Mol. Cells *29*, 145-151, February 28, 2010
DOI/10.1007/s10059-010-0023-1

Molecules and Cells

A New Antioxidant with Dual Functions as a Peroxidase and Chaperone in *Pseudomonas aeruginosa*

Byung Chull An, Seung Sik Lee, Eun Mi Lee, Jae Taek Lee, Seung Gon Wi[1], Hyun Suk Jung[2], Woojun Park[3], and Byung Yeoup Chung*

Thiol-based peroxiredoxins (Prxs) are conserved throughout all kingdoms. We have found that a conserved typical 2-Cys Prx-like protein (PaPrx) from *Pseudomonas aeruginosa* bacteria displays diversity in its structure and apparent molecular weight (MW), and can act alternatively as a peroxidase and molecular chaperone. We have also identified a regulatory factor involved in this structural and functional switching. Exposure of *P. aeruginosa* to hydrogen peroxide (H_2O_2) causes PaPrx to convert from a high MW (HMW) complex to a low MW (LMW) form, which triggers a chaperone to peroxidase functional switch. This structural switching is primarily guided by either the thioredoxin (Trx) or glutathione (GSH) systems. Furthermore, comparison of our structural data [native and non-reducing polyacrylamide gel electrophoresis (PAGE) analysis, size exclusion chromatography (SEC) analysis, and electron microscopy (EM) observations] and enzymatic analyses (peroxidase and chaperone assay) revealed that the formation of oligomeric HMW complex structures increased chaperone activity of PaPrx. These results suggest that multimerization of PaPrx complexes promotes chaperone activity, and dissociation of the complexes into LMW species enhances peroxidase activity. Thus, the dual functions of PaPrx are clearly associated with their ability to form distinct protein structures.

INTRODUCTION

The peroxiredoxins (Prxs), also known as thioredoxin (Trx)-dependent peroxidases and alkyl hydroperoxide reductase-C22 (AhpC) proteins, have received considerable attention in recent years as a new and expanding family of thiol-specific antioxidant proteins (Park et al., 2000). Prxs are abundant proteins in *Escherichia coli*, and many organisms produce more than one isoform of Prx. The Prxs use redox-active cysteines (Cys) to reduce peroxides and were originally divided into two categories, 1-Cys Prxs and 2-Cys Prxs, based on the number of conserved cysteine residues directly involved in catalysis (Chae et al., 1994).

It has been demonstrated recently that Prxs exhibit dual physiological functions as both a peroxidase and a molecular chaperone (Jang et al., 2004; Jeong et al., 2000; Moon et al., 2005; Wood et al., 2003). The molecular chaperone activity has received considerable attention in recent years as it represents a new function for thiol-specific antioxidant proteins. In general, upon exposure to oxidative stress or heat shock, the Prx protein structure switches from a LMW form with peroxidase activity to a HMW complex with molecular chaperone activity (Jang et al., 2004; Moon et al., 2005). The molecular chaperone recognizes and binds nascent polypeptide chains and partially folded protein intermediates, preventing their aggregation and misfolding. The oligomerization of Prx leads to an increase in surface hydrophobicity, which allows chaperone activity to increase.

In this study, we examined whether a prokaryotic Prx also undergoes structural and functional switching from a peroxidase to a molecular chaperone. Genetic analyses demonstrated that the prokaryotic 2-Cys Prx-like protein PaPrx from *Pseudomonas aeruginosa* PAO1 contains two conserved catalytic sites similar to the 2-Cys Prxs of other organisms. Our biochemical studies revealed that PaPrx can act alternatively as a Trx-dependent peroxidase and as a molecular chaperone. Furthermore, we show that reversible switching between the dual functions of this protein is triggered by oxidative stress in association with significant structural changes.

MATERIALS AND METHODS

Bacterial strains, media and materials

The bacterial strains *Pseudomonas aeruginosa* PAO1 and *E. coli* were grown aerobically at 30°C in LB medium (0.5% sodium chloride, 0.5% yeast extract and 1% tryptone) and were used to clone the *Prx* gene. Yeast Trx and thioredoxin reductase (TR) were prepared as described (Chae et al., 1994). Protein molecular size standards used in PAGE were purchased from ELPIS (Korea). Ampicillin, L-rhamnose, bovine serum albumin (BSA), hydrogen peroxide [H_2O_2; 30% (v/v)], NADPH were obtained from Sigma (USA).

Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea, [1]Bio-Energy Research Institute, Chonnam National University, Gwangju 500-757, Korea, [2]Division of Electron Microscopic Research, Korea Basic Science Institute, Daejeon 305-333, Korea, [3]Division of Environmental Sciences and Ecological Engineering, Korea University, Seoul 136-701, Korea
*Correspondence: bychung@kaeri.re.kr

Received August 5, 2009; revised October 29, 2009; accepted November 3, 2009; published online January 12, 2010

Keywords: chaperone, oxidative stress, peroxidase, peroxiredoxin, *Pseudomonas aeruginosa*

Cloning of *PaPrx* gene and expression in *E. coli*

The *PaPrx* gene was cloned from *Pseudomonas aeruginosa* PAO1 genomic DNA by polymerase chain reaction (PCR). Briefly, PCR reactions were carried out in 20 μl mixtures containing 10 ng of genomic DNA, 0.2 μM deoxyribonucleoside triphosphates (dNTPs), 20 pmol of each primer set for *PaPrx* (*Xho*I, 5′-ccgctcgagatgagcgtactcgta; *Hind*III, 3′-cccaagcttttaca-gcttgctggc), and 1 unit of Taq DNA polymerase (Promega, USA) in a standard PCR buffer under the following conditions: denaturation for one cycle at 94°C, 60 s; and 35 cycles at 94°C, 30 s; 50°C, 45 s; 72°C, 45 s, followed by one cycle at 72°C for 10 min. After PCR amplification, the *PaPrx* gene was subcloned into the *pGEM-T* vector, which was then transformed into *E. coli* DH5α cells. Constructs were confirmed by nucleotide sequencing and then the *PaPrx* fragment from *pGEM-T* was transferred into the *pRSETa* expression vector to create *pRSETa*::*PaPrx*.

