Dual Role of the Active-Center Cysteine in Human Peroxiredoxin 1: Peroxidase Activity and Heme Binding

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Abbreviations: PRX, peroxiredoxin; ROS, reactive oxygen species.

ABSTRACT

HBP23, a 23-kDa heme-binding protein identified in rats, is a member of the peroxiredoxin (Prx) family, the primary peroxidases involved in hydrogen peroxide catabolism. Although HBP23 has a characteristic Cys-Pro heme-binding motif, the significance of heme binding to Prx family proteins remains to be elucidated. Here, we examined the effect of heme binding to human peroxiredoxin-1 (PRX1), which has 97% amino acid identity to HBP23. PRX1 was expressed in *Escherichia coli* and purified to homogeneity. Spectroscopic titration demonstrated that PRX1 binds heme with a 1:1 stoichiometry and a dissociation constant of 0.17 μM. UV-vis spectra of heme-PRX1 suggested that Cys52 is the axial ligand of ferric heme. PRX1 peroxidase activity was lost upon heme binding, reflecting the fact that Cys52 is not only the heme-binding site but also the active center of peroxidase activity. Interestingly, heme binding to PRX1 caused a decrease in the toxicity and degradation of heme, significantly suppressing H_2O_2-dependent heme peroxidase activity and degradation of PRX1-bound heme compared with that of free hemin. By virtue of its cytosolic abundance (~20 μM), PRX1 thus functions as a scavenger of cytosolic hemin (<1 μM). Collectively, our results indicate that PRX1 has a dual role; Cys-dependent peroxidase activity and cytosolic heme scavenger.

1. INTRODUCTION

Heme (iron-containing protoporphyrin IX) is an essential molecular cofactor in electron transfer [1], oxygen metabolism [2] and oxidation reactions [3], Heme also acts as an effector molecule to modulate transcription [4,5], translation [6,7], and protein degradation [5,8]. Reflecting these diverse contributions of heme, hemoproteins are localized to various organelles, including the nucleus, endoplasmic reticulum, and plasma membrane [9,10]. Because heme biosynthesis is completed in mitochondria, heme must be trafficked to other organelles via the cytosol. Cytosolic heme-binding proteins, which bind heme loosely, are thought to contribute to heme trafficking. These include fatty acid-binding proteins (FABPs), glutathione S-transferases (GSTs), and heme-binding proteins with a molecular mass of 23 kDa (HBP23) [9,11]. Both GSTs and HBP23 have a Cys-Pro (CP) motif, which is one of the heme regulatory motifs and is found in a wide variety of proteins whose function is regulated by heme [12,13]. The Cys residue in the CP motif is a heme ligand. GSTs and HBP23 have relatively weak heme-binding capacities, with dissociation constants ($K_{d,heme}$) of ~0.1–1 μM, and 55 nM, respectively [14,15], which are a much larger than those of typical hemoproteins such as myoglobin ($K_{d,heme} \approx 10^{-7}$ μM) [16].

HBP23 is highly conserved to an antioxidant enzyme of the peroxiredoxin (Prx) family, in which the Cys in the CP motif constitutes the active center for reduction of hydrogen peroxide (H_2O_2) (Fig. S1). Members of the Prx (EC 1.11.1.15) family are ubiquitous peroxidases found in almost all kingdoms [17]. The active center of Prx proteins consists of two Cys residues, and one Cys residue is reactive with H_2O_2; thus, members of the Prx family are termed cysteine-dependent peroxidases to distinguish them from heme peroxidases such as horseradish peroxidase [18]. Prx1 is classified as a '2-Cys' Prx, whose two conserved cysteines are a hallmark of its peroxidase activity. 2-Cys Prx proteins contain an N-terminal peroxidatic Cys (Cys_P-SH) and a C-terminal resolving Cys (Cys_R-SH), both of which are contributed by CP motifs. Cys_P-SH is oxidized by H_2O_2 to cysteine sulfenic acid (Cys_P-SOH),

and then forms an intermolecular disulfide bond in a head to tail manner with Cys_R-SH from an adjacent monomer. Under physiological conditions, the disulfide linkage is reduced by NADPH-dependent thioredoxin and thioredoxin reductase to regenerate Cys_P-SH [19,20]. To the best of our knowledge, there are no other proteins in which the cysteine in the active center of enzymes also forms a CP motif, leading us to hypothesize that heme binding to Prx1 affects Prx1 cysteine-dependent peroxidase activity. However, the involvement of heme binding in the cysteine-dependent peroxidase activity of Prx1 remains to be elucidated.

