Human Peroxiredoxin 1 and 2 Are Not Duplicate Proteins

THE UNIQUE PRESENCE OF CYS\textsuperscript{83} IN Prx1 UNDERSCORES THE STRUCTURAL AND FUNCTIONAL DIFFERENCES BETWEEN Prx1 AND Prx2*”

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Human peroxiredoxins 1 and 2, also known as Prx1 and Prx2, are more than 90% homologous in their amino acid sequences. Prx1 and Prx2 are elevated in various cancers and are shown to influence diverse cellular processes. Although their growth regulatory role has traditionally been attributed to the peroxidase activity, the physiological significance of this function is unclear because the proteins are highly susceptible to inactivation by H\textsubscript{2}O\textsubscript{2}. A chaperone activity appears to emerge when their peroxidase activity is lost. Structural studies suggest that they may form a homodimer or doughnut-shaped homodecamer. However, little information is available whether human Prx1 and Prx2 are duplicative in structure and function. We noted that Prx1 contains a cysteine (Cys\textsuperscript{83}) at the putative dimer-dimer interface of decameric Prx1. These findings are consistent with the hypothesis that human Prx1 and Prx2 possess unique functions and regulatory mechanisms and that Cys\textsuperscript{83} bestows a distinctive identity to Prx1.

Human Prx1\textsuperscript{2} and Prx2 are highly homologous members of the peroxiredoxin protein family (1). Prx1 and Prx2 are shown to be elevated in several human cancers. Prx1 is elevated in oral, esophageal, pancreatic, follicular thyroid, and lung cancers (2–10). Both Prx1 and Prx2 are elevated in mesothelioma, breast, and head-and-neck cancers (11–14). Elevation of Prx1 correlates with resistance to chemotherapy in breast cancer (15). In contrast, down-regulation of Prx1 has been shown to sensitize lung cancer cells to radiation and reduce metastasis of lung cancer xenografts (16). Similarly, overexpression of Prx2 renders leukemia and stomach cancer cells resistant to chemotherapeutic agents (17, 18), and down-regulation of Prx2 sensitizes head-and-neck cancer cells to radiation (14) and gastric carcinoma cells to cisplatin (19). These studies suggest that both Prx1 and Prx2 confer resistance to therapy and promote an aggressive survival phenotype of the cancer cells.

The cell survival enhancing functions of Prx1 and Prx2 are generally attributed to their H\textsubscript{2}O\textsubscript{2} scavenging activities. The H\textsubscript{2}O\textsubscript{2} catalytic mechanism of Prx is unique among the peroxide-detoxifying enzymes. For example, glutathione peroxidase reduces peroxides while oxidizing glutathione as a co-substrate. The H\textsubscript{2}O\textsubscript{2} eliminating action of Prx1, on the other hand, is mediated by a transient inter-molecular disulfide bond formation between the catalytic Cys\textsuperscript{52} of one Prx1 molecule and the Cys\textsuperscript{173} residue of another Prx1 molecule (1). Likewise, the H\textsubscript{2}O\textsubscript{2} catalytic action of Prx2 is mediated through a disulfide bond formation between Cys\textsuperscript{51} and Cys\textsuperscript{172}. The formation of an inter-molecular disulfide bond between Prx1 and Prx2 does not appear to occur. Transiently formed disulfide bond is reduced back to Cys-SH by the action of disulfide oxidoreductase, thioredoxin. The Cys\textsuperscript{173} residue of Prx1 (Cys\textsuperscript{172} in Prx2) has traditionally been referred to as a “resolving” Cys. This is based on the fact that the transient inter-molecular disulfide bond formation between the Cys\textsuperscript{73/172}-SH of one Prx molecule and the Cys\textsuperscript{52/51}-SOH of another Prx molecule engaged in peroxide catalysis is a necessary step for reduction of the Cys\textsuperscript{52/51}-SOH back to Cys\textsuperscript{52/51}-SH. Despite the initial biochemical characterization of Prxs as peroxide-removing enzymes, the physiological significance of their peroxidase activity is unclear, because the catalytic Cys of a Prx is highly susceptible to loss of activity as a result of its overoxidation to sulfenic (–SO\textsubscript{2}H) or sulfonic acid.
acid (–SO₃H) during peroxide detoxification (20–22). When the catalytic Cys is overoxidized, the peroxidase activity is lost.

Structural studies from various species suggest that Prx proteins may exist as an α₂ homodimer or a doughnut-shaped (α₂)₅ homodecamer (23–25). Two molecules of reduced Prxs form an α₂ homodimer, which in turn serves as a building block for decamer formation. According to x-ray crystallographic studies, the inter-molecular disulfide bond that occurs transiently during the catalytic cycle requires local unfolding because the distance between the catalytic Cys and the resolving Cys is too far apart at ~13 Å (24, 25). The latter effect destabilizes the dimer-dimer (DD) interface and, by extension, the decameric structure. The stability of the DD interface is quickly restored when the disulfide bond is reduced back to Cys-SH by thioredoxin. This completes a cycle of H₂O₂ catalysis by the Prx protein. If the mechanics of unfolding or the conditions for disulfide formation are unfavorable for various reasons, the catalytic Cys becomes overoxidized to sulfenic (–SO₂H) or sulfonic acid (–SO₃H) and loses its H₂O₂ catalytic activity. The DD interface of an overoxidized Prx dimer, however, appears to be more stable than that of a fully reduced Prx dimer, and it may in fact lead to a more compact decamer (23–25). Recent studies suggest that high molecular weight Prxs may function as a molecular chaperone (26–28). The information above suggests that the redox status of the catalytic Cys and the molecular chaperone activities of Prxs may be closely linked.