His_6-fused PaPrx was purified using a native Ni-NTA column (Peptron, Korea) and eluted with a linear gradient of 200 to 500 mM imidazole in phosphate-buffered saline (PBS) buffer. After dialysis against 50 mM HEPES, pH 8.0, the protein concentration was measured using the Bradford method (Bradford, 1976), with BSA as the standard. The purity of the purified recombinant Prxs was determined to be > 99% based on SDS-PAGE.

Peroxidase activity assay

The thioredoxin-dependent peroxidase activity of purified Prxs was measured as described previously with minor modifications (Cheong et al., 1999; Kim et al., 2005; Lee et al., 1997). Total protein or fractions separated by SEC (F-1 and F-2) were incubated in 50 mM HEPES (pH 8.0) containing 200 μM NADPH, 3 μM yeast Trx, and 1.5 μM yeast TR. The reaction mixture was incubated at 30°C for 5 min, followed by the addition of a 10-μl aliquot of H_2O_2 at various concentrations. NADPH oxidation was monitored for the next 6 min by a decrease in absorbance at 340 nm as measured by a DU800 spectrophotometer equipped with a thermostatic cell holder (Beckmann, USA).

Molecular chaperone activity assay

The molecular chaperone activity was determined as described previously (Cheong et al., 1999; Hendrick and Hartl, 1993; Lee et al., 1997) by assessing the ability of recombinant Prxs to inhibit the thermal aggregation of substrate proteins (Jang et al., 2004; 2006; Moon et al., 2005). Briefly, 1 μM of malate dehydrogenase (MDH) was mixed with various concentrations of total PaPrx or protein fractions (F-1 and F-2) in a degassed 50 mM HEPES (pH 8.0) solution. The reaction mixture was incubated at 45°C for 15 min, and the increase of light scattering as a result of thermal aggregation of substrate proteins was monitored at 360 nm with a DU800 spectrophotometer equipped with a thermostatic cell holder (Beckmann, USA).

Size exclusion chromatography

SEC was performed at 25°C by fast protein liquid chromatography (FPLC) (AKTA; Amersham Biosciences) using a superdex 200 HR 10/30 column equilibrated at a flow rate of 0.5 ml/min at 25°C with 50 mM HEPES (pH 8.0) buffer containing 100 mM NaCl. Protein peaks (A_{280}) were isolated and concentrated using a Centricon YM-30 (MILLIPORE, USA).

Electron microscopy and single particle image processing

For negative staining, fractionated proteins were 50-fold diluted with 50 mM HEPES, pH 8.0. Following dilution, 5 μl of the final mixture was applied to a carbon-coated grid that had been glow-discharged (Harrick Plasma, USA) for 3 min in air, and the grid was negatively stained using 1% uranyl acetate. The same procedure was used for all specimens. For metal shadowing, the proteins were diluted about 10-fold with 50 mM HEPES, pH 8.0, and mixed with an equal volume of glycerol. The resulting mixture was sprayed onto freshly cleaved mica and then rotary shadowed with platinum at an angle of 6°. Grids were examined in a Philips CM120 electron microscope (FEI, USA) operated at 80 kV. Images were recorded on a 2Kx2K F224HD slow scan CCD camera (TVIPS, Germany) at a magnification of 65,000 (0.37 nm/pixel). Single particle image processing was carried out using SPIDER (Health Research Inc., USA), and averaged images were produced by alignment and classification of windowed particles from micrographs (Burgess et al., 2004). The number of particles used in the processing were 239 (F-1; PaPrx) and 248 (F-2; PaPrx).

Oxidative stress species-dependent structural switch of PaPrx in *P. aeruginosa*

To investigate the structural switch of PaPrx in cells exposed to H_2O_2, methyl viologen (MV) or gamma rays, cells were grown to an OD_{600} of 0.5, and then split into 10 ml aliquots for stress and recovery samples. H_2O_2 and MV were added at the indicated final concentrations for 30 min, and irradiation was performed at the indicated doses for 30 min. Stressed cells were harvested and resuspended in PBS. Crude extracts (8 μg) were dissolved in sample loading buffer and then resolved by native- and reducing-PAGE. Proteins were transferred to a nitrocellulose membrane and then analyzed by Western blot using a mouse anti-PaPrx antibody. Immunoreactive proteins were detected using a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody.

Observation of structural switch by oxidative stresses *in vivo*

P. aeruginosa cells were cultured in LB medium at 30°C in a shaking incubator. Cells were grown to mid-exponential phase (OD_{600} = 0.5) in 100 ml LB medium and split into 50 ml aliquots for stress and recovery sample preparation. H_2O_2 was added at a final concentration of 20 mM for 30 min. The stressed sample was immediately harvested. The recovery sample was inoculated into fresh LB medium at 30°C for 30 min and then harvested. Crude cell lysates were subjected to native, non-reducing or reducing-PAGE. The structural properties of PaPrx were analyzed by immunoblotting using anti-PaPrx antibody.