Here, we report the purification and characterization of human PRX1, which shares 97% amino acid identity with rat HBP23 (Fig. S1). Purified PRX1 bound to heme with a stoichiometry of 1:1 and exhibited a $K_{d,heme}$ of heme binding of 0.17 μM. A mutational study showed that Cys_P-SH, donated by one of the CP motifs, bound heme, leading to the loss of cysteine-dependent peroxidase activity. However, hemin peroxidase activity and H_2O_2-mediated hemin degradation of heme-PRX1 were significantly reduced compared with free hemin, properties that are beneficial for cells. Taken together, our data suggest that PRX1 acts as a "shelter" for free hemin that prevents the undesirable peroxidation of biomolecules, but at the cost of diminished cysteine-dependent peroxidase activity.

2. MATERIALS AND METHODS

2.1. Materials. All chemicals were purchased from Wako Pure Chemical Industries (Japan), Nacalai Tesque (Japan) and Sigma-Aldrich (USA), and were used without further purification.

2.2. Protein Expression and Purification. A full-length *PRX1* gene construct, codon optimized for *E. coli* expression, was purchased from Eurofins Genomics (Japan) and amplified by polymerase chain reaction. The amplified fragment was cloned into the modified pET-28b vector [21] (Merck Millipore, Germany) using a Gibson Assembly kit (New England Biolabs, UK). The *PRX1* expression plasmid was transformed into the *E. coli* BL21(DE3)

strain (Nippon Gene, Japan) and cultured at 37 °C in LB broth supplemented with 50 μg/mL kanamycin. After cultures reached an optical density at 600 nm of 0.6–0.8, expression of the His-tagged fusion protein was induced with 0.4 mM isopropyl *β*-D-thiogalactopyranoside. The cells were further grown at 37 °C for 4 hours and harvested by centrifugation. The pellet was suspended in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiothreitol (DTT) at pH 8.0. The suspension was further incubated for 30 minutes at 4 °C after adding 1 mg/mL lysozyme and DNase. The sample was disrupted by sonication and then centrifuged at 40,000 × g for 30 minutes. The resulting supernatant was loaded onto a HisTrap column (GE Healthcare, Sweden) pre-equilibrated with 50 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole (pH 8.0). The bound protein was eluted with 50 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole (pH 8.0) after washing with several column volumes. After cleavage of the His_6-tag using Turbo 3C protease (Accelagen, USA), the reaction mixture was again applied to a HisTrap column and the flow-through fraction was collected. Tag-cleaved PRX1 was then applied to a HiLoad 16/600 Superdex 200 gel-filtration column (GE Healthcare) pre-equilibrated with 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The protein concentration was estimated from the absorbance at 280 nm with a molar extinction coefficient (ε_{280}) of 18,450 $M^{-1}cm^{-1}$. Cysteine residue mutations were introduced using a PrimeSTAR mutagenesis basal kit from Takara Bio (Japan). Primers used for the construction of the clone and mutagenesis are shown in Table S1.

2.3. Absorption Spectroscopy. Absorption spectra were obtained using a V-660 UV-vis spectrophotometer (JASCO, Japan). Hemin binding studies were conducted by difference absorption spectroscopy. Hemin was dissolved in 0.1 M NaOH, and its concentration was determined on the basis of absorbance at 385 nm using a molar extinction coefficient (ε_{385}) of 58.44 $mM^{-1}cm^{-1}$. Aliquots of the hemin solution (1 mM) were added to both the sample cuvette containing 10 μM apo-PRX1 and the reference cuvette at 25 °C. Spectra were

recorded 3 minutes after the addition of hemin. The absorbance difference at 370 or 371 nm was plotted as a function of heme concentration, and the $K_{d,heme}$ was calculated using the quadratic binding equation [21].

