Catalase-like activity in vertebrate Prx1

Cysteine-independent catalase-like activity of vertebrate peroxiredoxin 1 (Prx1)

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Background: Peroxiredoxin (Prx) was previously known only as a Cys-dependent thioredoxin.

Results: Cys-independent catalase-like activity was observed in two vertebrate Prx1 proteins.

Conclusion: Prx1 possesses dual antioxidant activities with varied affinities towards H2O2.

Significance: This discovery extends our knowledge on Prx1 and provides new opportunities to further study the biological roles of this family of antioxidants.

ABSTRACT

Peroxiredoxins (Prxs) are a ubiquitous family of antioxidant proteins that are known as thioredoxin peroxidases. Here we report that Prx1 proteins from Tetraodon nigroviridis and humans also possess a previously unknown catalase-like activity that is independent on Cys residues and reductants, but dependent on iron. We have identified that the GVL motif was essential to the catalase-like (CAT) activity of Prx1, but not to the Cys-dependent thioredoxin peroxidase (POX) activity, and generated mutants lacking POX and/or CAT activities for individually delineating their functional features.

We discovered that the TnPrx1 POX and CAT activities possessed different kinetic features in reducing H2O2. The overexpression of wild-type TnPrx1 and mutants differentially regulated the intracellular levels of ROS and p38 phosphorylation in HEK-293T cells treated with H2O2. These observations suggest that the dual antioxidant activities of Prx1 may be crucial for organisms to mediate intracellular redox homeostasis.

Peroxiredoxins (Prxs or Prdxs) are a family of ubiquitous antioxidant enzymes known to be involved in sensing and detoxifying hydrogen peroxide (H2O2) and other reactive oxygen species (ROS) in all biological kingdoms (1-3). Mammalian Prxs also participate in the regulation of signal transduction by controlling the cytokine-induced peroxide levels (4-6). Humans and other mammals possess six Prx isoforms, including four typical 2-cysteine (2-Cys) Prxs (Prx1-4), an atypical 2-Cys Prx5 and a 1-Cys Prx6 (7-9). The thioredoxin peroxidase (POX) activity is the hallmark of Prx proteins. In the case of Prx1-4, the conserved N-terminal peroxidatic Cys residue (CysSH, corresponding to the Cys51 in the mammalian...
Prx1 is oxidized by H$_2$O$_2$ to cysteine sulfinic acid (C$_\text{P}$-SO$_2$H) and then resolved by a reaction with the C-terminal resolving Cys$^{172}$ (C$_\text{R}$-SH) in the adjacent monomer to form a disulfide bound Cys$^{51}$ and Cys$^{172}$. The disulfide linkage is reduced by NADPH-dependent thioredoxin (Trx)/thioredoxin reductase (TrxR) cycles to complete the Prx catalytic cycle in cells, or by a reducing agent such as dithiothreitol (DTT) commonly used in assaying POX activity (10-12). Alternatively, at least the C$_\text{R}$-SH and C$_\text{R}$-SH residues in Homo sapiens Prx1 (HsPrx1) can be glutathionylated in the presence of a small amount of H$_2$O$_2$, and deglutathionylated by sulfiredoxin (Srx) or glutaredoxin I (Grx I). C$_\text{P}$-SH may also be hyper-oxidized in the presence of excessive amount of H$_2$O$_2$ to form reversible sulfenic acid (C$_\text{P}$-SO$_2$H) that can be slowly recycled by Srx, or irreversible sulfenic acid (C$_\text{P}$-SO$_2$H$_2$), resulting in the loss of the POX activity and the formation of Prx1 decamers with protein chaperone function (13-17). Among these reactions, the rapid recycling of POX activity is responsible for the reduction of H$_2$O$_2$ and other ROS, while the other two appear to be involved in the regulation of Prx functions (18).

Although Prxs can be oxidized in multiple ways, all these POX activities rely on the Cys-dependent peroxidation cycles. However, in the present study, we unexpectedly observed that the Prx1 from the green spotted puffer fish Tetraodon nigroviridis (TnPrx1) was able to reduce H$_2$O$_2$ that was independent on the Cys peroxidation and in the absence of reducing agents. This Cys-independent activity observed in wild-type (WT) and site-mutated TnPrx1 proteins differs from the classic POX activity in Prxs, but resembles the catalase-like activity, making Prx1 as a dual antioxidant protein. For clarity, we denoted Cys-dependent POX and Cys-independent CAT-like activities in TnPrx1 as TnPrx1-POX and TnPrx1-CAT, respectively. We have determined detailed kinetic features on the TnPrx1-CAT activity, and also identified that the $^{117}$GVL$^{119}$ motif was essential to this activity. Using a HEK-293T cell transfection system, we showed that the TnPrx1-CAT participated in the regulation of H$_2$O$_2$ and H$_2$O$_2$-dependent phosphorylation of p38 in cells. Additionally, CAT activity was also confirmed in human Prx1 (HsPrx1), suggesting that the Cys-independent Prx1-CAT activity is conserved from fish to mammals.

**EXPERIMENTAL PROCEDURES**

Cloning and expression of recombinant Prx1 proteins—The Prx1 open reading frames (ORFs) of T. nigroviridis (TnPrx1) and Homo sapiens (HsPrx1) were amplified by RT-PCR from mRNA isolated from pufferfish kidney and Hela cells (corresponding to the GenBank accession numbers DQ003333 and NM_001202431, respectively), and cloned into the pET28a bacterial expression vector containing a Hisx6-tag at the N-terminus as described (19). TnPrx1 mutants were generated by site-directed mutagenesis by replacing all three Cys residues (i.e., Cys$^{52}$, Cys$^{71}$ and Cys$^{173}$) with Ser residues to eliminate POX activity (denoted by POX CAT$^-$), or the $^{117}$GVL$^{119}$ motif with $^{117}$HLW$^{119}$ to eliminated the CAT-like activity (POX CAT$^-$), or both (POX CAT$^-$) (see Table 1 for details on the genotypes of constructs). Recombinant Prx1 proteins were expressed in Escherichia coli and purified from the soluble fractions by Ni-TNA agarose beads-based chromatography and eluted with elution buffer containing 250 mM imidazole or as specified (19). Purified Prx1 proteins were subjected to SDS-PAGE analysis and stained with Coomassie bright blue. The protein purities were determined by densitometry using 1D Image Analysis Software with Kodak Gel Logic 200 Imaging System (Eastman Kodak Company, USA).

A reversible monomer-to-dimer transition system was established to evaluate the Cys-dependent formation of dimers, in which the purified recombinant proteins in form of monomers were first allowed to be oxidized to form dimers in air at 4 °C, and then the resulting protein dimers were reduced to monomers by the treatment of dithiothreitol (DTT, 50 mM or as specified) at room temperature for 10 min. The reduced and oxidized forms of Prx1 were detected by non-reducing SDS-PAGE.

