masses are indicated in kDa. (B) Schematic diagram of the disulfide bonds via Cys14 (star) or via Cys87 and Cys208 (triangle), the trypsin cleavage site (arrow) and the dimeric catalytic unit (broken rectangle).

Prx4 existed predominantly as disulfide-bonded dimers with small amount of monomers and high-molecular-mass species (Figure 2A, lane 5), which became truncated dimers and monomers after trypsin digestion (Figure 2A, lane 6). Treated with 100 μM H2O2, Prx4 molecules were mostly oxidized into high-molecular-mass species (Figure 2A, lane 7), and further trypsin digestion turned them into N-terminus-removed dimers linked via interchain disulfides between Cys87 and Cys208 (Figure 2A, lanes 4 and 8, represented as a broken rectangle in Figure 2B). This suggested that the monomer observed in the digestion product of intact Prx4 (Figure 2A, lane 6) was most likely to be generated from disulfide-bonded dimers via Cys14 (lane 5, represented as a broken ellipse in Figure 2B). Thus we characterized the interchain disulfide via Cys14 formed between two catalytic dimers. Mutation of Cys14 to serine did not change the decameric form of Prx4 in either the reduced or oxidized form, even at a concentration as low as 1 μM (Figure 3), suggesting that this covalent link is not necessary for the decamerization of Prx4. The mutant Prx4-ΔN, lacking the N-terminal 46 residues, was predominantly a decamer in a reduced state, but partially dissociated into dimers especially at lower concentrations after reaction with H2O2, suggesting that the flexible N-terminal region may play a role in stabilization of the Prx4 decamer in the oxidized state.

Figure 3 Oligomerization of Prx4, Prx4-C14S and Prx4-ΔN in reduced and oxidized forms determined by gel filtration

Reduced and oxidized samples were prepared by overnight incubation of 20 μM protein at 25°C with 5 mM DTT (A) and 40 μM H2O2 (B) respectively. Samples at the indicated concentrations were applied to a Superdex 200 HR 10/30 column and chromatographed at 0.5 ml/min using 50 mM Tris/HCl buffer (pH 7.6) containing 0.1 M NaCl with (A) or without (B) 5 mM DTT at 25°C. Molecular masses are indicated in kDa.

Locally unfolded conformation of oxidized Prx4

In the absence of DTT, Prx4 was crystallized in a different unit cell (Supplementary Table S1), and exhibits almost the same overall structure as the reduced one. However, the averaged 2Fo − Fc omit map of four chains in one asymmetric unit clearly showed that the region around Cys87 exists in mixed conformations (Figure 4). One is fully folded, quite similar to the reduced structure. The other is locally unfolded, in which, the first turn of helix α2 is no longer in a helical conformation and the C-terminal region in the partner subunit becomes invisible probably due to high flexibility. The unfolded helix α2 resembles...
the corresponding loop in the oxidized form of *Salmonella enterica* serotype Typhimurium AhpC (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/441/bj4410113add.htm), suggesting that this conformation may represent the oxidized form (Cys87–Cys208 bonded) of Prx4, although the disulfide was not traceable because of the poor electron density. Notably, Cys87 shifted ∼10 Å to the position that was occupied by the aromatic rings of the partner YF motif in the reduced structure, suggesting that the YF motif, which is absent from AhpC, has to move to allow the interchain disulfide formation between Cys87 and Cys208.

Oxidation of Cys87 by H2O2

Treatment with 1 mM H2O2 of reduced Prx4 crystal yielded a 2.4 Å structure (see Supplementary Table S1), the overall conformation of which looked almost no different from that of the untreated Prx4. However, the $F_o - F_c$ map using a model with all Cys87 residues in the reduced form clearly showed extra electron density near the $S\gamma$ atom of Cys87 in four out of five chains (Figure 5). The distance from the centre of the density to $S\gamma$, ∼1.8 Å, is shorter than that for a hydrogen bond with water or a H$_2$O$_2$ molecule. In the final model, Cys87 is assigned in the -SOH form in chains A and B, in the -SO$_2$H form in chains C and D, and remains reduced in chain E. All side-chain atoms of Cys87 in chains A, B, C, and D fit the $2F_o - F_c$ map very well with almost no extra electron density nearby and with reasonable B factors, except for a slight positive electron density around $S\gamma$ in Cys87 of chain E possibly from partial oxidation. Even if the possibility that all active sites existed in a mixture of oxidation states at some level cannot be excluded, our final model represents the major form well.