RESULTS

Sequence alignment and comparison

We aligned nine protein sequences encompassing representative AhpC proteins and Prxs from other prokaryotic and eukaryotic organisms (Fig. 1A). The alignment showed that these proteins each contain two perfectly conserved VCP tripeptides (highlighted in gray). Interestingly, the sequence homology between the PaPrx protein of a *P. aeruginosa* and the *P. putida* KT2440 Prx (PP1084) was 89%. Furthermore, we found that PaPrx was more structurally similar to PP1084 using consensus secondary structure prediction (data not shown). Taken together, these results suggest that the functional or enzymatic characteristics of PaPrx may be closer to prokaryotic 2-Cys Prxs, which have been shown to function as a peroxidase and a molecular chaperone, than to eukaryotic Prxs.

PaPrx exhibits dual functions as a peroxidase and a molecular chaperone

Recent evidence has indicated that Prx proteins have dual physiological functions as peroxidases and molecular chaper-

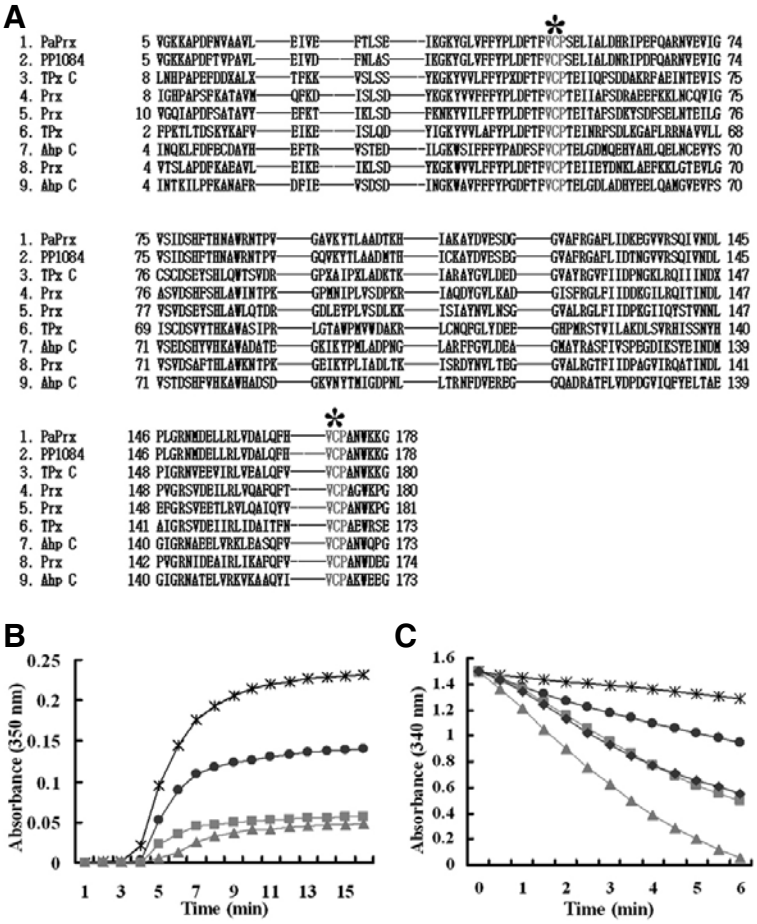

Fig. 1. Investigation of PaPrx enzymatic functions. (A) Alignment of the amino acid sequences of *P. aeruginosa* PaPrx (2-Cys Prx) with homologous AhpC and Prxs from several representative prokaryotes and eukaryotes. The highly conserved tripeptide (VCP) is related to the catalytic function and is designated by an asterisk. The abbreviations for the amino acid sequences of Prxs from various species are as follows: 1, PaPrx of *P. aeruginosa* PAO1; 2, PP1084 of *P. putida* KT2440; 3, TPx C of *C. fasciculata*; 4, Prx of *R. norvegicus*; 5, Prx of *P. purpurea*; 6, TPx of *E. cuniculi* GB-M1; 7, Ahp C of *E. faecalis*; 8, Prx of *Leptospira sp.*; 9, Ahp C of *P. acnes*. (B) The chaperone activity of recombinant PaPrx was measured by the aggregation of MDH at 43°C at different molar ratios: 1 vs. 0 PaPrx (-·-) in reaction buffer; 1 vs. 5 PaPrx (●-●); 1 vs. 10 PaPrx (■-■); and 1 vs. 1 yeast TPx (▲-▲). (C) The peroxide reductase activity of recombinant PaPrx from *P. aeruginosa* was measured using the yeast Trx system at different concentrations: without PaPrx (-·-) in reaction buffer; 2 μM PaPrx (●-●); 5 μM PaPrx (■-■); 10 μM PaPrx (▲-▲); and 5 μM yeast TPx (◆-◆). Recombinant PaPrx possesses distinct peroxidase and chaperone activities as shown by the rapid decrease of NADPH absorbance at 340 nm and the increase in MDH absorbance at 350 nm, respectively.