**Protein structure homology-modeling**—TnPrx1 protein structure homology-modeling was performed using a rat Prx1 (PDB ID: 1QQ2; 80% identity) as template. Global alignment of various structural models was performed by using PyMOL to produce various structural-model figures. Active site of TnPrx1 was predicted using an alpha shape algorithm to determine potential active sites in 3D.
protein structures in MOE site finder, and further mutation was designed to disturb the structure of active site. Site-directed mutants of TnPrx1 were constructed using the overlapping extension PCR strategy. Primers used in the experiments were shown in Table 2. All constructed plasmids were sequenced to verify the correct gene insertion and successful mutation.

**Enzyme activity assays**—The reduction of H$_2$O$_2$ by TnPrx1 and HsPrx1 was determined by a modified sensitive Co(II) catalysis luminol chemiluminescence assay as described (20). Briefly, the luminol-buffer cocktail was composed by 100 μL of luminol (100 mg mL$^{-1}$) in borate buffer (0.05 M, pH 10.0) and 1 mL Co(II)-EDTA (2 and 10 mg mL$^{-1}$, respectively, pH 9.0). Reactions started with mixing 50 μL of proteins (50 μg mL$^{-1}$) with 50 μL of a series of H$_2$O$_2$ solutions (0 – 500 μM) for 1 min at 25 °C, following by adding 1.1 mL of the luminol-buffer cocktail to stop the reaction. The same amount of phosphate buffered saline (PBS) was used to replace proteins in the control and for generating standard curves.

The intensity of emission was measured with an FB12 luminometer (Berthold Detection Systems, Pforzheim) and the maximum values were recorded. The kinetic parameters of Prx1 proteins were determined using the Michaelis-Menten and/or allosteric sigmoidal kinetic models. The production of oxygen was measured with an oxygen electrode (#341003038/ 9513468, Mettler Toledo). The reaction was performed in 4 mL of 600 μM H$_2$O$_2$ solutions, and the measurement was started by addition of proteins, POX$^*$CAT$^+$ dimers (0.32 μM) and POX$^*$CAT$^-$ monomers (0.64 μM), under soft stirring. Oxygen production rates were monitored at varied time points. Reactions with bovine catalase (8 nM) (Sigma-Aldrich) and BSA (6 μM) were used as positive and negative controls, respectively.

**Determination of enzyme properties**—Effect of pH on Prx1-CAT activity was evaluated by detecting the reduction of H$_2$O$_2$ in reactions carried out in 0.2 mM Na$_2$HPO$_4$/0.1 mM citrate buffer for pH 2.0 – 8.0 and 50 mM disodium pyrophosphate/NaOH buffer for pH 8.0 – 11.0, respectively. Effect of temperature was tested between 0 – 70 °C at pH 7.0. The thermal and pH stabilities were similarly assayed, except that concentrated Prx1 proteins were first treated at 0 °C for 1 h at varied temperatures, or 6 h under varied pH conditions, and then their specific activity determined under regular assay conditions (i.e., pH 7.0 at room temperature).

Specific activities were also assayed for iron-saturated proteins prepared by mixing proteins with FeCl$_3$ (1:100 molar ratio), followed by ultrafiltration (MWCO at10 kDa) to remove unbound iron. The role of iron in Prx1-CAT was further evaluated by iron chelation and rescue assays, in which TnPrx1 proteins were treated with 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron, 25 mM) and 2,2-dipirydyl (DP, 50 mM) at 4 °C overnight, followed by ultrafiltration. Chelator treated samples were then incubated with FeCl$_3$ (200 μM or as specified) at 4°C overnight, followed by ultrafiltration to remove unbound iron. The residual TnPrx1-CAT activities of iron-free and iron-rescued proteins were determined in standard reactions as described above. To confirm that iron was truly bound to TnPrx1, iron-rescued samples were subjected to extensive ultrafiltration with PBS, and the iron content was detected by the inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 8000DV, Perkin-Elmer) as described (21).

The effects of 7 other metals on the TnPrx1-CAT activity was tested, in which WT TnPrx1 dimers were treated with tiron/DP mixture, and then reconstituted individually by incubating them with Mg$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$ or Zn$^{2+}$ (Prx1:metal molar ratio = 1:5) at 4 °C overnight. The effect of two classic catalase inhibitors (DTT and 3-amino-1,2,4-triazole [3-AT]) on TnPrx1 was assayed by pretreating proteins with 10 mM 3-AT at 4 °C overnight or 1 mM DTT at 25 °C for 30 min. Bovine catalase was used as positive control. Enzyme activities were assayed as described above.

**Effects of WT TnPrx1 and mutants on intracellular ROS level and the phosphorylation of P38 MAPK**—The ORFs of WT and TnPrx1 mutants were subcloned into pCMV-Tag2B vector. Human embryonic kidney 293T cells (HEK-293T) were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco) in the presence of 5% CO$_2$. For transient transfection, cells were plated in 100-mm cell culture plates (1.4 × 10$^6$ cells/plate), grown overnight, and transfected with 17 μg of WT Prx1 or mutant plasmids using Fugene reagent.
Concentration of H₂O₂ increases from the sample treated with the highest dose of lipid peroxidation. Viable cells were then plated into 96-well collagen-coated plates (2×10⁴ cells/well), and treated with H₂O₂ at final concentrations between 0–850 µM for 60 min.

Intracellular fluorescence signals of oxidized DCFH at 0 and 1 h time points (T₀ and T₁) followed by the H₂O₂ treatment were measured with a Synergy H1 Hybrid Reader (BioTek, USA) (λₑₓ/λₑᵐ = 485/525 nm). The relative fluorescence signal for each sample (RF sample) was calculated using equations:

\[
RF = \frac{ΔF_{\text{sample}}}{ΔF_{\text{max}}} \quad (1)
\]

\[
ΔF_{\text{sample}} = (F_{T₁-T₀}/F_{T₀}) \times 100\% \quad (2)
\]

where ΔF max represented the fluorescence signal increases from the sample treated with the highest concentration of H₂O₂.

For evaluating the effect of TnPrx1 constructs on the phosphorylation of intracellular p38 MAPK, transfected cells treated with H₂O₂ (0–1200 µM) were collected and lysed, followed by Western blot analysis using antibodies against p38 and phosphorylated p38, respectively (Cell Signaling Technology). The immuno-reactive bands were visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL).

Statistical analysis— All experiments were performed independently for at least three times. Data were presented as the mean ± standard deviation of the mean (SD). Two-tailed Student’s t-test was used to assess statistical significance between experimental and control groups.

