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Crystal structures of human peroxiredoxin 6 in different oxidation states

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ABSTRACT

Peroxiredoxins (Prxs) are a family of antioxidant enzymes found ubiquitously. Prxs function not only as H_2O_2 scavengers but also as highly sensitive H_2O_2 sensors and signal transducers. Since reactive oxygen species are involved in many cellular metabolic and signaling processes, Prxs play important roles in various diseases. Prxs can be hyperoxidized to the sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$) forms in the presence of high concentrations of H_2O_2. It is known that oligomerization of Prx is changed accompanying oxidation states, and linked to the function. Among the six Prxs in mammals, Prx6 is the only 1-Cys Prx. It is found in all organs in humans, unlike some 2-Cys Prxs, and is present in all species from bacteria to humans. In addition, Prx6 has Ca^{2+}-independent phospholipase A_2 (PLA_2) activity. Thus far only the crystal structure of Prx in the oxidized state has been reported. In this study, we present the crystal structures of human Prx6 in the reduced (SH) and the sulfinic acid (SO_2H) forms.

1. Introduction

Peroxiredoxins (Prxs) are a family of antioxidant enzymes found ubiquitously in various cellular compartments, such as the cytosol, nucleus, mitochondria, and endoplasmic reticulum [1]. Based on the location and the number of conserved cysteine residues involved in catalysis they are divided into three classes: typical 2-Cys Prxs, atypical 2-Cys Prxs and 1-Cys Prxs. In humans, there are six Prxs (Prx1-6): Prx1-4 and Prx5 are categorized as typical 2-Cys and atypical 2-Cys, respectively, while Prx6 is categorized as the only 1-Cys Prx [2]. They all share the same basic catalytic mechanism, in which a redox-active cysteine (peroxidatic cysteine) in the active site is oxidized to a sulfenic acid by the peroxide substrate. However, the recycling of sulfenic acid back to a thiol distinguishes the three classes. The 2-Cys Prxs contain the peroxidatic- and resolving cysteines, which form a disulfide bond to reduce the oxidized form of the peroxidatic cysteine, while the 1-Cys Prxs do not possess the resolving cysteine. In 2-Cys Prxs, thioredoxin is the reductant while the 1-Cys Prxs are reduced by glutathione in the presence of glutathione *S*-transferase π [3]. Both 2-Cys Prxs and 1-Cys Prxs can be hyperoxidized to the sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$) forms in the presence of high concentrations of H_2O_2. However, while the hyperoxidized forms of 2-Cys Prxs can be reversed to restore peroxidase activity by the ATP-dependent enzyme sulfiredoxin, the hyperoxidation of 1-Cys Prxs cannot be restored [4]. Prxs are known to function not only as H_2O_2 scavengers but also as highly sensitive H_2O_2 sensors and signal transducers. Because reactive oxygen species are involved in many cellular metabolic and signaling processes, Prxs play important roles in various diseases, particularly in inflammation, cancer, and neurodegenerative diseases [5].

Structural studies have shown that all Prxs contain a thioredoxin fold with insertions forming additional secondary-structural elements. The peroxidatic cysteine, in the highly conserved Pro-$(Xxx)_3$-Thr/Ser-$(Xxx)_2$-Cys motif present in all Prx classes, is located in a narrow solvent-accessible pocket surrounded by highly conserved residues. While atypical 2-Cys Prx exists as a monomer, all other Prxs, *e.g.* typical 2-Cys Prxs and 1-Cys Prx, are found as homodimers with the C-terminus of one subunit reaching across the dimer interface to interact with the other subunit. Interestingly, some typical 2-Cys Prxs were found to form toroid-shaped complexes with a pentameric arrangement of dimers [6]. Oligomerization is reported to be redox-sensitive; either the reduced or over-

Abbreviations: Prx, peroxiredoxin; *h*Prx, Prx from human; GSH, glutathione; GST, glutathione *S*-transferase; PLA_2, phospholipase A_2; MR, molecular replacement.

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oxidized form of the enzyme favors the decameric state [7], and other factors such as post-translational modifications, ionic strength, and pH have been reported to affect the equilibrium as well. For example, in the case of Prx1, phosphorylation at Thr90 was reported to induce the high-molecular weight complex by exposing the hydrophobic region [8], and a recent study showed that conformational changes occur near the peroxidatic cysteine associated with pH-induced decamerization in Prx1 [9]. The oligomeric state of the structure is linked to the chaperone activity [10].

Prx6, the only 1-Cys Prx in mammals, is found in all organs in humans, unlike some 2-Cys Prxs, and is present in all species from bacteria to humans [11]. In addition, Prx6 is unique because it has Ca^{2+}-independent phospholipase A_2 (PLA_2) activity; Prx6 can reduce oxidized phospholipids at neutral pH and hydrolyze phospholipids at acidic pH [12]. The phospholipid oxidized by H_2O_2 binds to Prx6 at cytosolic pH ranges of 7.0–7.4, whereas the reduced phospholipid binds to Prx6 at a maximum of pH 4 [13]. Phospholipase A_2 activity is reportedly increased dramatically in hyperoxidation states [14]. Therefore, Prx6 is bifunctional, showing peroxidatic activity involving Cys47 as well as PLA_2 activity involving Ser32, His26, and Asp140 [15]. In the case of Prx6, the crystal structure of only the oxidized state was reported previously (PDB code: 1PRX) [16]. Because Prx6 has similar structural features as Prx1, possible oligomerization was suggested for Prx6 [11].

In this study, we present the crystal structures of human Prx6 in the reduced (SH) and the sulfinic acid (SO_2H) forms.