Further comparison reveals that the B factors of helix α2 with Cys87 in the -SO$_2$H form and its partner C-terminal region (chains C/G and D/F) are much lower than those in the -SOH and the reduced forms (Figure 5 and see Supplementary Figure S4A at http://www.BiochemJ.org/bj/441/bj4410113add.htm), showing much lower flexibility. Since similar differences in B factors were also observed before H$_2$O$_2$ treatment (Supplementary Figure S4B), such flexibility differences are not attributed to oxidation, but are most likely to be due to different solvent exposure in crystal packing. It should not be a coincidence that conformational changes happen during the catalytic cycle right at these regions with different flexibility. Clearly, the low flexibility of helix α2 in chains C and D as well as their partner C-terminal region (especially the YF motif) would inevitably restrict surrounding conformational changes and thus gave Cys87 more chance to be overoxidized. In this structure, the crystal packing endowed subunits in the decamer with diverse flexibility around these regions leading to distinct oxidation states of Cys87, and thus enabled us to address the necessity of conformational changes during the catalysis.

**Biochemical study of the reaction of Prx4 with H$_2$O$_2$**

The second-order rate constant for the reaction of Prx4 with H$_2$O$_2$ was determined to be $2.2 \times 10^7$ M$^{-1}$s$^{-1}$ (Figure 6A), indicating that Prx4 can remove H$_2$O$_2$ as efficiently as haem or selenium peroxidases and other Prxs do.

The peroxidase activity was also monitored in a reaction coupled to NADPH oxidation (Figure 6B). NADPH was oxidized with an initial reaction rate of ∼1 μM s$^{-1}$ in the presence of 2.5 μM Prx4 and 50 μM H$_2$O$_2$, the minimal amount for quantitatively monitoring the reaction in practice. When the H$_2$O$_2$ concentration increased, the oxidation rate of NADPH decreased, indicating loss of peroxidase activity of Prx4.

Consistent with the potential role of the YF motif discussed above, the mutant Prx4-ΔC with eight residues (227–234, including the YF motif) in the C-terminus deleted, although it retained the decamer structure (see Supplementary Figure S2) and almost full peroxidase activity at 50 μM H$_2$O$_2$, maintained the enzymatic activity at H$_2$O$_2$ concentrations up to 500 μM (Figure 6B), indicating decreased susceptibility to inactivation by overoxidation.

The dimeric mutant Prx4-T118E, although bearing the YF motif, also became more resistant to peroxide inactivation (Figure 6C). The crystal structure of Prx4-T118E was almost the same as that of the wild-type protein with a slight shift of α3 (see Supplementary Figure S1), suggesting that the resistance to inactivation of the dimeric mutant was unlikely to be caused by structural perturbation other than dissociation.

An antibody specifically recognizing the overoxidized peroxidatic cysteine residue [24] was used to confirm the above results (Figure 6C). Overoxidation of wild-type Prx4 intensified with increasing H$_2$O$_2$ concentrations, whereas the overoxidation of Prx4-ΔC and Prx4-T118E remained negligible, with a faint increase only at 500 μM H$_2$O$_2$.

**DISCUSSION**

H$_2$O$_2$ is generated as a by-product during disulfide bond formation in the ER driven by the Ero1/PDI system. How cells consume or use this reactive oxygen species is an open question. The present study focused on one of the three known ER-located peroxidases: Prx4. The reactivity of Prx4 with H$_2$O$_2$ (2.2 \times 10^7$ M$^{-1}$s$^{-1}$) is much higher than that of GPx8 [25], PDI [26] and other thiol proteins in the ER that we are aware of. Therefore H$_2$O$_2$ would preferentially react with Prx4, making Prx4 a good sensor and an efficient scavenger for low concentrations of H$_2$O$_2$ once it is present in the ER.

Prx4 expression was reported to be increased sharply during the last days of B-cell differentiation to accommodate increased generation of H$_2$O$_2$ paralleling IgM synthesis [3].