RESULTS

Cys-independent CAT activity in TnPrx1— Thioredoxin peroxidase (POX) was previously the only known enzyme activity in Prxs that relied on NADPH-dependent oxidoreduction between Trx and TrxR to maintain the continuation of their POX activity. In the absence of Trx/TrxR/NADPH or a reducing agent (e.g., DTT), the reactions stop after the formation of a C₅-C₉ disulfide bound, in which one pair of Prx monomers may only reduce two H₂O₂ molecules. Four to six H₂O₂ molecules may be reduced when they were hyper-oxidized without the formation of disulfide bounds.

Surprisingly, however, in the absence of a reducing agent, we observed that the recombinant WT TnPrx1 monomers (>99% purity in reduced status) were able to continuously reduce H₂O₂ molecules (Fig. 1A, B, C), implying the presence of non-POX oxidoreduction activity in TnPrx1. Similar activity was observed when TnPrx1 was fully oxidized to form dimers (Fig. 1B, D), confirming that the observed non-POX activity was independent on the status of Cys residues. The observed activity was not attributed to nonspecific background reactions as it was not observed in reactions containing no or denatured TnPrx1 (Fig. 1C, D, first and last columns). Additionally, we also detected the O₂ production (Fig. 1E, F), and the calculated ratio between the reduced H₂O₂ and the produced O₂ was 2.29:1, indicating that this activity was derived from a catalase-like (CAT) activity (i.e., 2 H₂O₂ → 2 H₂O + O₂), rather than the POX activity that only produces H₂O₂.

To fully rule out the possibility that a trace amount of contaminating catalase from *Escherichia coli* was present in the TnPrx1 preparations (despite of >99% purity) and contributed to the activity, we prepared TnPrx1 proteins under different elution stringency (i.e., imidazole at 150 to 300 mM) to allow various impurities (i.e., containing various amounts of contaminants). We confirmed again that the activity was derived from TnPrx1, as it was correlated with the amount of TnPrx1, rather than with the level of impurity (Fig. 1G, H).

The activity was iron-dependent, as it could be inhibited by ferrous/ferric chelators tiron and DP, and the addition of Fe³⁺ could not only increase the activity of untreated TnPrx1, but also reverse the inhibition by chelators (Fig. 1H, Fig. 2A). Fe³⁺ displayed low nanomolar level binding affinity with TnPrx1 (apparent *Kₐ* = 0.17 µM), and a ~1:1 (metal:Prx1) stoichiometry (Fig. 2C). To confirm the iron-TnPrx1 binding, we directly evaluated the iron content of recombinant TnPrx1 proteins under various conditions. Proteins were subjected to extensive ultrafiltration to remove unbound iron. The molecular ratio between iron and untreated iron (Promega). Blank pCMV-Tag2B vector was used as negative control. After 48 h of transfection, cells were washed with PBS, and incubated with 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 200 µM, Sigma) in serum-free medium at 37 °C for 30 min to allow uptake by cells and intracellular cleavage of the diacetate groups by thioesterase. Cells were washed with PBS to remove free DCFH-DA from the medium, and counted by trypan blue (0.4%) exclusion method. Viable cells were then plated into 96-well collagen-coated plates (2×10⁴ cells/well), and treated with H₂O₂ at final concentrations between 0–850 µM for 60 min.

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where ΔF max represented the fluorescence signal increases from the sample treated with the highest concentration of H₂O₂.

For evaluating the effect of TnPrx1 constructs on the phosphorylation of intracellular p38 MAPK, transfected cells treated with H₂O₂ (0–1200 µM) were collected and lysed, followed by Western blot analysis using antibodies against p38 and phosphorylated p38, respectively (Cell Signaling Technology). The immuno-reactive bands were visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL).

Statistical analysis— All experiments were performed independently for at least three times. Data were presented as the mean ± standard deviation of the mean (SD). Two-tailed Student’s t-test was used to assess statistical significance between experimental and control groups.
TnPrx1 protein was 0.64 (±0.002):1 (Fig. 2D). Treatment by chelators reduced the ratio to 0.08 (±0.003):1, whereas the addition of FeCl3 (200 μM) restored the ratio to 0.75 (±0.006):1 (Fig. 2D). These observations indicated that each TnPrx1 binds to one iron, and up to 75% of the recombinant TnPrx1 proteins were in active form.

Additionally, the effect of other metals, including Mg2+, Ca2+, Cu2+, Mn2+, Co2+, Ni2+ and Zn2+ on the CAT-like activity was tested, but no enhancement activity was observed (data not shown). The dependence on iron, but not on reducing agents and Cys residues were characteristic to catalases, further confirming that the observed activity was not derived from the POX activity of Prxs. Instead, it resembled a catalase that was previously unknown to Prxs. However, TnPrx1 was insensitive to the inhibitors of typical CATs, such as DTT and the irreversible inhibitor 3-AT (Fig. 2E, F), suggesting that Prx1 might represent a new class of CAT-like enzyme. Indeed, unlike typical CATs, TnPrx1 lacked the Soret absorbance peak unique to heme-containing moieties (data not shown), indicating that it is a heme-less metalloprotein, rather than a heme-containing protein.

In the presence of DTT, WT TnPrx1 displayed Michaelis-Menten kinetics on low concentrations of H2O2 (i.e., <100 μM) (Fig. 3A). The Km value was 2.2 μM that was comparable to the Km values previously reported for Prx1-POX activities that were typically much lower than 20 μM (7). At higher H2O2 concentrations (>50 μM), TnPrx1 exhibited allosteric kinetics suggesting a positive cooperativity, i.e., \( K_{\text{app}} = 214 \) μM and Hill coefficient \((n) = 3.4 \). In the absence of DTT, however, TnPrx1 showed no or little activity until [H2O2] reached to >50 μM \( (K'_{\text{app}} = 168 \) μM, \( n = 4.7 \) \) (Fig. 3A; Table 1). The data were in agreement with the notion that TnPrx1 possessed both POX and CAT activities, as the activities with DTT (POX + CAT) were higher than those without DTT (CAT only) by a relatively constant rate (i.e., 2.5 S-1, determined by a "Michaelis Menten + allosteric sigmoidal" model).

Since CAT activity was described for the first time in a Prx1 of fish origin, we wanted to know whether it was also present in mammalian Prx1. We expressed recombinant human Prx1 (HsPrx1) and performed similar assay with or without a reducing agent. Our data supported that HsPrx1 was also bifunctional by possessing POX and CAT activities with kinetic parameters comparable to those of TnPrx1 (i.e., \( K'_{\text{app}(-\text{DTT})} = 347 \) μM, \( n_{(-\text{DTT})} = 10.1 \), \( K'_{\text{app}(+\text{DTT})} = 342 \) μM, and \( n_{(+\text{DTT})} = 8.8 \), respectively) (Fig. 3E; Table 1). Although Prxs from more species need to be examined to make a firm conclusion, the data here suggest that the CAT-like activity is likely conserved among vertebrate Prx1 from fish to mammals.