Human Prx1 and Prx2 are 91% homologous and 78% identical in their amino acid sequences. Are Prx1 and Prx2 duplicate proteins or do they have unique functions and regulatory mechanisms? Although Prx1 and Prx2 have been studied independently in a number of cell and animal systems, little information is available regarding this fundamental question. Studies of Prx1 knock-out (KO) and Prx2KO mice illustrate the complexity of addressing this deceivingly simple question. One of the major phenotypes of Prx1KO was uncontrolled cell proliferation and the development of tumors in certain cell types (29). This striking observation led to the suggestion that Prx1 may act as a tumor suppressor. This is contradictory to the proposed role of Prx1 in promoting cell survival, treatment resistance, and malignant progression of various cancer cells (1–16). Absence of Prx2, on the other hand, resulted in splenomegaly and malignant progression of various cancer cells (1–16). Absence of Prx1, on the other hand, resulted in splenomegaly and malignant progression of various cancer cells (1–16). Prx2KO mice. Obviously, reduced peroxide removal resulting from the absence of either Prx1 or Prx2 cannot offer an answer to the drastically different phenotypes observed in these studies. The above information and findings from various human cancer cells and tissues, however, clearly indicate the following: 1) Prx1 and Prx2 may not be identical in function and are distinguishable by their regulatory mechanisms; 2) maintaining an adequate level of these proteins may be important in keeping normal cell proliferation/apoptosis in check; 3) the functions of Prx1 and Prx2 may be regulated in a cell type- and tissue context-dependent manner; and 4) their functions would need to be tested (and interpreted) in light of the various genetic and environmental factors that could affect the molecular behavior of Prx1 and Prx2.

When we examined the amino acid sequences of human Prx1 and Prx2, we noted that in addition to the well characterized catalytic Cys (Cys²² in Prx1 and Cys²¹ in Prx2) and resolving Cys (Cys¹⁷³ in Prx1 and Cys¹⁷² in Prx2) residues, there is a Cys⁸³ that is unique to Prx1 and not found in Prx2 (Fig. 1). According to available structural information from various Prx species (24, 25), the Cys⁸³ of human Prx1 appears to be located at the putative DD interface. The goal of this study was to investigate whether there is a functional and/or structural difference between human Prx1 and Prx2 at the protein level, and if so, whether Cys⁸³ in Prx1 contributes to the difference. Our approach was to employ defined experimental systems and to compare the molecular behaviors of these two proteins side by side. We purified recombinant human Prx1 and Prx2 proteins and evaluated their peroxidase and molecular chaperone activities. We also examined the biochemical and structural characteristics of the Cys⁸³ → Ser substituted Prx1 (Prx1C83S) with those of wild type Prx1 and Prx2. Based on the results from a combination of mutagenesis, biochemical, computer modeling, mass spectrometry, and x-ray crystallographic studies, we conclude that Prx1 and Prx2 are not redundant proteins and that the Cys⁸³ of Prx1 plays a critical role in bestowing a distinctive identity on Prx1, which is not shared by Prx2.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dithiothreitol (DTT), iodoacetamide (IAA), NADPH, hydrogen peroxide, citrate synthase, and insulin were obtained from Sigma. Tris-(2-carboxyethyl)phosphine hydrochloride was from Invitrogen. Malate dehydrogenase (MDH) was from Roche Applied Science.
Expression and Purification of Recombinant Proteins—The Prx1/pET-17b and Prx2/pET-17b vectors containing the entire coding regions of human Prx1 and Prx2 were constructed. Site-directed mutagenesis of Cys46 to Ser43 was performed to generate the PrxC83S with the QuikChange mutagenesis kit (Stratagene) using the Prx1/pET-17b as a template. Recombinant human Prx1, Prx2, and PrxC83S proteins were purified by sequential ion exchange chromatography and size exclusion chromatography as described previously (31). Briefly, the cell extract was loaded onto DEAE-Sepharose (GE Healthcare) and equilibrated with 20 mM Tris-Cl (pH 7.5). The proteins were dialyzed with 50 mM sodium phosphate buffer (pH 6.5). The dialyzed proteins were loaded onto SP-Sepharose (GE Healthcare) and equilibrated with 50 mM sodium phosphate buffer (pH 6.5). The bound proteins were eluted with a linear gradient of sodium chloride. The fractions containing Prx1, Prx2, or PrxC83S were pooled and stored at −80 °C. Unless otherwise specified, all proteins were treated with 0.5 mM DTT for 30 min prior to their use. DTT was removed with Hitrap desalting column (5 mL; GE Healthcare). Full-length clones of yeast thioredoxin (γTrx) and yeast thioredoxin reductase (γTrR) were obtained from yeast genomic DNA (Clontech). The γTrx and γTrR proteins were purified as described previously (32).