2.4. Detection of Cysteine-Dependent Peroxidase Activity. The activity of PRX1 was determined by measuring the amount of dimerization after the reaction with H_2O_2 using non-reducing SDS-PAGE. The reaction was initiated by mixing H_2O_2 (30 μM) with PRX1 (10 μM) at 25 °C, and then stopped 5 minutes after initiating the reaction by adding catalase to remove excess H_2O_2. Subsequently, 5×SDS loading buffer containing 60 mM Tris-HCl, 25% (v/v) glycerol, 2% (w/v) SDS and 0.1% (w/v) bromophenol blue (pH 6.8) was added, followed by incubation for 10 minutes at room temperature. Samples were analyzed on 12.5% polyacrylamide gels. H_2O_2 and catalase concentrations were determined spectrophotometrically using ε_{240} of 43.6 M^{-1} cm^{-1} and ε_{405} of 324 mM^{-1} cm^{-1}, respectively.

2.5. Heme Peroxidase Activity Assay. Heme peroxidase activity was determined spectrophotometrically by measuring co-oxidation of the substrate by H_2O_2 [22]. The assay was performed in 0.5 mL of reaction mixture containing 360 μM H_2O_2, 1.25 mM 4-aminoantipyrine, 86 mM phenol, and 1.5 μM hemin or heme-PRX1 at 25 °C. The reaction was initiated by adding H_2O_2, and antipyrilquinoneimine absorbance at 512 nm was monitored using a V-660 UV-vis spectrophotometer for 30 minutes at 1-minute intervals.

2.6. H_2O_2-Mediated Hemin Degradation. The hemin-degradation reaction was monitored by UV-vis spectroscopy. Following addition of 30 μM H_2O_2 to 10 μM hemin or heme-PRX1 in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4), the spectrum was recorded at 1-minute intervals for 30 minutes. Soret band peaks at 386 and 370 nm correspond to free hemin and PRX1-bound hemin, respectively. The data were normalized by subtracting the zero time

point value from subsequent time points.

3. RESULTS

3.1. Expression and Purification of PRX1. Human PRX1 was expressed in *E. coli* strain BL21(DE3) and purified. The purified PRX1 protein had an apparent molecular mass of 22 kDa and was estimated to be ~95% pure by SDS-PAGE (Fig. S2A). Three major peaks on the size-exclusion chromatogram, with elution times of 54.8, 76.7 and 88.0 minutes corresponded to a decamer, dimer and monomer, respectively, based on molecular masses estimated from the migration of bands against standard proteins (Fig. S2B). Molecular mass of the fraction eluted at 45.3 minutes was much larger than 669 kDa, indicating that this fraction is a soluble aggregate (Fig. S2B). The monomeric form of PRX1 was used in subsequent analyses, because it was a major component of the purified protein, and the dimeric form was inactive to H_2O_2 (Fig. S3A). The decameric form was highly active (Fig. S3A), but the amount was too small (Fig. S2B). Furthermore, the importance of the decameric form of PRX1 remains to be controversial. Although Prx is known as a heme-binding protein [15], the UV-vis spectrum of purified PRX1 had almost no absorption in the visible region, indicating that it was devoid of heme (Fig. S3B).

3.2. Heme-Binding Properties of PRX1. To confirm the heme-binding ability of PRX1, we performed heme-titration experiments. Difference absorption spectra obtained by subtracting the spectrum for free heme from that of PRX1-bound heme at different concentrations are shown in Fig. 1A. A plot of the absorbance difference versus heme concentration at 371 nm suggested that PRX1 binds heme with a 1:1 stoichiometry (Fig. 1A, inset). Because the titration curve was not completely saturated even in the presence of 3 equivalents of heme, the binding stoichiometry of heme to PRX1 was confirmed using the pyridine hemochrome

method, which also yielded a value of 1:1 (Fig. S4). The $K_{d,heme}$ of PRX1 for heme calculated from equation 1 was 0.17 ± 0.03 μM, which is slightly larger than that for rat HBP23 (55 nM) [15]. The difference spectrum showed a prominent peak at 413 nm. Because the plot of absorbance difference at 413 nm was monotonously increased, the emergence of this peak suggests non-specific heme binding. However, deconvolution of the Soret band of the purified heme-PRX1 after removal of excess of heme by gel filtration showed no peaks at 413 nm, indicating that the amount of the nonspecific heme binding is negligible for our experiment.