To further validate TnPrx1-CAT activity, we performed a site-directed mutagenesis and constructed a mutant by replacing all three Cys residues with Ser residues to completely eliminate its Cys-dependent POX activity. The resulting mutant (POX−CAT+) was unable to form dimers as expected (Fig. 3G, H), but still capable of converting H2O2 to O2 (Fig. 1E, F). The POX CAT+ mutant displayed virtually identical sigmoidal curves when assayed with and without DTT that resembled that of WT TnPrx1 without DTT, as well as similar kinetic parameters (i.e., \( K_{\text{app}(-\text{DTT})} = 211 \) μM, \( n_{(-\text{DTT})} = 3.7 \), \( K_{\text{app}(\text{DTT})} = 227 \) μM, and \( n_{(+\text{DTT})} = 3.3 \)) (respectively) (Fig. 3B; Table 1). These observations confirmed that the observed TnPrx1-CAT activity was truly independent on the Cys residues and reducing agent.

**Potential active site for the CAT-like activity in TnPrx1**— The discovery of a previously unknown Prx1-CAT activity prompted us to search for the functional motif. By examining a previous reported structure of rat Prx1 (PDB ID: 1QQ2), and homology-based modeling of TnPrx1, we observed a flexible loop consisting of six residues, Gly117–119, Phe127 (rat Prx1), or Tyr127 (TnPrx1), Ile142 and Ile144 at the dimer interface, in which a H2O2 molecule could well fit into a pocket formed by the highly conserved 117GVL119 residues (Fig. 4). To test whether this pocket might contribute to the TnPrx1-CAT activity, we generated a TnPrx1 construct by replacing 117GVL119 with 117HLW119 (denoted by POX−CAT+) to alter the pocket structure. Indeed, the mutant POX−CAT−lost CAT-like activity (i.e., no activity without DTT in the reactions), but retained only DTT-dependent POX activity that followed Michaelis-Menten kinetics characteristic to Prx1-POX activity (\( K_m = 4.15 \) μM) (Fig. 3C; Table 1).

To further dissect individual TnPrx1-POX and Prx1-CAT activities, we generated a double-
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mutation (POX CAT•), in which all Cys residues and 117GVL119 were replaced by Ser and 117HLW119, respectively. As expected, this double-negative mutant lost both POX and CAT activities and was unable to reduce H2O2 regardless of whether DTT was present or not (Fig. 3D). Among all the mutants tested, POX CAT• also displayed expected iron-dependency, in which iron chelators inhibited its activity that could be restored by adding iron (Fig. 2B), whereas the two CAT− mutants (i.e., POX• CAT− and POX− CAT−) only retained low activity (6% vs. WT) that were unaffected by iron chelators and iron (data not shown). Additionally, the TnPrx1-CAT activity tolerated low temperature more than pH, as it was able to retain virtually constant peak activity between 0 – 40 °C, but only retained peak activity at ~pH 7.0 (Fig. 5).

Collectively, these observations confirm that TnPrx1 possesses both POX and CAT activities, and the residues 117GVL119 are critical to Prx1-CAT activity. TnPrx1-POX acted on H2O2 with much higher affinity (Km = 4.15 μM), but had a relatively low maximal activity (kat = 0.23 s−1) with a wider range of H2O2 levels (Table 1; Fig. 3C); whereas Prx1-CAT acted on H2O2 with lower affinity (K′(−DTT) = 210.7 μM), but had a much higher activity (kat = 2.3 s−1) (Table 1).

Implication of TnPrx1-CAT in regulating ROS level and signaling—The physiological roles of TnPrx1-CAT activity were investigated using a mammalian cell transfection system. First, we transfected and overexpressed HEK-293T cells with various TnPrx1 constructs, and examined the effects in regulating intracellular ROS (iROS) in response to H2O2 treatment. The expression of TnPrx1 constructs in transfected cells was confirmed by qRT-PCR (Fig. 6A, B). We observed a general trend that cells overexpressing CAT− proteins (i.e., WT and POX• CAT−) had lower iROS levels than those expressing CAT+ proteins (i.e., blank vector, POX•CAT+ and POX− CAT−) in response to the treatment of 150 – 600 μM exogenous H2O2 (Fig. 6C).

Second, since H2O2 was known to also function as a signaling molecule, particularly in regulating kinase-driven pathways (22), we tested whether Prx1-CAT-associated regulation of intracellular H2O2 affected the phosphorylation of p38 that played a central role in the p38 mitogen-activated protein kinase (MAPK) signaling pathway. In HEK-293T cells transfected with blank or double-negative (POX• CAT−) plasmids, there were low background levels of phosphorylated p38 (p-p38) in the absence of H2O2 stimulation (0 μM) (Fig. 6D). The levels of p-p38 in cells treated with 225 – 1,200 μM H2O2 displayed a bell-curve that peaked in the 525 – 900 μM H2O2 groups, which was comparable to previously reported data (23). When CAT+ constructs (i.e., WT and POX CAT+) were overexpressed, a considerable delay of phosphorylation of p38 was observed, as p-p38 was significantly (p<0.05) up-regulated in cells challenged with H2O2, starting at 525 μM, peaked in the 900 – 1,200 μM.

On the other hand, in cells overexpressing POX• CAT− TnPrx1, no significant delay of p38 phosphorylation was observed, as p-p38 was only significantly up-regulated in cells challenged with H2O2, starting at 375 μM, peaked around 750 μM and declined at 1,200 μM (Fig. 6D), whose pattern was similar to that of blank or double-negative group. Although further studies are needed to fully dissect the physiological roles of individual Prx1-POX and Prx1-CAT activities in cells and in vivo, these observations provide primary evidence on the involvement of the TnPrx1-CAT activity in regulating ROS-mediated p38 signaling pathway when cells were incubated with high micromolar to low millimolar level of H2O2.

DISCUSSION

Eukaryotic cells contain a complex system to detoxify and regulate H2O2 and other reactive oxygen species. These include small molecules, such as ascorbic acid, β-carotene, glutathione, and α-tocopherol, and various enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and peroxiredoxin (Prx) (24). Some of these enzymes or isoforms are mainly cytosolic (e.g., Prx1, Prx2, Prx5, Prx6, SOD1 and GPx1), while others may be compartmentalized (e.g., catalase in peroxisome, SOD2 and Prx3 in mitochondria, while SOD3, GPx3 and Prx4 in plasma), which constitutes a precise antioxidant network for the defense against various oxidative stresses in the diverse cellular activities (4,25,26).