Peroxidase Activity Assay—The thioredoxin-dependent peroxidase activity of the purified Prxs was measured as described previously with minor modifications (32). A total of 1.1 μg each of Prx1, Prx2, or PrxC83S was incubated in 50 mM Hepes (pH 7.0) containing 200 μM NADPH, 3 μM γTrx, and 1.5 μM γTrR. The reaction mixture was incubated at 30 °C for 5 min, followed by the addition of a 10-μl aliquot of H2O2 at various concentrations. NADPH oxidation was monitored for the next 10 min by monitoring the absorbance at 340 nm. The reaction was terminated with the addition of 0.6 mM IAA to block the residual SH group. The reaction was allowed to go in the dark for 5 min and then quenched by adding 100 nmol of Tris(2-carboxyethyl)phosphine hydrochloride. An aliquot of 100 ng of Prx from each reaction mixture was taken and subjected to Western blot analysis with Prx1, Prx2, or Prx-SO2/H2O2 antibodies (Lab Frontier Life Science Institute, Seoul, Korea).

Gel Filtration Chromatography—A total of 0.45 mg each of Prx1, PrxC83S, or Prx2 protein was loaded onto a Superdex 200 column (1.6 × 60 cm) equilibrated with buffer containing 50 mM sodium phosphate, pH 7.0, and 0.1 M NaCl. Thymoglobulin (699 kDa), aldolase (150 kDa), bovine serum albumin (67 kDa), and bovine trypsinogen (24 kDa) were used as molecular weight standards.

Intrinsic Tryptophan Fluorescence Spectroscopy—Intrinsic Trp fluorescence spectra were recorded using an LB-45 spectrofluorometer (PerkinElmer Life Sciences) with an excitation wavelength of 295 nm. Samples of Prx1, PrxC83S, or Prx2 protein were used at a concentration of 10 μg/ml in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM NaCl. The excitation and emission band passes were set at 3 nm. Spectra were monitored from 300 to 500 nm at room temperature.

Homology Modeling—A model of decameric human Prx1 was built based on the atomic structure of well-characterized bacterial Prx protein, AhpC (Protein Data Bank code 1YEP). A molecular operating environment (version 2005.06, Chemical Computing Group, Montreal, Canada) running on a G5 dual 2.7-GHz PowerPC workstation was used for homology modeling. The best intermediate model was energy-minimized to remove unfavorable van der Waals contacts. PyMOL (34) was used for analysis and illustration purposes.

Intact Protein Analysis by MALDI-TOF Mass Spectrometry—Samples of Prx1, PrxC83S, or Prx2 in water, 0.1% trifluoroacetic acid were mixed with a solution of 3,5-dimethoxy-4-hydroxycinnamic acid, sinapinic acid matrix. Five pmol each of the proteins was applied to a sample plate. The MALDI microMX™ mass spectrometer (Waters, Milford, MA) was operated in positive linear mode (post acceleration dynode, 5 kV; sample period, 1 ns). Data acquisition was performed over the m/z ranges of 25,000–250,000. Mass spectra were calibrated by the default method and verified by using bovine serum albumin for external calibration.

In-solution Digestion of Recombinant Proteins with Trypsin—Samples of Prx1, PrxC83S, or Prx2 proteins were diluted to 1 μM concentration with 10% acetonitrile, 40 mM ammonium bicarbonate buffer. After brief sonication to dissolve the samples, 200 μl each of protein solution was transferred to Eppendorf tubes. For some experiments, the protein solution was reduced with 20 μl of DTT (50 mM in 10% acetonitrile, 40 mM ammonium bicarbonate buffer) at 60 °C for 30 min. After it was cooled to room temperature, iodoacetamide solution was added (20 μl, 200 mM in 10% acetonitrile, 40 mM ammonium bicarbonate buffer), and the reaction mixture was incubated at room temperature for 30 min in the dark. A solution of sequencing grade trypsin (10 μg of trypsin dissolved in 500 μl of 10% acetonitrile, 40 mM ammonium bicarbonate buffer) was added to the mixture to achieve an enzyme/protein ratio of 1:10 (w/w). The solution was digested at 37 °C for 16 h.

MALDI-TOF Analysis of Trypsin-digested Peptides—After trypsin digestion, the peptide mixtures were dried in a Speedvac.
concentrator and dissolved in water containing 0.1% trifluoroacetic acid. Samples were mixed 1:1 (v/v) with 10 mg/ml α-cyano-4-hydroxycinnamic acid in 400:400:200 (v/v) acetonitrile/ethanol/water. A total of 1.3 µl of sample/matrix mixture was spotted onto a MALDI target plate, allowed to dry in air at room temperature, and analyzed on a MALDI micro MX™ mass spectrometer. The machine was operated in a positive reflectron mode with an accelerating voltage of 25 kV, and a mass spectrometer. The machine was operated in a positive ion mode with a source temperature of 120 °C and a cone voltage of 45 eV in V-mode. The collision energies were set at 10 and 30 V for MS and MS/MS scans, respectively. The MS/MS spectra were obtained in a data-dependent acquisition mode.