ones, and the latter function has received considerable attention in recent years as it represents a new role for these thiol-specific antioxidant proteins (Jang et al., 2004; Jeong et al., 2000; Moon et al., 2005; Wood et al., 2003). To investigate whether the 2-Cys Prx from *P. aeruginosa* possesses both peroxidase and molecular chaperone activities, we performed a series of experiments *in vitro*. First, we analyzed the chaperone activity of PaPrx by testing its ability to suppress thermal aggregation of a model substrate, MDH, at 43°C. Incubating MDH with increasing concentrations of PaPrx produced a concomitant concentration-dependent decrease in MDH aggregation. At a 10:1 subunit molar ratio of PaPrx to MDH, MDH aggregation was completely suppressed. This suggests that PaPrx can indeed act as an efficient molecular chaperone, although PaPrx's chaperone activity was 10-fold lower than that of yeast Trx-dependent Prx (TPx) (Fig. 1B). We then analyzed the peroxidase activity of PaPrx by testing its ability to reduce H_2O_2 using the yeast Trx and TR system (Fig. 1C). We found that PaPrx exhibited high H_2O_2 catabolic peroxidase activity, similar to (Fig. 1C). Taken together, these results show that PaPrx functions both as a peroxidase and a molecular chaperone, though its chaperone activity was significantly lower than that of the positive control (yeast TPx, Jang et al., 2004).

Recombinant PaPrx forms differently sized HMW protein structures *in vitro*

Since several Prxs form HMW complexes with masses of 230 to 500 kDa (Hirotsu et al., 1999; Schröder et al., 2000), we determined the molecular masses of purified PaPrx by SEC (Fig. 2A). Two major peaks were detected. The minority of PaPrx molecules were contained in the first fraction (F-1), which contained the largest multimer complexes, while a majority were in the second fraction (F-2) (Fig. 2A). The molecular sizes of the F-1 proteins were too great to penetrate the pores of a 10% native-polyacrylamide gel, and thus proteins_were retained at the top of the separating gel (Fig. 3A, F-1). In contrast, F-2 proteins formed partial multimer complexes with molecular masses ranging from about 40 to 230 kDa (Fig. 3A, F-2). These results suggest that PaPrx forms more oligomeric complexes than HMW complexes, consequently, consistent with its strong peroxidase activity.

The dual functions of recombinant PaPrx are regulated by its protein structure *in vitro*

A conserved feature of molecular chaperones is their tendency to associate into dimers, trimers, and higher oligomers in a reversible fashion (Chang et al., 2002; Haley et al., 1998; Hendrick and Hartl, 1993; Ito et al., 2001). Recently, studies of several typical 2-Cys Prxs have shown that they produce homo multimeric complexes and that the dual function of Prxs as a peroxidase and chaperone is closely associated with the degree of protein polymerization and dissociation (Chuang et al., 2006; Jang et al., 2004; 2006; Lee et al., 2009; Moon et al., 2005; Nooren and Thornton, 2003a; 2003b).

In this study, we have shown that PaPrx forms HMW complexes ranging in size from 40 to 1,000 kDa (Figs. 2A and 3A), and functions both as a peroxidase and as a molecular chaperone *in vitro* (Figs. 1B and 1C). We next investigated whether

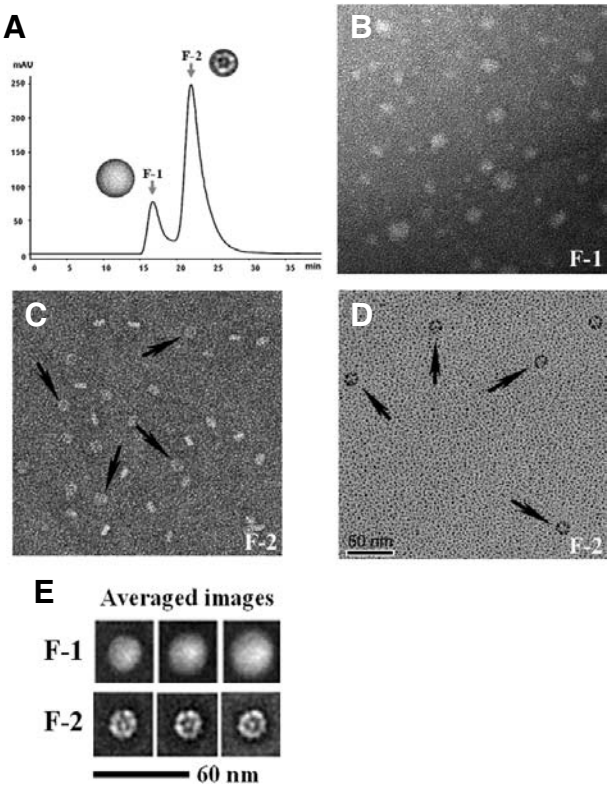

Fig. 2. Protein structure analysis using SEC and EM. (A) SEC analysis of PaPrx. SEC was performed using a superdex 200 HR 10/30 column as described in the "Materials and Methods". The separated proteins were divided and pooled into two fractions (F-1 and F-2) for further analysis. Structural analysis of classified PaPrx fractions was performed using EM (B-D). (B) Negatively stained fields of fractionated proteins taken from HMW fractions (F-1). Globular-shaped oligomers were apparent. (C, D) Negatively stained images (left panel) and metal shadowed (right panel) of middle MW fractions (F-2). Black arrows indicate individual ring-shaped-like complexes found in the middle MW fractions (F-2). The scale bar in (D) represents 60 nm in all fields (B-D). (E) Projected images of the class averages revealed spherical particles with diameters ranging from 8 to 40 nm. The globular-shaped oligomers appeared in the F-1 fractions, while the ring-shaped structures were easily detected in the F-2 fractions of PaPrx.