Cells are known to rely heavily on Prxs in scavenging H2O2 and other ROS molecules. In fact, they are the third most abundant proteins in
Our data indicated that TnPrx1- CAT activity was iron. However, not heme-related, but dependent on mononuclear heme-binding is non-essential to their functions. Reported to be able to bind heme (36), although hydroperoxide reductase C (AhpC) was also a bacterial 2-Cys peroxiredoxin alkyl heme-binding protein 23 kDa (HBP23) (10,35), and Mammalian Prx1 was previously identified as (33).

Cys-dependent POX activity was near the Cys residues but distant from the GVL site (31-33). This suggests that Prx1 contained two independent H$_2$O$_2$ binding sites, agreeing with previous reports that the H$_2$O$_2$-binding site for the Cys-dependent POX activity was near the Cys$^{51}$ and Cys$^{172}$ residues but distant from the GVL site (31-33).

Catalases are heme-containing enzymes (34). Mammalian Prx1 was previously identified as heme-binding protein 23 kDa (HBP23) (10,35), and a bacterial 2-Cys peroxiredoxin alkyl hydroperoxide reductase C (AhpC) was also reported to be able to bind heme (36), although heme-binding is non-essential to their functions. Our data indicated that TnPrx1-POX activity was not heme-related, but dependent on mononuclear iron. However, the exact iron-binding site remains to be determined. Sequence analysis indicates that Prx1 proteins from T. nigroviridis and mammals contain a 2-His-1-carboxylate facial triad-like motif (e.g., motif $^{81}$HX$_2$HX$_3$HE$_{121}$ in TnPrx1) that is conserved in mononuclear non-heme iron enzymes (37). Additionally, a Trp$^{87}$ residue is also present at the motif. Aromatic residues, particularly Trp and Tyr, are known to be enriched at the Fe-sites of iron-proteins (38). The involvement of aromatic residues in redox catalysis and/or electron transfer is not yet fully understood, but their capability to mediate electron transfer reactions makes them most suitable for tunneling electrons to/from redox sites (38). On the other hand, the putative facial triad is not in the immediate proximity with the GVL motif. Therefore, its involvement in iron-binding and the mechanism of iron-mediated electron transfer for the Prx1-CAT activity need to be verified by further structure-based analysis.

The identity and similarity of vertebrate Prx1 are over 77% and 88% (Table 3), and the active site of CAT activity is completely conserved among Prx1 proteins, suggesting that CAT activity may be a ubiquitous function of Prx1 family members. The confirmation of reductant-independent H$_2$O$_2$-binding site for the Cys-dependent POX activity indicates that this new function is likely conserved at least in some vertebrates. Furthermore, $^{117}$GVL$^{119}$ are conserved in Prx1-3, while $^{117}$GIV$^{119}$ in Prx4. Although the Prx5 and Prx6 share low similarity with Prx1-4, they have similar three-dimensional structures (39) suggesting that CAT activity might be present in other Prxs, at least Prx1-3. It might also explain why some parasites and cyanobacteria do not contain catalase and GPx, but have diverse Prx homologies (29,40).

The intracellular concentrations of H$_2$O$_2$ and other ROS molecules in vivo are not precisely known, but may range from sub- to lower micromolar levels in various prokaryotic and eukaryotic cells. However, intracellular H$_2$O$_2$ levels may rise to the order of 100 μM in phagocytes, and the transient H$_2$O$_2$ levels may reach to >200 μM in brain cells (41,42). Moreover, appropriately stimulated polymorphonuclear leukocytes (PMN) and monocytes can produce up to 1.5 nmol of H$_2$O$_2$ in 10$^5$ cells per hour (which is roughly equivalent to >350 to 450 mM of H$_2$O$_2$ if it is not removed and accumulated per hour, given their cell sizes at ~330 and 420 fL) (43,44). In the present study, we have shown that Prx1 acts mainly (if not only) as POX under low level H$_2$O$_2$ environment with high affinity and relatively low capacity ($K_a$ and $k_{cat}$ at ~2.23 – 4.15 μM and ~0.23 s$^{-1}$), but as both POX and CAT when H$_2$O$_2$ level reaches to ~50 μM or...
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higher, in which the latter behaves as an allosteric enzyme with 10 times higher activity than the former (\(K_m\) and \(k_{cat}\) at \(\approx 210 \mu M\) and 2.3 s\(^{-1}\)). In vitro transfection experiments also confirmed the notion, as HEK-293T cells overexpressing WT TnPrx1 and mutant retaining CAT activity were capable of scavenging more iROS than those overexpressing mutants lacking CAT or both POX and CAT activities at low to middle micromolar \(H_2O_2\) levels (Fig. 6C). The levels of exogenous \(H_2O_2\) to produce significant effects on cellular activities such as on the phosphorylation of p38 in cells transfected with various TnPrx1 mutants were at \(\approx 375 – 1050 \mu M\) or higher (Fig. 6D), which was corresponding to \(\approx 50 – 150 \mu M\) intracellular \(H_2O_2\) based on the model predicting that intracellular \(H_2O_2\) concentrations was \(\approx 7\)-fold or even 10-100 folds lower than that applied exogenously (42,45,46). The corresponding intracellular levels of \(H_2O_2\) fell within the levels for physiologically relevant signaling (i.e., \(15 – 150 \mu M\)) (46).

Collectively, these features enable Prx1 to function on a wider range of ROS concentrations than many other proteins in the cytosol, in which Prx1-POX acts on sub-to lower micromolar iROS normally present in cells, whereas Prx1-CAT (probably along with GPx and classic CAT enzymes) acts on moderate to higher micromolar iROS concentrations that are present in certain types of cells (e.g., some brain and immune cells) and/or required for \(H_2O_2\) signaling (Table 1)

However, it is noticeable that, although TnPrx1 and HsPrx display CAT-like activity, their catalytic efficiencies are \(\approx 100\)-fold smaller than those of regular CATs (i.e., \(k_{cat}/K_m^{Prx1-CAT}\) at \(\approx 10^4\) M\(^{-1}\) s\(^{-1}\) vs. \(k_{cat}/K_m^{CAT}\) at \(\approx 10^6\) M\(^{-1}\) s\(^{-1}\)), which raises a question whether Prx1-CAT function is critical to organisms, as a higher level of iROS may be quickly scavenged by regular CAT. Prx1 is a cytosol protein, whereas native CATs are typically present in peroxisomes. Data-mining the Multi-Omics Profiling Expression Database (MOPED) (https://www.proteinspire.org/) also reveals that human Prx1 is much more abundant than CAT in most cells/tissues (Fig. 7A). Therefore, we speculate that the CAT-like activity in Prx1 and possibly in other Prxs may act as one of the first line of scavengers for cytosolic ROS. Prx-CAT may also play more critical role in scavenging and/or regulating ROS in certain cells and tissues that are deficient, or contain extremely low levels of CAT. For example, in human bone, oral epithelium and retina, the CAT protein levels are 132-, 45- and 36-fold less than Prx1 (i.e., 13 vs. 1,730, 55 vs. 2,490, and 110 vs. 4,020 ppm, respectively). Some cancer cells might also take advantage of the Prx1-CAT activity, as the expressions of CAT were deficient or highly down-regulated in many of cancer cells (47), whereas those of Prx1 were up-regulated in cancer cells including breast, lung and urinary cancers and hepatocellular carcinoma (48). The down- and up-regulation of CAT and Prx1 was also clearly supported by comparing the MOPED protein expression profiles between cancer and non-cancer cells (Fig. 7). Additionally, we also confirmed by qRT-PCR that the mRNA level of CAT in HEK-293T cells was \(\approx 50 – 200\)-fold less than that of Prx1 (Fig. 6A, B).