**RESULTS**

Comparison of Peroxidase and Molecular Chaperone Activities of Prx1 and Prx2—To compare the H₂O₂ catalytic activities of human Prx1 and Prx2, the standard peroxidase reaction was carried out by using the yeast Trx system (yTrx, yTR, and...
NADPH) as an electron donor. The activities were evaluated by monitoring the decrease in absorbance at 340 nm ($A_{340}$) because of the oxidation of NADPH. As shown in Fig. 2, A and B, the peroxidase activities of both Prx1 and Prx2 diminished progressively in a time-dependent manner as the concentration of $H_2O_2$ was increased from 0.2 to 2.0 mM, as evidenced by the increases of $A_{340}$ values. However, the inactivation of Prx1 was much more severe than that of Prx2 at all $H_2O_2$ concentrations. The peroxidase activity of Prx2 was fairly well maintained with a concentration of $H_2O_2$ as high as 2.0 mM. Even at 600 s after starting the reaction, Prx2 was still capable of reducing $H_2O_2$, as evidenced by the appreciable decrease of an $A_{340}$ value. Although the activities of Prx1 at 0.2 mM and 2.0 mM $H_2O_2$ were reduced to 36.5 ± 0.4 and 24.8 ± 0.2%, respectively, Prx2 activities remained at levels of 55.8 ± 1.1 and 35.7 ± 0.6% at those same respective concentrations of $H_2O_2$. In fact, the activity of Prx2 at 2.0 mM $H_2O_2$ was comparable with that of Prx1 at 0.2 mM $H_2O_2$. The degree of inactivation of Prx1 was greater than that of Prx2 at all concentrations with a p value of less than 0.001. The greater sensitivity of Prx1 to $H_2O_2$-induced inactivation was confirmed by probing with a Prx-SO$_2$H antibody that specifically recognizes the overoxidized (i.e. inactivated) catalytic Cys. Although the sulfenic/sulfonic (-SO$_2$/3H) form of Prx1 was detectable at all concentrations of $H_2O_2$, the overoxidation of Prx2 was visible only at a level of $H_2O_2$ greater than 1 mM (Fig. 2C).

Next, we compared the molecular chaperone activities of Prx1 and Prx2 by using MDH as a substrate. As shown in Fig. 3A, Prx1 displayed a robust capacity to suppress the thermal aggregation of MDH. The molecular chaperone activity of Prx1 increased in a concentration-dependent manner, peaking at 12 $\mu$M. In contrast, the chaperone activity of Prx2 was considerably less than that of Prx1 on an equimolar basis (Fig. 3B). Similar results were obtained when citrate synthase or insulin was used as a substrate (data not shown).

Effect of Cys$^{83}$ to Ser$^{83}$ Substitution of Prx1 on Peroxidase and Molecular Chaperone Activities—To investigate a possible role of Cys$^{83}$ in influencing the function of Prx1, the Cys$^{83}$ residue of Prx1 was replaced by Ser$^{83}$. Fig. 4A shows the peroxidase activity of Prx1C83S in the presence of increasing concentrations of $H_2O_2$. It is clear that the peroxidase function of Prx1C83S was robust and not as easily inactivated as the wild type Prx1 during $H_2O_2$ catalysis. In contrast to the wild type Prx1, a significant peroxidase activity was observed in Prx1C83S at all $H_2O_2$ concentrations. The activity of Prx1C83S at 2.0 mM $H_2O_2$ remained as high as 47 ± 1.0% at 600 s after starting the reaction. The peroxidase activities of Prx1 at 1.0 and 2.0 mM $H_2O_2$ are shown for comparison; the peroxidase activities of Prx1C83S were significantly greater than those of wild type Prx1 at both concentrations ($p < 0.001$). The resistance of Prx1C83S to overoxidation by $H_2O_2$ was confirmed by the negative Western blot data with a Prx-SO$_2$/3H antibody (Fig. 4B). Prx1C83S also appeared to have completely lost its ability to suppress the thermal aggregation of MDH (Fig. 4C). The chaperone activities of wild type Prx1 at 8 and 12 $\mu$M are shown for comparison.

The Oligomeric Status of Prx1 Differs Significantly from That of Prx2 and Prx1C83S—The results above suggest that the molecular properties of Prx1C83S are significantly changed from those of the wild type Prx1, and in a direction that resembles the characteristics of Prx2. Because the structure of Prxs may be closely linked to the peroxidase and molecular chaperone activities, we next carried out gel filtration chromatography to examine the oligomeric status of Prx1, Prx1C83S, and Prx2. The elution profile of Prx1C83S and Prx2 was significantly different from that of Prx1 (Fig. 5A). The position of Prx1 at its peak corresponded approximately to a molecular mass of 340 kDa, which is somewhat greater than the expected molecular mass (220 kDa) of a decameric structure. The position of Prx1C83S was similar to that of Prx2. The apparent molecular masses of Prx1C83S and Prx2 at their peaks were 57 and 67 kDa, respectively. These values were also slightly higher than the expected molecular mass of a dimer (44 kDa). Considering that hydrodynamic radius influences the elution pattern of a protein on gel filtration chromatography, it is likely that the differences between the expected and observed molecular masses resulted from the differential compactness of these proteins in solution. Nonetheless, the results strongly suggested the presence of differences in the oligomerization characteristics of Prx1 compared with that of Prx1C83S or Prx2.