The heme-binding environment was next investigated using UV-vis absorption spectroscopy. PRX1 was reconstituted with a 1.2-fold excess of heme, and then unbound heme was removed using a gel-filtration column. Absorption spectra of heme-reconstituted PRX1 are shown in Fig. 1B. The Soret absorption maximum of ferric PRX1 was 370 nm, and the visible maxima were 521 and 653 nm. The far blue-shifted Soret peak at ~370 nm is known as a signature of a five-coordinate high-spin heme with an axial thiol ligand [23,24], as shown in Table 1, indicating that PRX1 binds heme through Cys.

3.3 Determination of the Heme-Binding Site. To specify the heme-binding residue, we performed site-directed mutagenesis of Cys. To this end, we replaced each of the four cysteine residues in PRX1 with Ala (Cys71, Cys83 and Cys173) or Ser (Cys52). Because the Ala mutant of Cys52 showed a strong tendency to aggregate, only Cys52 was replaced with Ser. Heme-titration experiments for all mutants were performed (Fig. 1C), and the difference spectra for PRX1 mutants versus free heme are shown in Fig. S5. The $K_{d,heme}$ values for PRX1 mutants C71A, C83A and C173A were 0.033, 0.050 and 0.14 μM, respectively, which are the same or slightly higher than that for wild-type PRX1. In contrast, the $K_{d,heme}$ for the C52S mutant could not be calculated owing to the drastic decrease in the absorption difference at 370 nm. These results clearly demonstrate that the heme-binding site is Cys52, which is identical to the active center of the cysteine-dependent peroxidase activity.

3.4. Effects of Cysteine-Dependent Peroxidase Activity on Heme Binding. We next investigated the effects of heme binding on the cysteine-dependent peroxidase activity of PRX1. The reaction of PRX1 with H_2O_2 results in formation of an intermolecular disulfide bond with an antiparallel conformation [25], as verified by non-reducing SDS-PAGE [26]. A gel image of PRX1 after incubation with or without H_2O_2 is shown in Fig. 2. In the absence of H_2O_2 treatment, PRX1 showed a band at ~22 kDa, whereas H_2O_2 treatment resulted in the appearance of a new band at ~40 kDa, corresponding to disulfide-bonded PRX1. This band shift suggests that PRX1 forms an intermolecular disulfide bond upon reaction with H_2O_2. The cysteine-dependent peroxidase activity of monomeric PRX1 seemed to be lower than that estimated (Fig. 2). This might be because some amount (< 40%) of the active center of monomeric PRX1 was oxidized during gel filtration. When the same assay was performed for heme-reconstituted PRX1, the 40 kDa-band did not appear, indicating that heme-PRX1 remained without a covalent bond, even after treatment with H_2O_2. These results show that heme binding to PRX1 inhibits dimer formation through the S-S bridge, implying a loss of cysteine-dependent peroxidase activity.

3.5. Functional Characterization of Heme-PRX1. The above results prompted us to examine the functional role of heme binding to PRX1. It is well known that free heme is toxic to cells owing to heme-mediated oxidation. To elucidate the significance of heme binding to Cys52 in PRX1, we first investigated H_2O_2-dependent heme peroxidase activity. Heme peroxidase activity was monitored under steady state conditions using 4-aminoantipyrine as a reducing substrate and phenol as an electron donor. The absorbance at 512 nm from the product, antipyrilquinoneimine, was plotted against time (Fig. 3A) [22]. The absorbance developed linearly for approximately 15 minutes, owing to the peroxidase activity of free hemin, as reported previously [22]. Complexation with heme reduced the heme peroxidase

activity of PRX1 by up to 55%, as shown by the limited increase in the absorbance at 512 nm. These data suggest that heme peroxidase activity is effectively inhibited in heme-reconstituted PRX1.

