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Dual role of the active-center cysteine in human peroxiredoxin 1: Peroxidase activity and heme binding

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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

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

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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 h 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 min 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 min. 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 min 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 min 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 min 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 min at 1-min 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-min intervals for 30 min. 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 min 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

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