We also examined the intrinsic tryptophan fluorescence profiles of Prx1, Prx1C83S, and Prx2 (Fig. 5B). The intensities of the Trp fluorescence spectra of Prx1 were much lower than those of
Prx1C83S and Prx2. Lower intensities in the Prx1 Trp spectra are indicative of a greater propensity to form an oligomeric structure (38). Consistent with the result obtained from the gel filtration study, the Trp fluorescence spectra indicated that the tertiary packing property of Prx1 is significantly different from that of Prx1C83S or Prx2.

Molecular Mass Determination by Mass Spectrometry Reveals That Prx1 Is Present as a Decamer, whereas Prx2 and Prx1C83S Are Primarily in a Dimeric Form—MALDI-TOF mass spectrometry was employed to determine the molecular masses of the intact Prx1, Prx2C83S, and Prx2 proteins in high resolution. The molecular mass/charge (m/z) values of 219,746.17, 110,201.47, 73,554.93, and 55,373.06 represent the singly, doubly, triply, and quadruply charged Prx1 decamer, respectively, further corroborating that Prx1 is present predominantly as a decamer (Fig. 6A). The minor peaks at m/z of 44,202.26 and 882,902.29 represent the singly charged Prx1 dimer and tetramer ion, respectively. We found that the decamer signature of Prx1 is completely lost in Cys83 to Ser83-substituted Prx1. As shown in Fig. 6B, Prx1C83S is present predominantly in a dimeric form. The intense signal shown at m/z value of 44,000.22 represents a Prx1C83S dimer with a charge state of +1. The weak signal of the Prx1C83S tetramer ion is detected at m/z value of 88,286.76. Consistent with the functional similarity between Prx1C83S and Prx2, Prx2 is also present primarily as a dimer (Fig. 6C). The intense peak at m/z of 43,627.27 is a Prx2 dimer with a charge state of +1. Similar to Prx1C83S, a weak signal of the Prx2 tetramer ion (m/z 87,373.28) was also detected. The molecular mass determination of intact protein by MALDI-TOF mass spectrometry is highly accurate. The % errors of all the experimental m/z values were less than 0.5% of the theoretical values (Table 1), except for the singly charged Prx1 decamer ion in which the % error of experimental mass is 0.55%.

The Cys83 of Prx1 Is Located at the Dimer-Dimer Interface—To gain insight as to how Cys83 may impact on the structural and functional characteristics of Prx1, we carried out a homology modeling study. Because the crystal structure of human Prx1 has not been determined, the atomic structure of the well characterized bacterial Prx, AhpC (Protein Data Bank code 1YEP), was used as a basis for homology modeling. Our modeling results indicated that the
DD interface of human Prx1 decamer contains a local 2-fold symmetry axis close to Cys\(^{83}\) of each peptide chain. The Cys\(^{83}\) residue in either \(x_1 = -180^\circ\) or \(x_1 = -60^\circ\) conformation packs at the DD interface in a manner similar to the packing of AhpC. The distance between the two Cys residues is at (Cys\(^{83}\) at \(x_1 = -180^\circ\)) or slightly above (Cys\(^{83}\) at \(x_1 = -60^\circ\)) the sum of van der Waals radii, thus ensuring a tight fit and perhaps entropic gain because of the burial of hydrophobic surfaces. Fig. 7 illustrates the DD interface of the Prx1 decamer model with the Cys\(^{83}\) side chain in \(x_1 = -180^\circ\) conformation. The presence of the Asp\(^{79}\) and Asp\(^{47}\) pairs at the DD interface does not appear to be destabilizing, because the side chains are likely be shielded by water molecules at the interface as is the case with the AhpC crystal structure. Consequently, repulsive negative charges at the DD interface or energy costs for burial of charged side chains are probably of little concern.

The Cys\(^{83}\) Residue of Prx1 Is Oxidized under an Ambient Atmosphere—Because of the specific location of Cys\(^{83}\) at the DD interface, we questioned whether the disulfide bond between the dimer units contributes to the preferential decamer structure of Prx1. Although the distance between the two dimer Cys\(^{83}\) sulfur atoms (2.42 Å) is slightly longer than the ideal disulfide bond distance of 2.03 Å (39), a Cys\(^{83}\)–Cys\(^{83}\) disulfide bond appears to be plausible with some adjustment to the backbone conformation in this region. We looked for a Cys\(^{83}\)–Cys\(^{83}\)-containing peptide ion or the MS/MS fragments of such ion by using MALDI-TOF and Q-TOF mass spectrometry, respectively. We did not find corresponding molecular masses under the experimental conditions that we tested. Nonetheless, when we analyzed the tryptic digests of Prx1 by MALDI-TOF, the 68–92 peptide that contains the reduced Cys\(^{83}\) was not detected, indicating that Cys\(^{83}\) may be oxidized under an ambient atmosphere. As shown in Fig. 8A, except for the 68–92 residue, the Cys\(^{83}\) containing the 38–62 peptide ion (\(m/z\) 3036.7607) and the Cys\(^{173}\) containing the 169–190 peptide ion (\(m/z\) 2349.2420) were clearly detected. The fact that the 68–92 ion (\(m/z\) 2751.6653) was clearly detected in Prx1C83S (Fig. 8B) further corroborated the idea that the loss of the 68–92 peptide signal in the wild type Prx1 may be because of the oxidation of Cys\(^{83}\).