The Prx-CAT function might also explain how some invertebrates lacking CAT and GPx regulate high levels of intracellular ROS. For example, some parasitic helminths (e.g., Fasciola hepatica and Schistosoma mansoni) and roundworms (e.g., filarial parasites), as well as some protozoa (e.g., Plasmodium sp.) are deficient of CAT and GPx, but possess highly expressed Prx genes (29,40).

In summary, we observed a CAT-like activity in the pufferfish and human Prx1 proteins that were independent on Cys residue and reductants, but dependent on non-heme mononuclear iron. TnPrx1-CAT activity was capable of regulating intracellular ROS and the ROS-dependent phosphorylation of p38 in transfected HEK-293T cells. These newly discovered features extended our knowledge on Prx1 and provided a new opportunity to further dissect its biological roles.
Conflict of interest-The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions-CCS, WRD, GZ, JZS contributed to the experimental design. CCS and WRD performed most experiments and data analysis. JZ constructed a cysteine mutant TnPrx1 (POX\(^{-}\)CAT\(^{+}\)) and performed protein expression. LN cloned the Prx1 gene of Tetraodon nigroviridis and Homo sapiens. CCS, WRD, GZ, JZS participated in manuscript preparation. LXX, GZ, JZS reviewed and edited the manuscript. All authors read and approved the final manuscript.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Verification of the catalase-like activity (CAT) of Prxl and mutants. (A) Expression and purification of soluble TnPrxl protein. Lanes: 1, protein markers; 2, crude cell lysate; 3, flow-through; 4-5, 40 mM imidazole wash; 6-8, eluted recombinant protein (250 mM imidazole); (B) Reductive dissociation of TnPrxl dimer induced by DTT. Lanes: 1, protein markers; 2-8, proteins treated with different concentrations of DTT (0, 0.1, 0.5, 1, 5, 10 and 50 mM); (C-D) Activities of TnPrxl in monomers (C) and dimers (D) in the absence of a reducing agent by detecting the reduction of H$_2$O$_2$ using a luminol chemiluminescence assay after incubation with 300 μM H$_2$O$_2$ for 10 min at 25 °C. The bands in red dashed box denoted the TnPrxl monomers and dimmers used in the corresponding assays. Theoretical values represented the maximal reduction of H$_2$O$_2$ possibly achieved by the oxidation of 3 TnPrxl Cys residues in a given amount of TnPrxl protein in the absence of reductants or redox recycling. hd = heated denatured TnPrxl protein; (E-F) Detection of O$_2$ production in reactions containing H$_2$O$_2$ and various protein constructs (i.e., 0.32 μM POX$^+$/CAT$^+$ dimers, 0.64 μM POX$^+$ CAT$^+$ monomers, 8 nM catalase and 6 μM BSA) with oxygen electrode technique; (G-H) Gradient elution of TnPrxl protein with varied concentrations of imidazole in elution buffer (G), and their corresponding activity by measuring the reduction of H$_2$O$_2$ with or without the addition of extra iron (200 μM) by luminol chemiluminescence (H). Activity was normalized to μmol of H$_2$O$_2$ reduced per min per gram of TnPrxl protein. Data are representative of at least three independent experiments. The error bars represent standard deviations (SDs), and statistical significances between experimental and control groups were determined by Student’s t-test. *** = p <0.001.

FIGURE 2. Iron-dependency and inhibition of TnPrxl and mutants determined by measuring the reduction of H$_2$O$_2$ by a luminol chemiluminescence assay. (A-B) Effects of iron chelators (25 mM 4,5-dihydroxy-1,3-benzene disulfonic acid [tiron] and 50 mM 2,2-dipyridyl [DP]) on the CAT activity of WT TnPrxl and mutants, and restoration of the activity by the addition of Fe$^{3+}$ (200 μM). Residual activities were expressed as the percent activity (vs. untreated WT TnPrxl); (C) Dose-dependent WT TnPrxl activity on Fe$^{3+}$. Residual activities were expressed as the percent activity (vs. WT TnPrxl treated with 200 μM Fe$^{3+}$); (D) Molar ratio between TnPrxl protein and bound iron determined by ICP-OES (inductively coupled plasma optical emission spectroscopy). TnPrxl was treated as specified, followed by extensive washes with water by ultrafiltration prior to ICP. Bovine catalase and PBS were used as controls; (E-F) Effects of catalase inhibitors 3-Amino-1,2,4-triazole (3-AT) and dithiothreitol (DTT) on the CAT activity of WT TnPrxl and mutants. Catalase was used as positive control. Residual activities were expressed as the percent activity (vs. untreated WT TnPrxl). Data are representative of at least three independent experiments. The error
bars represent standard deviations (SDs), and statistical significances between experimental and control groups were determined by Student’s $t$-test. ** = $p < 0.01$, *** = $p < 0.001$.

FIGURE 3. Kinetic features of Prx1 proteins. (A-E) Enzyme kinetics curves for pufferfish Prx1 (TnPrx1 WT and mutants) and human Prx1 (WT HsPrx1) with or without DTT. (F) Structural comparison of the potential cavity of wild-type Prx1 protein (yellow) and its mutant (warm pink) in mesh form. The image is a merged model of the two Prx1 proteins; (G-H) The dimeric versus monomeric status of TnPrx1 proteins in non-reducing or reducing SDS-PAGE, respectively. All TnPrx1 proteins were treated with monomer-to-dimer transition protocol prior to the assays.

FIGURE 4. Structural comparison between rat Prx1 (PDB ID:1QQ2) (A) and TnPrx1 determined by homology-modelling (B). Structural models were represented in surface forms prepared using PyMOL software (www.pymol.org). The amino acids located at the dimer interface were shown in colors. The pockets containing the $^{117}$GVL$^{119}$ motif in rat Prx1 and TnPrx1 were highlighted in yellow.