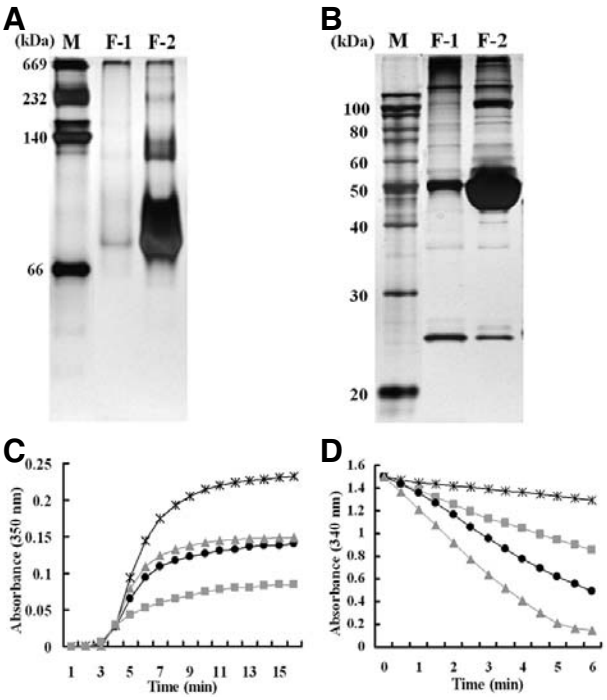

Fig. 3. Protein structure-dependent regulation of peroxidase and chaperone activities. SEC fractions were separated on a 10% native-PAGE (A) or 12% non-reducing-PAGE (B). PaPrx protein was stained with Coomassie Brilliant Blue R-250. Lane F-1, F-1 fraction of PaPrx; lane F-2, F-2 fraction of PaPrx. (C) Chaperone activity assays of the two PaPrx fractions (F-1 and F-2) were performed by measuring the aggregation of MDH at 43°C. The chaperone activity of PaPrx was measured at different molar ratios: 1 vs. 0 PaPrx (-·-) in reaction buffer; 1 vs. 5 total PaPrx (●-●); 1 vs. 5 F-2 fraction of PaPrx (▲-▲); and 1 vs. 5 F-1 fraction of PaPrx (■-■). (D) Peroxidase activity assays of the two PaPrx fractions (F-1 and F-2) were performed with the yeast Trx system. The peroxidase activity of the PaPrx fractions was measured at different concentrations: 0 μM PaPrx (-·-) in reaction buffer; 5 μM total PaPrx (●-●); 5 μM F-1 fraction of PaPrx (■-■); and 5 μM F-2 fraction of PaPrx (▲-▲).

the PaPrx fractions separated by SEC differed in their specific activities (Fig. 2A). The F-1 and F-2 fractions showed opposite trends in their chaperone and peroxidase activities. The HMW protein complexes (F-1) exhibited high chaperone but low peroxidase activity, whereas the LMW fraction (F-2) displayed high peroxidase and low chaperone activity, compared to total protein activity (Figs. 3C and 3D). These results suggest that protein multimerization promotes chaperone activity, and dissociation of the complexes into LMW species enhances their peroxidase activity. Thus, the dual functions of PaPrx are clearly associated with their ability to form distinct protein structures.

Electron microscopy analysis of the protein structures of fractionated PaPrx

The critical factors that govern the oligomeric assembly of 2-Cys Prxs are still a controversial issue (Chauhan and Mande, 2001; Kitano et al., 1999). Our SEC and native-PAGE analysis indicated that PaPrx contained predominantly oligomeric MW complex structures and few HMW complexes (Figs. 2A and 3A). Previous reports and our results suggest that Prxs multimerization promotes chaperone activity, and dissociation of the complexes into LMW species enhances peroxidase activity.

To investigate whether the dual functions of PaPrx are associated with their ability to form distinct protein structures, we performed a comparative analysis of HMW complex structures in the SEC F-1 fraction and LMW structures in the SEC F-2, using EM. EM of the negatively stained protein fractions revealed two different structural configurations, spherical and irregularly shaped small particles. The spherically shaped particles observed in the F-I fraction were subjected to rotational and translational alignment (Fig. 2B). Interestingly, the projected images of the class averages revealed spherical particles with diameters ranging from 15 to 40 nm (Fig. 2E, F-1). The oligomeric structures of the proteins in the F-2 fraction were also analyzed by EM and image processing. Ring-shaped structures were easily detected in the F-2 fraction (Figs. 2C and 2D). The projected images of the class averages revealed irregularly shaped small particles with diameters ranging from 8 to 12 nm, possibly reflecting the number of Prx molecules in