![FIGURE 6. MALDI-TOF mass spectrum of intact human Prx1, Prx1C83S, and Prx2 proteins. The oligomeric status of Prx1 (A), Prx1C83S (B), and Prx2 (C) was determined by measuring the accurate molecular masses of the intact proteins by MALDI-TOF mass spectrometry.](image)

**TABLE 1**

|                | Dimer [M + H]\(^{+}\) | Tetramer [M + H]\(^{+}\) | Decamer [M + H]\(^{+}\) | Decamer [M + 2H]\(^{2+}\) | Decamer [M + 3H]\(^{3+}\) | Decamer [M + 4H]\(^{4+}\) |
|----------------|------------------------|--------------------------|--------------------------|-----------------------------|-----------------------------|-----------------------------|
| Prx1           | Theoretical 44,192.55   | 88,385.11                | 220,962.79               | 110,482.39                  | 73,655.26                   | 55,241.70                   |
|                | Experimental 44,202.26  | 88,290.29                | 219,746.17               | 110,201.47                  | 73,554.93                   | 55,373.06                   |
|                | % error 0.02           | 0.11                     | 0.25                     | 0.14                        | 0.24                        |
| Prx1C83S       | Theoretical 44,160.42   | 88,320.85                | 220,802.12               | 110,402.06                  | ND\(^{a}\)                  | ND\(^{a}\)                  |
|                | Experimental 44,000.22  | 88,286.76                | ND\(^{a}\)               | ND\(^{a}\)                  | ND\(^{a}\)                  | ND\(^{a}\)                  |
|                | % error 0.36           | 0.04                     | ND\(^{a}\)               | ND\(^{a}\)                  | ND\(^{a}\)                  | ND\(^{a}\)                  |
| Prx2           | Theoretical 43,756.48   | 87,512.95                | 218,782.38               | 109,392.19                  | 72,928.46                   | 54,696.60                   |
|                | Experimental 43,627.27  | 87,373.28                | ND\(^{a}\)               | ND\(^{a}\)                  | ND\(^{a}\)                  | ND\(^{a}\)                  |
|                | % error 0.30           | 0.16                     | ND\(^{a}\)               | ND\(^{a}\)                  | ND\(^{a}\)                  | ND\(^{a}\)                  |

\(^{a}\) ND indicates not detected.
the 38–62, 68–92, and 169–190 residues of Prx1 and Prx1C83S are shown in Table 2.

To test whether the disappearance of the Cys\textsuperscript{83}–containing peptide in Prx1 is because of the oxidation of Cys\textsuperscript{83}, an aliquot of Prx1 was incubated with DTT, and the thiol (–SH) moieties were alkylated with IAA. The MALDI-TOF analysis revealed that the 68–92 residue with the \( m/z \) of 2881.6497 was fully restored in the DTT-treated and -alkylated wild type Prx1 digests. Fig. 8C shows an overlay of the isotopic clusters of the alkylated 68–92 peptide ion of Prx1 with or without DTT treatment. In contrast, when the 68–92 peptide ion of Prx1C83S was alkylated with or without DTT treatment, no difference was observed. Fig. 8D displays an overlay of the isotopic clusters of the alkylated 68–92 of Prx1C83S at \( m/z \) values of 2808.6533 (with DTT) and 2808.5066 (without DTT). These results further confirmed that the oxidation of Cys\textsuperscript{83} contributes to the loss of the 68–92 ion signal of wild type Prx1.

The Cys\textsuperscript{83}–Cys\textsuperscript{83} Disulfide Bond Is Present at the Dimer-Dimer Interface of Prx1 Decamer—X-ray crystallographic studies were conducted to determine the three-dimensional structure of human Prx1 protein. The crystal structure determined at 3.2 Å (\( R \)-factor value of 0.299 and \( R \)-free value of 0.328) clearly demonstrated that human Prx1 is present as a decamer, in which five homodimers associate around a local 5-fold rotational axis forming a doughnut-shaped oligomer (Fig. 9A). The crystallographic data also provided direct evidence that the Cys\textsuperscript{83} residues at the DD interfaces are all involved in the formation of disulfide bridges across dimers. The electron density maps of the five interfaces show the presence of disulfide bonds at DD interfaces (Fig. 9B), demonstrating that Prx1 is locked into a decameric form through interfacial disulfide bond formation. One homodimeric Prx1 molecule is shown in Fig. 9C, pointing out the locations of interfacial Cys\textsuperscript{83}–Cys\textsuperscript{83} disulfide bonds. The locations of the Cys\textsuperscript{52} and Cys\textsuperscript{172} residues are shown for reference. Given that the Cys\textsuperscript{52} residue is approximately 13 Å apart from the Cys\textsuperscript{172} in each homodimer unit, it is unlikely that the Cys\textsuperscript{52} and Cys\textsuperscript{172} are linked by a disulfide bond. The overoxidation status of Cys\textsuperscript{83} could not be ascertained from the crystallographic information.