Although free heme is toxic to biological macromolecules, heme itself must be protected against degradation by oxidation during intracellular trafficking. Thus, we next compared the sensitivity of heme to H_2O_2-mediated degradation (Fig. 3B). The addition of H_2O_2 to free hemin resulted in a rapid decrease in the Soret band at 386 nm, as reported previously [22], indicating that hemin degradation occurred. In contrast, when hemin was complexed with PRX1, the decrease in absorbance at 370 nm was much slower than that for free hemin. Because heme-PRX1 did not exert the cysteine-dependent peroxidase activity (Fig. 2), the observed slow degradation of heme bound to PRX1 is not owing to consumption of H_2O_2 as a cysteine-dependent reaction. Thus, we supposed that the accessibility of H_2O_2 to heme is reduced upon binding to PRX1. Thus, these results indicate that PRX1 protects heme from degradation by H_2O_2.

4. DISCUSSION

4.1 Heme Coordination Environment of PRX1. Although HBP23 is frequently mentioned as a candidate of cytosolic heme-binding proteins, knowledge of its heme-binding site and the functional significance of its heme binding have been limited, motivating our characterization of the human homolog, PRX1. Heme titration experiments showed that PRX1 binds 1 equivalent of heme (Fig. 1A) and the Soret band for ferric heme-PRX1 strongly suggested Cys-coordinated environment (Fig. 1B). Indeed, a mutational study demonstrated that Cys52 is responsible for heme binding (Fig. 1C). Indeed, the $K_{d,heme}$ of PRX1 was 0.17 μM (Fig. 1A), a value within the range of cytosolic heme concentrations [27]. Such a moderate heme affinity of PRX1 suggests that PRX1 can serve as a heme transporter to cytosolic apoproteins.

The UV-vis absorption spectrum of heme-PRX1 displayed a Soret band at 370 nm, which is different from that reported previously for HBP23 (λ_{max} ≈ 408 nm) [15]. Gel-filtration chromatography showed that PRX1 exists in monomeric, dimeric, and decameric states (Fig. S2B). When decameric PRX1 was reconstituted with heme, the Soret band appeared at 415 nm (Fig. S6), implying that the previously reported Soret maximum [15] is derived from the protein in a mixture of several oligomeric forms. Such a difference indicates that oligomerization status might influence the heme-binding environment of PRX1.

Despite the presence of two CP motifs in PRX1, only Cys52 is a favorable heme-binding site. A closer look at the crystal structure of human PRX4 reveals that this preference is largely derived from structural features around Cys52 as described below (corresponding to Cys_P-SH). Importantly, Cys_P-SH forms two hydrogen bonds with the guanidinium group of Arg128 and the backbone amide group of Pro45 (Fig. S7). These hydrogen bonds cause a downward shift in the pK_a of the side chain in Cys52 from 8.5, the value for the free cysteine, to approximately 6 [28]. Such an environment would make Cys highly active even at a neutral pH. Thus, Cys52 is suitable for heme binding.

4.2. Suppression of Hemin Toxicity by PRX1. Heme-bound PRX1 loses cysteine-dependent peroxidase activity (Fig. 2), because the heme occupies the active center. This property would seem to be undesirable for PRX1 to function as an antioxidant enzyme. However, PRX1 is highly abundant in cells, with an estimated concentration of 20 μM [29,30]. In contrast, the intracellular levels of free heme must be lower than 1 μM [11], because heme oxygenase-1, with a $K_{d,heme}$ of 0.84 μM, is capable of degrading excess heme under heme-replete conditions [31,32]. Furthermore, PRX1 is extremely reactive with H_2O_2, with a rate constant of ~10^7–10^8 $M^{-1}s^{-1}$, which is comparable to that of catalase and selenium-containing glutathione peroxidase [33]. It is therefore conceivable that, given its molar excess, PRX1 has sufficient capacity for heme binding to serve an antioxidant role.

The heme-binding residue Cys52 is evolutionary conserved in all members of the Prx family. Importantly, heme is an indispensable molecule for all organisms owing to its regulatory functions. This fact allows us to speculate that scavenging heme is a common role for PRX1. Moreover, PRX1 binds heme not only to prevent it from interacting with H_2O_2, but also to suppress H_2O_2-dependent heme peroxidase activity (Fig. 3A) and H_2O_2-mediated heme degradation (Fig. 3A). This implies that PRX1 acts as "heme shelter" to protect both biomolecules and heme itself from undesired oxidation (Fig. 4). Therefore, we propose that PRX1 has a dual role, in the first case acting as a heme shelter and in the second protecting against oxidative stress through its cysteine-dependent peroxidase activity (Fig. 4).