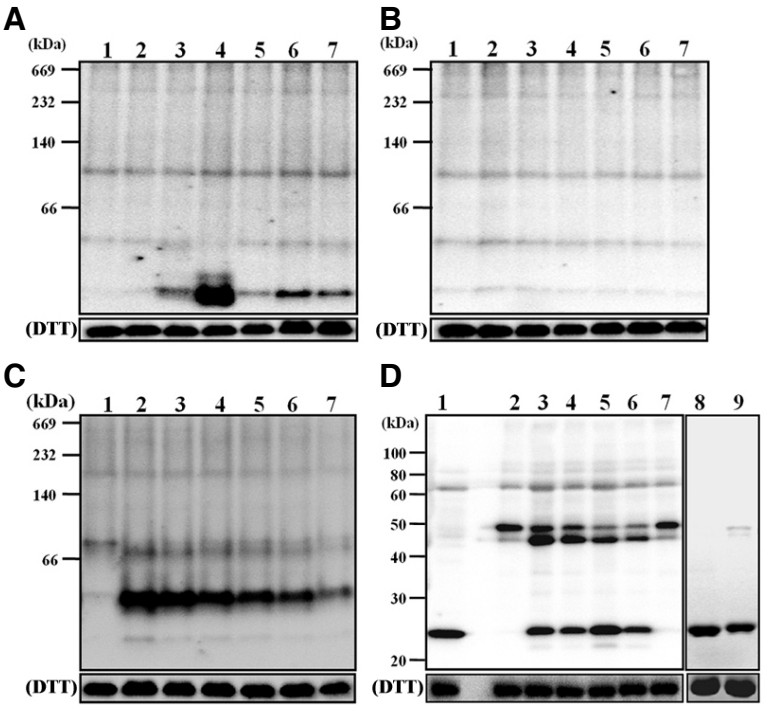

Fig. 4. Oxidative stress-dependent structural changes of PaPrx. The structural changes of PaPrx in *P. aeruginosa* cells exposed to H_2O_2, MV, or gamma rays were investigated. (A) H_2O_2 and MV were added at the indicated final concentrations for 30 min. Cells were exposed to different conditions: lane 1, untreated; lane 2, 0.5 mM H_2O_2; lane 3, 10 mM H_2O_2; lane 4, 50 mM H_2O_2; lane 5, 0.5 mM MV; lane 6, 10 mM MV; and lane 7, 50 mM MV. (B) Irradiation was performed at the indicated doses for 30 min: lane 1, untreated; lane 2, 30 Gy; lane 3, 60 Gy; lane 4, 90 Gy; lane 5, 120 Gy; lane 6, 150 Gy; and lane 7, 200 Gy. (C) Cells were challenged with 20 mM H_2O_2 for 30 min and then allowed to recover for 60 min without H_2O_2. The structural changes in PaPrx were analyzed by native-PAGE using cells collected at 15 min intervals. Cells were exposed to different conditions: lane 1, untreated; lanes 2 and 3, exposure to 20 mM H_2O_2 for 15 and 30 min; and lanes 4 to 7, recovery without H_2O_2 for 15, 30, 45, and 60 min. (D) To understand the structural change mechanism, crude proteins were extracted from untreated cells and then exposed to various components (100 mM DTT, 1 mM NADPH, 10 mM H_2O_2 for 30 min at 30°C in the presence or absence of the yeast Trx or GSH systems), and the structural changes in PaPrx were analyzed by non-reducing PAGE. Lane 1, cell extract with DTT; lane 2, cell extract without components; lane 3, cell extract with 1 mM NADPH; lane 4, cell extract with 1 mM NADPH and 10 mM H_2O_2; lane 5, cell extract with 1 mM NADPH, 10 mM H_2O_2, and the yeast Trx system; lane 6, cell extract with 1 mM NADPH, 10 mM H_2O_2, and the yeast GSH system; lane 7, cell extract with 10 mM H_2O_2; lane 8, recombinant PaPrx proteins with 1 mM NADPH, 10 mM H_2O_2, and the yeast Trx system; and lane 9, recombinant PaPrx proteins with 1 mM NADPH, 10 mM H_2O_2, and the yeast GSH system. The Trx and GSH systems contained Trx-TR and GSH-GR. PaPrx was detected using an anti-PaPrx antibody by Western blot.

each particle (Fig. 2E, F-2). Thus, both the SEC and EM results suggest that PaPrx predominantly forms oligomeric complex structures. Taken together, the results of the structural analyses (native and non-reducing PAGE analysis, SEC analysis, and EM observations) and enzymatic analyses (peroxidase and chaperone assay) indicate that the formation of oligomeric HMW complex structures increases PaPrx chaperone activity. Moreover, the relatively low level of HMW structures relative LMW oligomeric forms is consistent with the low chaperone and high peroxidase activity of PaPrx (Jang et al., 2004; Moon et al., 2005).

Oxidative stress-induced PaPrx structural changes in *P. aeruginosa*

Typical 2-Cys Prxs are obligate homodimers containing two identical active sites (Alphey et al., 2000; Hofmann et al., 2002; Schröder et al., 2000; Wood et al., 2002). Recently, studies of several typical 2-Cys Prxs have revealed that dramatic changes in their oligomeric states (dimers and decamers) are linked to alterations in the redox state that occur during the catalytic cycle (Braford, 2004; Kim et al., 2005; Wood et al., 2003).

We analyzed the structural changes of PaPrx that occurred *in vivo* in *P. aeruginosa* cells exposed to various oxidative stresses. Cells were cultured with or without various oxidative stresses, and crude extracts were subjected to Western blot analysis under native conditions (Figs. 4A and 4B). PaPrx proteins in untreated *P. aeruginosa* cells formed various multimeric structures. Methyl viologen (MV)-treated *P. aeruginosa* cells exhibited a low conversion of PaPrx into LMW forms, although conversion was concentration-dependent up to 50 mM. In contrast, gamma ray treatment did not cause PaPrx structural switching. However, when cells were challenged with different H_2O_2 concentrations for 30 min, the various multimeric structures were immediately converted into LMW forms. Moreover, treatment with 20 mM H_2O_2 induced conversion of the majority of the HMW complexes into LMW proteins, but the LMW proteins returned to their original structures within 30 min following the removal of H_2O_2 (Fig. 4C).