Structural analysis of Prx4 in different redox states in the present study has shed light on the reaction of Prx4 with H$_2$O$_2$. We detected for the first time the reactive -SH form, the intermediate -SOH form and the overoxidized -SO$_2$H form of the peroxidatic Cys87 within one Prx4 decamer after the reaction with H$_2$O$_2$, providing solid structural evidence for peroxidation and overoxidation of Prx4 by H$_2$O$_2$. Cys87 reacts with H$_2$O$_2$, forming Cys-SOH, followed by unravelling of the first turn of helix α2 to free Cys87, allowing it to form a disulfide with the resolving Cys208 from the partner subunit, which was initially ∼13 Å away.
motif in ball-and-stick representation. The Fo the indicated concentrations for 5 min. Aliquots of 5 Western blotting with a Prx-SO2/3H-specific antibody and rabbit anti-Prx4 serum.

The region around Cys87 and the YF motif in the C-terminal region from the partner chain are shown in a ribbon representation coloured according to the B factor distribution, with Cys87 and the YF motif in ball-and-stick representation. The Fo − Fc maps (green) surrounding the oxygen atoms (red) covalently bound to 5′F of Cys87 are contoured at 3.5σ.

Interestingly, dimeric Prx4-T118E, bearing the YF motif and a similar conformation to the wild-type protein in the reduced state, is resistant to overoxidation. In this respect, dimeric AhpC with a corresponding mutation showed increased resistance to inactivation only by high concentrations of H2O2 [28]. Therefore decamerization may be another structural feature for overoxidation of Prx4, besides the YF motif. The dimeric structures of typical 2-Cys Prxs resolved to date are found all in locally unfolded conformation with the C-terminal region unobserved in X-ray crystallography [27]. This implies that the region around the peroxidatic cysteine residue and the partner C-terminus in the dimeric state are more flexible than in the decameric state, thus endowing the peroxidatic cysteine residue more freedom to form interchain disulfide bonds with the resolving cysteine residue, keeping away from overoxidation.

Generally, the oligomeric states of typical 2-Cys Prxs, such as AhpC [29], are redox-sensitive, and the disulfide formation at the active site weakens the decamer [7, 27]. In contrast, oxidized Prx4 exists in a rather stable decameric form, probably stabilized by its unique flexible N-terminal region.

The YF motif above the active site in the reduced state was proposed to limit the active-site dynamics [7, 8, 27]. In the present study, we clearly observed that the peroxidatic Cys87 in oxidized Prx4 moved and occupied the position of the partner YF motif in the reduced state, and this positional clash had not been reported previously to our knowledge, even in the structural superposition of the reduced and oxidized rat Prx1, which has been used as an example to explain the susceptibility of 2-Cys Prxs to overoxidation [7, 8, 27]. The characterized positional clash of Cys87 and its partner YF motif provided a straightforward explanation for the restricting role of the YF motif on the active site in Prx4. The flexibility around Cys87 and its partner C-terminal region is critical for conformational changes during the reaction of Prx4 with H2O2. By using Prx4 structural analysis, Western blotting and peroxidase assay, we also demonstrated that, in the presence of high concentrations of H2O2, Cys87 can be overoxidized, leading to inactivation. Deletion of the eight C-terminal residues (including the YF motif) increased resistance to overoxidation.

Figure 5 Different redox states of Cys87 in Prx4 treated with H2O2

The region around Cys87 and the YF motif provided a straightforward explanation for the restricting role of the YF motif on the active site in Prx4. The flexibility around Cys87 and its partner C-terminal region is critical for conformational changes during the reaction of Prx4 with H2O2. By using Prx4 structural analysis, Western blotting and peroxidase assay, we also demonstrated that, in the presence of high concentrations of H2O2, Cys87 can be overoxidized, leading to inactivation. Deletion of the eight C-terminal residues (including the YF motif) increased resistance to overoxidation.

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Figure 6 Reaction of Prx4 with H2O2

(A) Formation of HRP compound I was recorded under competition with Prx4 (left), and the second-order rate constant was determined by plotting $\{F(1 - F)\} k_{HRP}$ against Prx4 concentrations (right). Data are expressed as means ± S.D. (n = 3). (B) NADPH oxidation was carried out at 25 °C in 50 mM Hepes (pH 7.0) with 1 mM EDTA, 2 μM Trx reducease, 5 μM Trx, 2.5 μM Prx4 proteins and H2O2 at different concentrations as indicated, and monitored by absorbance changes at 340 nm. (C) Prx4 proteins at 2.5 μM were incubated with H2O2 at the indicated concentrations for 5 min. Aliquots of 5 μM from each reaction were subjected to Western blotting with a Prx-SO2/3H-specific antibody and rabbit anti-Prx4 serum.