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FIGURE LEGENDS

Figure 1: Heme titration and electronic absorbance spectra of the heme-PRX1 complex. (A) Absorption difference spectra of heme binding to PRX1 following stepwise addition of heme (2–30 μM) to PRX1 (10 μM) versus buffer blank in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). Inset: Absorbance difference at 371 nm as a function of heme concentration. (B) UV-vis absorption spectra of heme-PRX1. (C) Effects of PRX1 mutations (C52S (●), C71A (■), C83A (▲) and C173A (♦)) on heme binding.

Figure 2: Dimerization assay. Apo- or holo-monomeric PRX1 (10 μM) was treated with 30 μM H_2O_2 for 5 minutes at 25 °C in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The reaction was stopped by adding 0.1 μM catalase to quench excess H_2O_2, after which PRX1 was resolved by non-reducing SDS-PAGE and stained with Coomassie Brilliant Blue. The band at ~60 kDa corresponds to catalase.

Figure 3: Functional characterization of heme-bound PRX1. (A) Peroxidase activity of hemin (●) and heme-PRX1 (○). (B) H_2O_2-mediated degradation of hemin (●) or heme-PRX1 (○).

Figure 4: Schematic representation of the dual function of PRX1 as a cysteine-dependent peroxidase and a heme-binding protein. *RER: rough endoplasmic reticulum.

TABLE

Table 1. Absorption maxima of the heme-PRX1 complex compared with those of other heme proteins

Protein	Ligand	Soret (nm)	Visible (nm)	Reference
PRX1	Cys	370	521, 653	This study
Bach1 (Type 2)	Cys	371	521, 541, 650	[23]
Irr[a]	Cys	372	ND[e]	[24]
$P450_{cam}$ (+cam)[b]	Cys	391	ND	[34]
CBS[c]	Cys/His	428	ND	[35]
CooA	Cys/Pro[d]	424	541, 566	[36]

[a]Iron response regulator protein; [b]*d*-camphor-bound $P450_{cam}$; [c]cystathionine-β-synthase; [d]N-terminal proline binds to heme; [e]not determined.