DISCUSSION

Human Prx1 and Prx2 have been studied independently in a number of cell and animal systems and have been shown to influence the survival, proliferation, and treatment response of cancer cells. In this study, we demonstrated that human Prx1 and Prx2 possess unique functions and regulatory mechanisms. Prx1 displays a greater molecular chaperone activity than Prx2 as assessed by its ability to inhibit the thermal aggregation of substrate proteins such as MDH. The peroxidase activity of Prx1, on the other hand, is more susceptible to inactivation by \( \text{H}_2\text{O}_2 \) than that of Prx2. Consistent with this finding, the catalytic Cys residue of Prx1 is more prone to overoxidation than that of Prx2. Amino acid sequence analysis indicates that there is a cysteine residue at position 83 that is unique to Prx1. Our mutagenesis, biochemical, mass spectrometry, computer modeling, and x-ray crystallographic results are congruent with the hypothesis that the redox-sensitive Cys\textsuperscript{83} of human Prx1 plays an important role in modifying the molecular characteristics of Prx1. The substitution of Cys\textsuperscript{83} to Ser\textsuperscript{83} (Prx1C83S) results in dramatic changes in the structural and functional properties of human Prx1 in a direction that aligns more closely to human Prx2.

Our results show that compared with Prx1, the peroxidase activity of Prx2 is not as easily inactivated as Prx1 during a catalytic cycle. Although our studies are done using purified proteins in cell-free systems, we propose that Prx2 may be physiologically better suited as a peroxidase enzyme. This hypothesis is consistent with the recent study demonstrating the \( \text{H}_2\text{O}_2 \) catalytic activity of Prx2 in regulating PDGF signaling. Prx2, but not Prx1, translocates to the membrane and eliminates \( \text{H}_2\text{O}_2 \) that was generated upon PDGF receptor activation in response to PDGF binding to the receptor (40). The catalytic Cys residue of Prx2 is not overoxidized in this condition. According to the available x-ray crystallographic information and the results presented in this study, the regional structure surrounding the active site Cys appears to differ between Prx1 and Prx2; the catalytic Cys in Prx1 appears to be surrounded by several hydrophobic residues (41), whereas access to the active site Cys in Prx2 appears to be restricted by Phe\textsuperscript{81} of the adjacent Prx2 dimer (23). This may account in part for the resistance of Prx2 to overoxidation during the catalytic cycle. Because the \textit{de novo} synthesis of \( \text{H}_2\text{O}_2 \) during PDGF signaling is likely occur in a very restricted region localized around the PDGF receptor, a preferential translocation of Prx2 from the cytosol to the membrane may also contribute to the peroxidase function of Prx2 during PDGF signaling.

The Cys\textsuperscript{83} residue of Prx1 is located at the DD interface. Sequence alignment indicates that Cys\textsuperscript{83} is conserved only in mammalian Prx1, including human, bovine, rat, and mouse, but...
FIGURE 8. Analysis of digested human Prx1 and Prx1C83S by MALDI-TOF mass spectrometry. A, Prx1 protein was digested with trypsin, and MALDI-TOF mass spectrometry was performed in a positive reflectron mode. The locations of the peptide ions containing the Cys\(^{52}\) and Cys\(^{173}\) residue are marked. B, MALDI-TOF mass spectra of the trypsin-digested Prx1C83S. The locations of the peptide ions containing the Cys\(^{52}\), Cys\(^{71}/\text{Ser}^{83}\), and Cys\(^{173}\) are marked. C, Cys residues in Prx1 were alkylated with IAA followed by the presence (+DTT/+IAA) or absence (−DTT/+IAA) of DTT treatment. The protein was digested with trypsin and subjected to MALDI-TOF mass spectrometry, and the natural isotopic patterns of the Cys\(^{83}\) containing peptide ion were obtained. Note the restoration of the Cys\(^{83}\)-containing peptide ion with the m/z value of 2881.6497 in the DTT-treated sample (+DTT/+IAA). The theoretical m/z value of Cys\(^{52}/\text{Cys}^{83}\)-alkylated 68–92 peptide ion is 2881.4221. D, natural isotopic patterns of the alkylated Cys\(^{83}\)-containing peptide ion at 2808.6533 (+DTT/+IAA) were overlaid with those of the 2808.5066 ion (−DTT/−IAA). The theoretical m/z value of Cys\(^{72}\)-alkylated 68–92 Prx1C83S peptide ion is 2808.4208.
not in bacteria, yeast, or parasite orthologs of Prxs (Fig. 10). We propose that Cys83 may have been gained later in evolution, perhaps for the functional specialization of Prx1 in mammalian species. Our results indicate that Cys83 may increase the affinity between the dimers at the DD interface, thereby enabling the formation of a decamer as a preferred structure. How might the substitution of Cys83 to Ser83 decrease the stability of the DD interface? A disulfide bridge across the DD interface may be a possibility. Our crystallographic analysis of the molecular structure of human Prx1 demonstrated the presence of Cys83–Cys83-linked disulfide bond at each of the five DD interfaces of the decameric Prx1. Although our mass spectrometry studies provided an accurate molecular mass corresponding to the Prx1 decamer, we were not able to detect the Cys83–Cys83-linked peptide ion under the mass spectrometry conditions that were tested. It is possible that our inability to obtain a Cys83–Cys83 peptide ion or its fragments could result from deformation of the peptide during the gas phase ionization by some unknown mechanisms. Because the nonbonded contact distance is very close (the nonbonded S–S distance is 2.42 Å at $\chi_1 = -180^\circ$ or $\sim 4.0$ Å at $\chi_1 = -60^\circ$) with a possible entropic gain of a hydrophobic surface, we cannot exclude the possibility that Prx1 could pack tightly at the DD interface without having to form a Cys83–Cys83 covalent linkage across the dimers. A Ser83 substitution is likely to alter the DD interface in at least two ways. First, it makes the surface less hydrophobic and more polar. Second, the van der Waals radius of oxygen (in Ser83) is 1.40 Å, opposed to 1.85 Å for sulfur (in Cys83). Thus, Ser will render the DD packing less tight. These consequences would have a destabilizing effect in Ser83-substituted Prx1 and make decamer formation energetically less favorable.