AUTHOR CONTRIBUTION

Xi Wang and Chih-chen Wang conceived the project. Xi Wang, Likun Wang, Xi’e Wang performed the experiments. Likun Wang and Fei Sun solved the structures. Xi Wang, Likun Wang, Fei Sun and Chih-chen Wang analysed data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4

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Figure S1  Structural comparison of Prx4 and Prx4-T118E in the reduced state
Prx4-T118E was crystallized as a decamer presumably due to the high protein concentration and the packing preference. Chain A of Prx4 (cyan) and Prx4-T118E (orange) were taken as representative, while well superimposed with the root mean square deviation of 0.301 over 196 residues. (A) Close-up view of the dimer–dimer interface. Distances (Å) between the corresponding carbon atoms in the main chain of the two structures are indicated. Only a slight shift (≈0.4 Å) of α3 in Prx4-T118E was identified. (B) Close-up view of the active site.

Figure S2  Gel-filtration profiles of Prx4, Prx4-T118E and Prx4-D1
Chromatography on a Superdex 200 HR 10/30 column was performed at 0.5 ml/min using 50 mM Tris/HCl buffer (pH 7.6) containing 0.1 M NaCl and 2 mM DTT at 25 °C. Molecular-mass markers were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa). The apparent molecular masses of Prx4, Prx4-T118E and Prx4-D1 were determined to be 246, 55 and 288 kDa respectively.

Figure S3  Superposition of the unfolded helix α2 of Prx4 (cyan) with that of oxidized AhpC (magenta, PDB code 1YEP)

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The co-ordinates and structural factors for Prx4 in three different redox forms and Prx4-T118E were deposited in the PDB with codes 3TKP, 3TKQ, 3TKS and 3TKR respectively.
Figure S4  Crystal packing of Prx4 with (A) or without (B) H$_2$O$_2$ treatment

The structures are shown in the cartoon coloured with respect to $B$ factors. Chains are indicated by capital letters and arrows, and Cys$^{87}$ and YF motifs are shown as spheres.
Catalysis and inactivation of human Prx4

### Table S1 Statistics for data collection, processing and structural refinement

Corresponding parameters for the highest-resolution shell are shown in parentheses. $R_{merge} = \Sigma_h |I_h| - <I_h>^2/\Sigma_h <I_h>^2$, where $<I_h>$ is the mean intensity of the reflection h. $R_{merge} = \Sigma_h |F_h| - |F_c|/\Sigma_h |F_h|$. $R_{merge}$ = R factor for a selected subset (5%) of the reflections that was not included in prior refinement calculations. PF, Photon Factory; RMSD, root mean square deviation; SSFR, Shanghai Synchrotron Radiation Facility.

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<tr>
<td>Space group</td>
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<tr>
<td>Cell dimensions</td>
<td>a, b, c (Å)</td>
<td>a, b, c (Å)</td>
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<tr>
<td>α, β, γ (°)</td>
<td>90.0, 103.1, 90.0</td>
<td>90.0, 102.5, 90.0</td>
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<tr>
<td>Resolution (Å)</td>
<td>50.00–2.50 (2.59–2.50)</td>
<td>50.00–2.40 (2.49–2.40)</td>
</tr>
<tr>
<td>$R_{merge}$</td>
<td>0.054 (0.300)</td>
<td>0.054 (0.300)</td>
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<tr>
<td>&lt;I&gt;/&lt;sI&gt;</td>
<td>17.0 (2.5)</td>
<td>11.6 (1.7)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.9 (99.8)</td>
<td>97.2 (94.0)</td>
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<tr>
<td>Redundancy (%)</td>
<td>2.5 (2.4)</td>
<td>3.1 (2.5)</td>
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<td>Refinement</td>
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<td>Resolution (Å)</td>
<td>45.12–2.49</td>
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<tr>
<td>$R_{work}/R_{free}$ (%)</td>
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<td>18.3/23.2</td>
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<td>RMSD angles (°)</td>
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<tr>
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