Figure

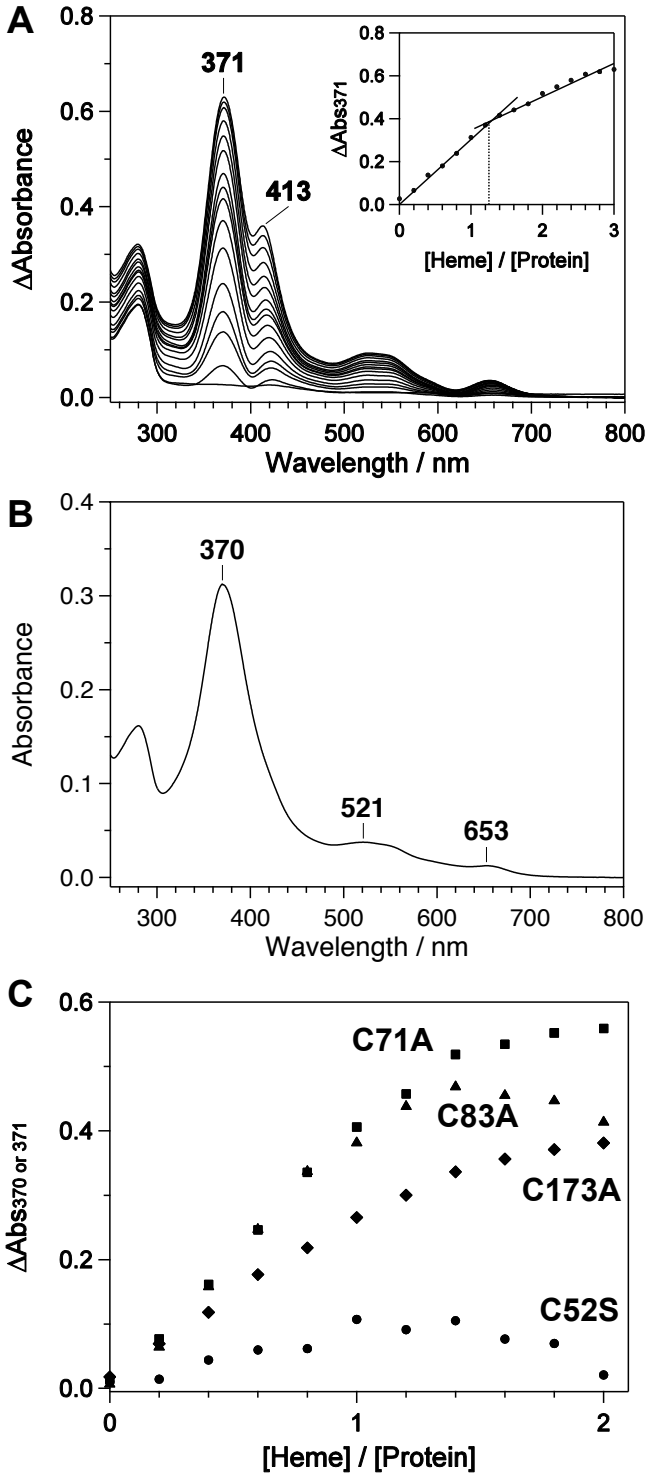

Figure 1

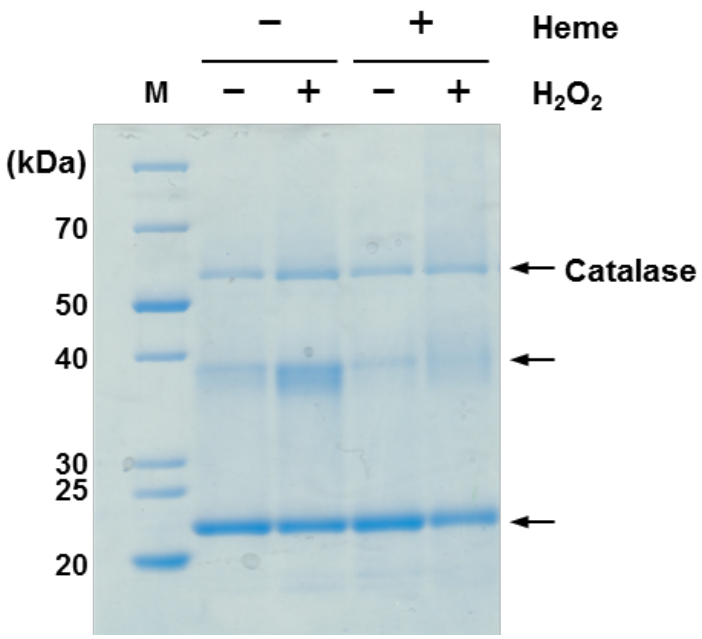

Figure 2

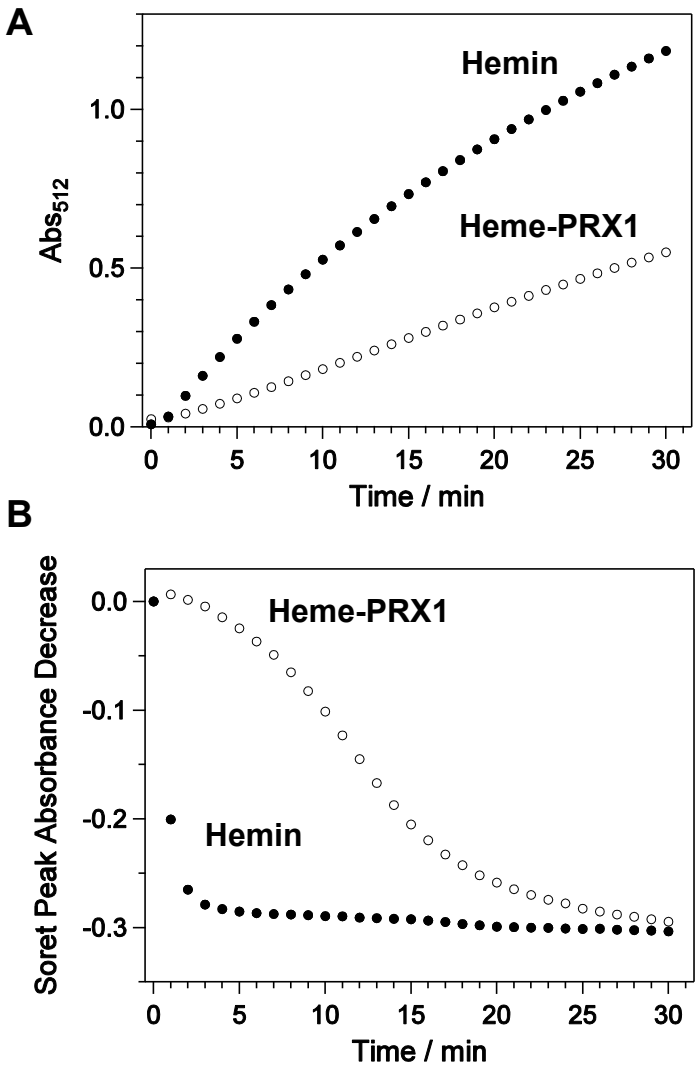
A
Hemin
Heme-PRX1
Abs512
1.0
0.5
0.0
0
5
10
15
20
25
30
Time / min
B
Heme-PRX1
Hemin
Soret Peak Absorbance Decrease
0.0
-0.1
-0.2
-0.3
0
5
10
15
20
25
30
Time / min