We then investigated molecules that modulate PaPrx structural switching. The addition of dithiothreitol (DTT) as a reductant or NADPH suggested that PaPrx could be reduced using NADPH as an electron donor (Fig. 4D, lanes 1 and 3). Although we were unable to detect any H_2O_2-induced structural changes in PaPrx *in vitro* (Fig. 4D, lane 7), we were able to detect structural changes in PaPrx using a Trx system containing either Trx-TR or glutathione (GSH)-glutathione reductase (GR). In addition, H_2O_2 stimulated Trx and GSH system-dependent structural changes in PaPrx *in vivo* (Fig. 4D, lane 4). These results suggest that Trx and GSH are essential for the formation of LMW PaPrx forms *in vivo* and *in vitro* (Fig. 4D, lanes 5, 6, 8, and 9). Taken together, these data suggest that H_2O_2 induces a reversible change in PaPrx protein structure *in vivo* from oligomeric MW complexes into LMW forms in an H_2O_2 concentration dependent fashion. Dissociation of the complexes into LMW species enhances PaPrx peroxidase activity (Fig. 3D) and may serve to protect *P. aeruginosa* cells from H_2O_2 stress.

A well-conserved feature of molecular chaperones is their tendency to associate reversibly as dimers, trimers, and HMW oligomers (Chuang et al., 2006; Hendrick and Hartl, 1993; Jang et al., 2004), and these alterations are required for their chap-

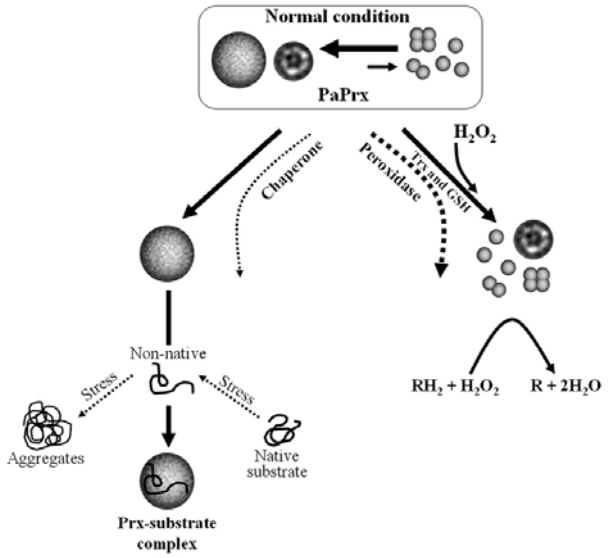

Fig. 5. (A) Model of oxidative stress-dependent structural and functional switching of PaPrx from a molecular chaperone to a peroxidase. Under normal conditions, PaPrx exists principally as oligomers and HMW complexes within cells. The two structural classes of Prx proteins are formed by two independent pathways, one of which is H_2O_2-insensitive and one that is H_2O_2-sensitive. In the H_2O_2-insensitive pathway, the HMW complexes act as super-chaperones, which offer a high level of protection to substrate proteins against oxidative stress. In the H_2O_2-sensitive pathway, oxidative stress induces HMW complexes to switch to LMW forms via the Trx and GSH systems.

erone activity (Haley et al., 1998). The structural conversion of several 2-Cys Prxs from LMW to HMW complexes is regulated by the Trx system and oxidative stresses (Jang et al., 2004; Kim et al., 2005; Moon et al., 2005). In contrast, significant amounts of PaPrx of *P. aeruginosa* were converted from MW complexes into LMW forms by H_2O_2 in the presence of the Trx and GSH systems (Fig. 4D), while other oxidative stresses did not affect PaPrx dissociation or oligomerization rates.

DISCUSSION

The 2-Cys Prx proteins are members of a ubiquitous family of peroxidases that exhibit antioxidant activity as peroxidases, function as molecular chaperones, and participate in redox-sensitive signaling (Jang et al., 2004; Wood et al., 2003). Most 2-Cys Prxs have been found to form condition-dependent oligomeric structures, although the physiological relevance of the association or dissociation of these proteins was unclear. A PaPrx found in *P. aruginosa* was investigated in this study and found to be a typical prokaryotic 2-Cys Prx, as it functioned as both a peroxidase and a molecular chaperone. Moreover, these functions were regulated by dynamic changes in the oligomeric structure (Figs. 2 and 3). We also discovered that these structural changes are sensitively regulated by H_2O_2. PaPrx formed non-regular oligomeric structures, which were converted into monomers by H_2O_2 treatment *in vivo*, although *in vitro*, recombinant PaPrx exhibited various multimeric structures and did not respond to H_2O_2. However, when PaPrx was reduced by either the Trx or GSH systems, disassociation of the oligomeric structure and transition to monomers was observed both *in vivo* and *in vitro* (Fig. 4).

These observations allow us to draw a comprehensive model that shows how PaPrx can perform dual functions as a peroxidase and chaperone during oxidative stress (Fig. 5). In this model, PaPrx reversibly changes its protein structure *in vivo* from HMW complexes to LMW species by two different pathways. At low concentrations of ROS under normal conditions, PaPrx exists principally in oligomer and HMW complex forms in the cell. Two classes of PaPrx with different structures are formed by two independent pathways, H_2O_2-insensitive and H_2O_2-sensitive. In the H_2O_2-insensitive pathway, the HMW complexes act as super-chaperones, which offer a high level of protection to substrate proteins against oxidative stress. In the H_2O_2-sensitive pathway, the HMW complexes significantly switch to LMW forms through Trx and GSH systems. The LMW PaPrx functions predominantly as a Trx-dependent peroxidase that catalyzes the removal of H_2O_2, while the HMW protein acts as a super-chaperone that effectively prevents the denaturation of protein substrates from stresses. These dual functions of PaPrx can reversibly switch when subjected to oxidative stress *in vivo,* and this reversion is accompanied by a structural change in PaPrx from high to LMW protein species.