FIGURE 5. The effect of pH and temperature on catalase-like activity and stability of TnPrx1 wild-type (POX$^+$CAT$^+$) and mutant (POX CAT$^+$) proteins. A. The effect of temperature on residual Prx1-CAT activity. The activity assay was performed at pH 7.0 and at various temperatures. B. The effect of temperature stability of Prx1-CAT. All the proteins were incubated at pH 7.0 and at various temperatures for 1 h and then estimating the residual activity. C The effect of pH on residual Prx1-CAT activity. The activity assay was performed at room temperature and at various pH values. D. The effect of pH stability of Prx1-CAT. The proteins were incubated with various pH at 4 °C for 6 h and then measuring residual activity.

FIGURE 6. Involvement of TnPrx1 constructs in regulating intracellular ROS and ROS-mediated phosphorylation of p38 MAPK in transfected HEK-293T cells. (A-B) The expression of various TnPrx1 constructs in transfected cells were confirmed by qRT-PCR in comparison with those of endogenous HsPrx1 and catalase genes. The relative levels of Prx1 transcripts (HsPrx1 only in blank control, or HsPrx1+TnPrx1 in transfected cells) were determined using a pair of primers derived from regions conserved between fish and mammalian Prx1 genes (Table 2). Fold changes of Prx1 and catalase transcripts were expressed in relative to the catalase transcripts in the blank control (A) or to the transcripts of their own genes (B). (C-D) Effects of TnPrx1 constructs on intracellular ROS and ROS-mediated phosphorylation of p38 MAPK in transfected cells treated with exogenous H$_2$O$_2$ as determined by DCFH fluorescence assay and Western blot analysis, respectively. In the Western blot analysis, antibody to human GAPDH was used as control (lower panel of D). Representative data from one of the three or more independent experiments were shown. The error bars represent standard deviations (SDs), and statistical significances between experimental and control groups were determined by Student’s $t$-test. * = $p < 0.05$.

FIGURE 7. Summary of Prx1 and catalase (CAT) protein levels in various human cells and tissues. (A) Relative expression levels of Prx1 and CAT proteins in cancer and non-cancer samples. Each set of three dots above the same X-axis point represent the levels of Prx1, CAT and total (CAT+Prx1) from the same sample. (B) Comparison of the Prx1 and CAT protein levels in cancer and non-cancer samples by plotting those of Prx1 (X-axis) against CAT (Y-axis). Data were derived from the Multi-Omics Profiling Expression Database (MOPED) developed by the Kolker Laboratory (http://www.kolkerlab.org and https://www.proteinspire.org/MOPED/mopedviews/proteinExpressionDatabase.jsf).
**TABLE 1.** Kinetic parameters of wild-type (WT) TnPrx1 and various mutants deficient in thioredoxin peroxidase (POX) and/or catalase-like (CAT) activities on H\textsubscript{2}O\textsubscript{2} in comparison with those reported for mammalian glutathione peroxidases (GPxs) and catalases in literature.

| Constructs | Genotype | DTT | $K_m$ or $K'$ (μM) | $k_{cat}$ (s\(^{-1}\)) | $k_{cat}/K_m$ (×10\(^4\) M\(^{-1}\) s\(^{-1}\)) | $n$ \(^2\) |
|------------|----------|-----|-------------------|-----------------|-----------------|-------|
| TnPrx1 (POX'CAT') \(^3\) | WT pufferfish Prx1 | − | 168 ± 8.8 | 1.8 ± 0.11 | ~1.0 | 4.7 ± 0.33 |
| | | + | 214 ± 11.7 (Overall) | 2.5 ± 0.36 (Overall) | ~1.1 (Overall) | 3.4 ± 0.34 |
| | | | 2.23 ± 0.03 (POX) | 0.21 ± 0.01 (POX) | ~8 (POX) | (Overall) |
| TnPrx1 (POX'CAT') | All 3 Cys → Ser | − | 211 ± 7.1 | 2.3 ± 0.17 | ~1.1 | 3.7 ± 0.42 |
| | | + | 227 ± 8.3 | 2.6 ± 0.26 | ~1.1 | 3.3 ± 0.46 |
| TnPrx1 (POX'CAT') \(^{17}\)GVL → \(^{17}\)HLW | − | ~250 | 0.013 ± 0.002 | | |
| | + | 4.15 ± 0.6 | 0.23 ± 0.01 | ~6 | |
| TnPrx1 (POX'CAT') | All 3 Cys → Ser and \(^{17}\)GVL → \(^{17}\)HLW | − | ~250 | 0.016 ± 0.001 | | |
| | + | ~250 | 0.016 ± 0.002 | | |
| HsPrx1 (WT) | WT human Prx1 | − | 347 ± 13.8 | 3.9 ± 0.16 | ~1.1 | 10.1 ± 1.8 |
| | | + | 343 ± 11.2 | 4.0 ± 0.06 | ~1.1 | 8.8 ± 1.0 |
| Mammalian GPx \(^4\) | NS | NS | 2×10\(^{2}\) – 2×10\(^{4}\) | 10\(^1\) – 10\(^2\) | ~10\(^4\) | |
| Mammalian catalase \(^4\) | NS | NS | 10\(^4\) – 10\(^5\) | 10\(^4\) – 10\(^5\) | ~10\(^2\) | |

\(^1\) Activity assayed with or without the reducing agent DTT (100 μM). In the absence of DTT, only CAT-like activity plus a basal level of H\textsubscript{2}O\textsubscript{2} consumption by oxidizing same molar amount of Cys residues in TnPrx1 and HsPrx1; \(^2\) $n$ = Hill coefficient; \(^3\) Parameters in the presence of DTT were given for overall activity (i.e., POX + CAT) determined by allosteric sigmoidal model and for POX activity only determined by Michaelis-Menten model at the lower range of H\textsubscript{2}O\textsubscript{2} concentrations (see curves in Figure 3A inset); \(^4\) Data acquired from [http://www.brenda-enzymes.org/](http://www.brenda-enzymes.org/). NS = Not suitable. All data are presented as the mean values ± standard deviations (SDs) of each group.
### TABLE 2. List of primers and their applications.