2. Materials and methods

2.1. Expression and purification of human Prx6

Human Prx6 cDNA (SwissProt entry P30041) was amplified by PCR using the forward primer 5′-GCCGTGACGGAATTCATGCCCG-GAGGTCTGCTTC-3′ (EcoRI site underlined) and reverse primer 5′-GCTAGGCATCTCGAGTCAAGGCTGGGGTGTGT- AG-3′ (XhoI site underlined). The PCR product was cloned into the *Escherichia coli* expression vector pET28a (Novagen, Madison, WI, USA) containing a hexahistidine tag to the N-terminus. The recombinant vector was transformed into the *E. coli* BL21 (DE3) expression strain. A single colony of transformants was selected and inoculated in Luria–Bertani medium containing ampicillin (50 μg mL^{-1}). The cells were grown at 37 °C until the optical density reached $OD_{600} = 0.6$ and were then induced with 1 mM isopropyl-β-*d*-thiogalactopyranoside. Cell growth continued for 20 h at 18 °C before harvesting the cells by centrifugation and resuspension in lysis buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol). After sonication and centrifugation, the supernatant containing 6X His-tagged Prx6 was loaded onto a nickel-nitrilotriacetic acid affinity chromatography (GE Healthcare Life Sciences, Little Chalfont, UK) equilibrated with 50 mM Tris at pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol. Proteins were further purified by gel filtration chromatography on a Superdex 75 26/60 column (GE Healthcare Life Sciences) equilibrated with 20 mM HEPES at pH 7.0, 2 mM EDTA, and 1 mM 1,4-dithio-DL-threitol. The sulfinic acid form was prepared by adding 10 mM H_2O_2 to the purified Prx6 followed by final gel filtration with 20 mM HEPES at pH 7.0, 2 mM EDTA and 1 mM 1,4-dithio-DL-threitol.

2.2. MALDI-TOF/MS analysis

In order to confirm the oxidation state of the prepared Prx6 samples, we carried out mass analysis by MALDI-TOF/MS (Voyager DE-PRO, Applied Biosystems, Foster City, CA, USA). Both samples were loaded onto an SDS-15% (w/v) polyacrylamide gel and the bands were cut from the gel. The gel slices were washed three times with 25 mM ammonium bicarbonate and 50% acetonitrile solution for 1 h and dried prior to digestion by trypsin (12.5 ng μL^{-1}) (Promega) in 25 mM ammonium bicarbonate for 16 h at 37 °C. The peptides from the gel were extracted with 60% acetonitrile solution containing 0.5% trifluoroacetic acid. After evaporation of the solvent, the peptides were dissolved in 0.05% trifluoroacetic acid and 5% acetonitrile solution. One microliter of the peptide mixture was mixed with the same volume of the matrix solution of 5 mg mL^{-1} α-cyano-4-hydroxy-cinnamic acid solution in 50% acetonitrile containing 0.1% trifluoroacetic acid, and the mass values of peptides were determined. The results are shown in Fig. 1.

2.3. Crystallization, data collection and processing

Initial screening for the crystallization condition was performed using a Hydra II Plus One (MATRIX Technology) robotics system and 96-well Intelli plates (Hampton Research, Aliso Viejo, CA, USA) at 295 K, and the hits were further optimized using the hanging drop method. Diffraction-quality crystals for the reduced and sulfinic acid forms were obtained by mixing equal volumes of 22 mg mL^{-1} of Prx6 with a reservoir solution containing 100 mM magnesium formate, 18% (v/v) polyethylene glycol 3350, and 100 mM HEPES at pH 7.5 and 20% (v/v) PEG 8000, respectively. The X-ray diffraction data for the two forms were collected at 100 K in a liquid nitrogen stream using synchrotron radiation (Pohang Light Source, Pohang, Korea). Both crystals belonged to the space group $P2_12_12_1$ with six molecules per asymmetric unit and had unit cell dimensions of a = 94.353 Å, b = 106.349 Å, c = 165.504 Å, and $\alpha = \beta = \gamma = 90°$ for the reduced form and a = 94.419 Å, b = 106.539 Å, c = 167.915 Å, and $\alpha = \beta = \gamma = 90°$ for the sulfinic acid form. Crystals diffracted to 2.5 Å and 2.9 Å resolution, respectively. The data were processed and scaled using the HKL2000 program suite [17]. The statistics are summarized in Table 1.

2.4. Structure determination and refinement

The reduced and sulfinic acid form structures were solved by molecular replacement method using the program MOLREP of the CCP4 suite using Prx 6 from *Arenicola marina* (PDB code: 2V2G) as a search model. All attempts using human Prx6 (PDB code: 1PRX) as the search model failed to yield a solution. Model building was conducted using the programs Coot and refined with the programs CNS [18], REFMAC [19], and PHENIX [20]. After manual rebuilding, the water molecules were placed into the electron density map, resulting in the final model. The stereochemical quality of the final model was confirmed using the program PROCHECK [21]. Figures were prepared using the program PyMOL [22]. The coordinates have been deposited in the Protein Data Bank (see Table 1).

3. Results and discussion

3.1. Overall structure of Prx6

Crystal structures of human Prx6 in the reduced and sulfinic acid forms were determined and refined to final R-values of 23.5% (R_{free} = 29.3%) and 25.3% (R_{free} = 27.1%) at 2.5 and 2.9 Å resolution, respectively. Statistics on data collection and refinement are summarized in Table 1. MALDI-TOF analysis showed that the two samples prepared were in two different states with peaks at 1339.5884 and 1371.6680 for the reduced and sulfinic acid form, respectively, as shown in Fig. 1*A*. The electron density maps calculated using atoms within an 8.0 Å radius and Cys47 omitted show clear density of the reduced and the sulfinic acid form of the catalytic Cys47 (Fig. 1*B*).

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