Figure 3

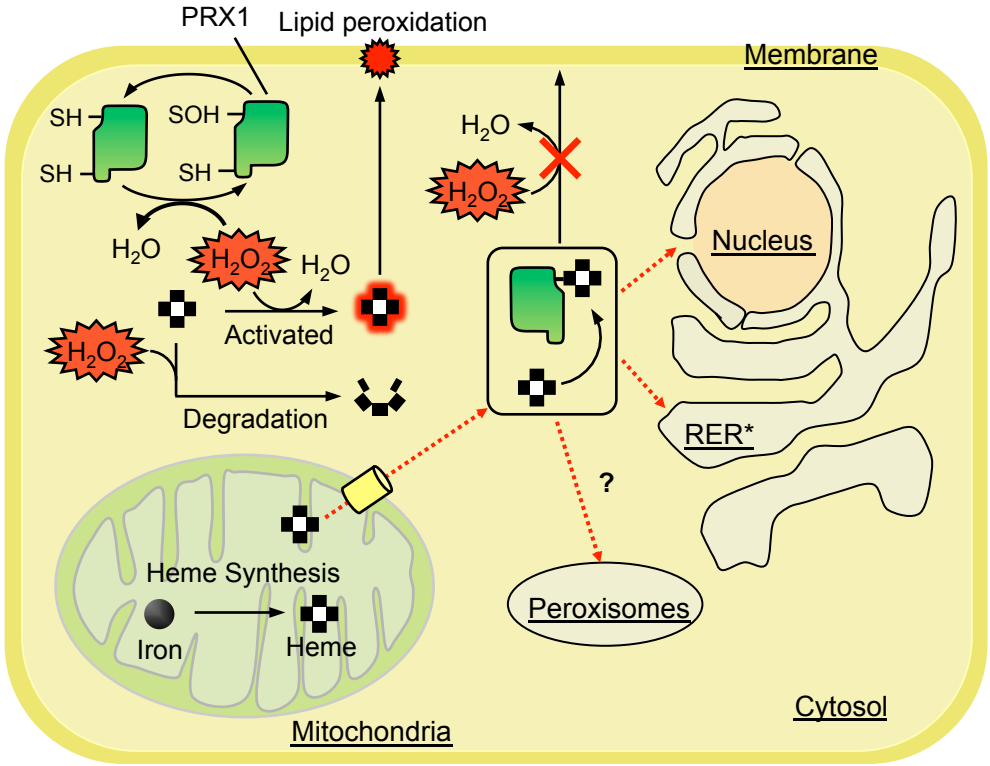
PRX1
Lipid peroxidation
Membrane
SH
SOH
SH
SH
H_2O
H_2O_2
H_2O
Activated
H_2O_2
Degradation
H_2O
H_2O_2
Nucleus
RER*
?
Heme Synthesis
Iron
Heme
Peroxisomes
Mitochondria
Cytosol

Figure 4