How might the structural property of Prx1 explain its molecular chaperone activity and greater sensitivity to inactivation by $\text{H}_2\text{O}_2$? According to the previously proposed mechanism (24, 25), the $\text{H}_2\text{O}_2$ catalytic cycle of Prxs requires a decamer to dimer transition through local unfolding of the loops containing the catalytic Cys52 and the Cys173. If Cys83 stabilizes the DD interface of Prx1 and discourages local unfolding, the active site Cys engaged in a catalytic cycle would not be able to form an inter-molecular disulfide bond and its subsequent reduction by

| Residues | Peptide sequence | Theoretical [M + H]$^*$ | Experimental [M + H]$^*$ |
|----------|------------------|-------------------------|-------------------------|
| Prx1     | 38–62 (K)YVVFPYPLDFTVCPEIIAFSDR(A) | 3036.4900 | 3036.7607 |
|          | 68–92 (K)KLCQGAVYDSDHCHLAWVNTKR(K) | 2767.3800 | ND$^a$  |
|          | 169–190 (K)HGEVFCAEGKPGSDTTKPPDVQK | 2349.1700 | 2349.2420 |
| Prx1C83S| 38–62 (K)YVVFPYPLDFTVCPEIIAFSDR(A) | 3036.4900 | 3036.8372 |
|          | 68–92 (K)KLCQGAVYDSDHCHLAWVNTKR(K) | 2751.3134 | 2751.6653 |
|          | 169–190 (K)HGEVFCAEGKPGSDTTKPPDVQK | 2349.1700 | 2349.4114 |

$^a$ND indicates not detected.
thioredoxin to complete the cycle. As a result, Prx1 is more prone to overoxidation, which in turn may lead to a more stable and compact oligomeric structure. This property may explain why Prx1 is a better molecular chaperone than Prx2. An interesting analogy appears to exist in the isoforms of glutathione peroxidase (Gpx). Although several Gpx isoforms act primarily as an antioxidant peroxidase, the phospholipid hydroperoxide Gpx (PHGpx) forms a capsule around sperm mitochondrion in the testis (42, 43). The PHGpx in the capsule is oxidatively cross-linked and enzymatically inactive. Because both Prx and Gpx systems utilize NADPH as an ultimate reducing source, the functions of the two peroxidase systems can be complementary or inter-dependent in certain cell types and tissues. Similar to the Prx system, functional specialization of the Gpx system might have been developed during evolution.

Lines of evidence suggest that overoxidation of the catalytic Cys may allow a mechanism of structural and functional switching of Prx from a peroxidase enzyme to a molecular chaperone. This hypothesis is consistent with the behavior of Prx1 in interacting physically with various cellular proteins. Using a yeast two-hybrid system, the interaction of Prx1 with the Src homology 3 domain of c-Abl, the Myc Box II domain of c-Myc, and the macrophage inhibiting factor has been demonstrated (44–46). We recently reported that Prx1 suppresses radiation-induced JNK signaling and apoptosis in lung cancer cells (31). Our results demonstrated that the JNK inhibitory effect is mediated through the interaction of Prx1 with the GSTpi-JNK complex, thereby preventing JNK release from the complex. The interaction of Prx1 with growth regulatory and signaling proteins may be responsible for the wide range of effects attributed to Prx1 (29, 47–50). Whether Prx2 behaves similarly to Prx1 in this respect is presently unknown.

In addition, the regulation of Prx1 and Prx2 expression appears to vary widely among different cell types. In the brain, Prx1 is expressed in astrocytes, whereas Prx2 is expressed in neurons (51). Prx1 is preferentially expressed in the Leydig cells, whereas Prx2 predominates in the Sertoli cells in the testis (52). These observations indicate that the cell type- and tissue-specific expression of Prx1 and Prx2 would also contribute to their respective activities in certain cells and tissues, but not in others. In this study, we provide...
Structural and Functional Differences of Prx1 and Prx2

evidence to support the existence of inherent structural and functional differences between Prx1 and Prx2 at the protein level. We show that the differences between the two highly homologous proteins are attributable in part to the unique presence of Cys^{83} in Prx1. The significance of this study is underlined by the realization that the differential molecular characteristics of Prx1 and Prx2 would continue to influence their molecular behaviors in various biological systems and also under a diverse environmental and genetic context. The information obtained in this study would provide a conceptual framework for further studies to delineate the physiological (or pathophysiological) functions of Prx1 and Prx2.

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