| Primer            | Sequences (5'-3')                  | Application   |
|-------------------|------------------------------------|--------------|
| TnPrx1-EcoRI-F    | GAATTCTAGGCTGAGGCAAGCTC            | Cloning      |
| TnPrx1-XhoI-R     | CTCAGAGGTGCTTGGAGAAGAATCTTTTG      | Cloning      |
| TnPrx1-Ser52F     | TTCACTTTGTGTCCCCCCTGAAG            | Mutation     |
| TnPrx1-Ser52R     | CTTCAGTGGGGGACACAAGGGTGAAG         | Mutation     |
| TnPrx1-Ser71F     | CGAAAAATTGGATCCGAGGTCATCG          | Mutation     |
| TnPrx1-Ser71R     | CGATGAAGCTCGATCCAAATTTTCGG         | Mutation     |
| TnPrx1-Ser173F    | GCATGGAGAAGTTCCTGCCGCG            | Mutation     |
| TnPrx1-Ser173R    | GCCGCGAGGGGAAACTTCTCCATGC         | Mutation     |
| TnPrx1-HLW-F      | CAATCTACAGACTACCCTATGGAAGGAAGGAGG | Mutation     |
| TnPrx1-HLW-R      | CCTTCGTCTCTTCCCTTCAATGTTGAGTCTGTAGAGATG | Mutation   |
| HsPrx1-F          | GCTGATAGGAGATGTCTCCAGGAA          | Cloning      |
| HsPrx1-R          | GCCAACTCAGGCCATTCCTACC            | Cloning      |
| HsPrx1-EcoRI-F    | CCGGAATTCATGTCTTCCAGGAAATGCTAAATTG | Expression  |
| HsPrx1-XhoI-R     | CGATCGAGCTTCTGCTTGAGAATATTC       | Expression  |
| Prx1-assay-F      | TCACCTTTGTTGCCCCACGGAGAT          | qRT-PCR      |
| Prx1-assay-R      | CACCTCCCCCTGGGTGTCAATGGAAC        | qRT-PCR      |
| Catalase-assay-F  | TACCTGTGAACTTGCTCCCTACCGTGC       | qRT-PCR      |
| Catalase-assay-R  | CATAGAATGCCCCAGCTGAGTAAC          | qRT-PCR      |
**TABLE 3.** Percent amino acid identity and similarity of vertebrate Prx1

|                | Rat  | Mouse | Bovine | Platypus | Chicken | Zebrafinch | Lizard | Frog | Zebrafish | Catfish | Tetraodon | Fugu | Rainbow trout |
|----------------|------|-------|--------|----------|---------|------------|--------|------|------------|---------|-----------|------|----------------|
| *(Human)*      | 97   | 95    | 96     | 92       | 88      | 87         | 86     | 84   | 81         | 84      | 79        | 80   | 82            |
| *(98)*         | (98) | (98)  | (98)   | (94)     | (94)    | (94)       | (94)   | (92) | (93)       | (89)    | (90)      | (92) |               |
| *Rat*          | 96   | 96    | 91     | 88       | 87      | 86         | 86     | 85   | 81         | 84      | 79        | 79   | 82            |
| *(100)*        | (97) | (97)  | (93)   | (93)     | (92)    | (91)       | (92)   | (88) | (89)       | (91)    |           |     |               |
| *Mouse*        | 95   | 90    | 87     | 87       | 85      | 82         | 79     | 82   | 77         | 77      | 79        | 79   | 82            |
| *(97)*         | (97) | (93)  | (93)   | (94)     | (93)    | (92)       | (92)   | (88) | (89)       | (91)    |           |     |               |
| *Bovine*       | 90   | 87    | 88%    | 85       | 84      | 80         | 83     | 79   | 79         | 79      | 80        | 80   | 82            |
| *(97)*         | (93) | (93)  | (93)   | (93)     | (91)    | (92)       | (92)   | (89) | (90)       | (92)    |           |     |               |
| *Platypus*     | 87   | 87    | 89     | 83       | 83      | 80         | 82     | 80   | 80%        | 81      |           |     |               |
| *(95)*         | (95) | (95)  | (94)   | (92)     | (94)    | (90)       | (91)   | (93) |           | (93)    |           |     |               |
| *Chicken*      | 97   | 90    | 83     | 83       | 89      | 85         | 85%    | 85   | 85%        | 85      |           |     |               |
| *(99)*         | (97) | (93)  | (92)   | (96)     | (92)    | (93)       | (93)   | (93) |           | (93)    |           |     |               |
| *Zebrafinch*   | 89   | 83    | 82     | 88       | 84      | 84         | 84     | 84   | 84         | 84      |           |     |               |
| *(97)*         | (92) | (93)  | (96)   | (92)     | (93)    | (93)       | (93)   | (93) |           | (93)    |           |     |               |
| *Lizard*       | 82   | 81    | 82     | 83       | 83      | 83         | 80     |      |            | (92)    |           |     |               |
| *Frog*         | 77   | 80    | 77     | 77       | 77      | 77         | 77     |      |            | (91)    |           |     |               |
| *Zebrafish*    | 90   | 83    | 84     | 84       | 87      |            |        |      |            | (95)    |           |     |               |
| *Catfish*      | 87   | 88    | 89     |            | (93)    | (94)       | (94)   |      |            | (93)    |           |     |               |
| *Tetraodon*    | 98   | 88    |        |           | (99)    | (94)       |        |      |            | (94)    |           |     |               |
| *Fugu*         | 88   |        |        |           | (95)    |            |        |      |            | (95)    |           |     |               |
Figure 1

A

B

C

D

E

F

G

H

Catalase-like activity in vertebrate Prxl

Figure 1

A

B

C

D

E

F

G

H

Catalase-like activity in vertebrate Prxl
Figure 2

A

B

C

D

E

F

Catalase-like activity in vertebrate Prx1

K_{d} = 0.17 \pm 0.02 \mu M

Protein Untreated = Protein+3-AT

Protein Untreated = Protein+DTT
Figure 3
Figure 4

A

B
Figure 5

A. Optimum Temperature

B. Temperature Stability

C. Optimum pH

D. pH Stability
Figure 6

A

B

C

D

HEK Blank
Wild-Type
POX-CAT
POX-CAT
POX-CAT

Catalase
TnPrx+HsPrxs

Relative mRNA level

1,000
100
10
1.0
1.0
1.0

0.1

HEK Blank
Wild-Type
POX-CAT
POX-CAT
POX-CAT

Catalase
TnPrx+HsPrxs

Relative ROS (%)

120
100
80
60
40
20
0

300
600
900

H₂O₂ Treatment (µM)

Wild-Type
POX-CAT
POX-CAT
Blank

p38
p38
p38
p38

H₂O₂ (µM)

225 250 375 500 625 750 900 1,000

H₂O₂ (µM)

225 250 375 500 625 750 900 1,000

Relative intracellular ROS (%)
Figure 7

A

Expression levels ($\times 10^2$ ppm)

Cancer  Non-cancer

B

Human catalase ($\times 10^3$ ppm)

Human Prx1 ($\times 10^3$ ppm)

Non-cancer  Cancer

Non-cancer CAT  Non-cancer Prx1  Cancer CAT  Cancer Prx1  Total (CAT+Prx1)
Cysteine-independent catalase-like activity of vertebrate peroxiredoxin 1 (Prx1)
Cen-Cen Sun, Wei-Ren Dong, Jing Zhao, Li Nie, Lin-Xin Xiang, Guan Zhu and Jian-Zhong Shao

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