In conclusion, the 2-Cys Prxs may help protect host cells against various stresses. The dual functions of 2-Cys Prxs in modulating ROS concentrations and preventing protein aggregation may allow PaPrx to serve as a potent stress sensor and chaperone that enables *P. aeruginosa* to survive and persist in extreme environments (Arrgio, 1998; Papp et al., 2003).

ACKNOWLEDGMENT

This project was carried out under the Nuclear R&D Program by the Ministry of Science and Technology (http://www.mest.go.kr), Republic of Korea.

REFERENCES

Alphey, M.S., Bond, C.S., Tetaud, E., Fairlamb, A.H., and Hunter, W.N. (2000). The structure of reduced tryparedoxin peroxidase reveals a decamer and insight into reactivity of 2 Cys-peroxiredoxins. J. Mol. Biol. *300*, 903-916.

Arrigo, A.P. (1998). Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. Biol. Chem. *379*, 19-26.

Barford, D. (2004). The role of cysteine residues as redox-sensitive regulatory switches. Curr. Opin. Struct. Biol. *14*, 679-686.

Burgess, S.A., Walker, M.L., Thirumurugan, K., Trinick, J., and Knight, P.J. (2004). Use of negative stain and single-particle image processing to explore dynamic properties of flexible macromolecules. J. Struct. Biol. *147*, 247-258.

Chae, H.Z., Robison, K., Poole, L.B., Church, G., Storz, G., and Rhee, S.G. (1994). Cloning and sequencing of thiol-specific antioxidant from mammalian brain: alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. Proc. Natl. Acad. Sci. USA *91*, 7017-7021.

Chang, T.S., Jeong, W., Choi, S.Y., Yu, S., Kang, S.W., and Rhee, S.G. (2002). Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. J. Biol. Chem. *277*, 25370-25376.

Chauhan, R., and Mande, S.C. (2001). Characterization of the *Mycobacterium tuberculosis* H37Rv alkyl hydroperoxidase AhpC points to the importance of ionic interactions in oligomerization and activity. Biochem. J. *354*, 209-215.

Cheong, N.E., Choi, Y.O., Lee, K.O., Kim, W.Y., Jung, B.G., Chi, Y.H., Jeong, J.S., Kim, K., Cho, M.J., and Lee, S.Y. (1999). Molecular cloning, expression, and functional characterization of a 2Cys-peroxiredoxin in Chinese cabbage. Plant Mol. Biol. *40*, 825-834.

Chuang, M.H., Wu, M.S., Lo, W.L., Lin, J.T., Wong, C.H., and Chiou, S.H. (2006). The antioxidant protein alkylhydroperoxide reductase of *Helicobacter pylori* switches from a peroxide reductase to a molecular chaperone function. Proc. Natl. Acad. Sci. USA *103*, 2552-2557.

Haley, D., Horwitz, J., and Stewart, P.L. (1998). The small heat

shock protein, αB-crystallin, has a variable quaternary structure. J. Mol. Biol. *277*, 27-35.
Hartl, F.U. (1996). Molecular chaperone in cellular protein folding. Nature *381*, 571-580.
Hendrick, J.P., and Hartl, F.U. (1993). Molecular chaperone functions of heat shock proteins. Annu. Rev. Biochem. *62*, 349-384.
Hirotsu, S., Abe, Y., Okada, K., Nagahara, N., Hori, H., Nishino, T., and Hakoshima, T. (1999). Crystal structure of a multifunctional 2-Cys peroxiredoxin heme-binding protein 23 kDa/proliferation-associated gene product. Proc. Natl. Acad. Sci. USA *96*, 12333-12338.
Hofmann, B., Hecht, H.J., and Flohé, L. (2002). Peroxiredoxins. Biol. Chem. *383(3-4)*, 347-364.
Ito, H., Kamei, K., Iwamoto, I., Inaguma, Y., Nohara, D., and Kato, K. (2001). Phosphorylation-induced change of the oligomerization state of alpha B-crystallin, J. Biol. Chem. *276*, 5346-5352.
Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.K., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W., et al. (2004). Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. Cell *117*, 625-635.
Jang, H.H., Chi, Y.H., Park, S.K., Lee, S.S., Lee, J.R., Park, J.H., Moon, J.C., Lee, Y.M., Kim, S.Y., Lee, K.H., et al. (2006). Structural and functional regulation of eukaryotic 2-Cys peroxiredoxins including the plant ones in cellular defense signaling mechanisms against oxidative stress. Physiol. Plant. *126*, 549-559.
Jeong, W.J., Cha, M.K., and Kim, I.H. (2000). A new member of human Tsa/AhpC as thioredoxin-dependent thiol peroxidase. J. Biochem. Mol. Biol. *33*, 234-241.
Kim, K.S., Choi, S.Y., Kwon, H.Y., Won, M.H., Kang, T.C., and Kang, J.H. (2002). Aggregation of α-synuclein induced by the Cu, Zn-superoxide dismutase and hydrogen peroxide system. Free Radic. Biol. Med. *32*, 544-550.
Kim, J.A., Park, S., Kim, K., Rhee, S.G., and Kang, S.W. (2005). Activity assay of mammalian 2-cys peroxiredoxins using yeast thioredoxin reductase system. Anal. Biochem. *338*, 216-223.
Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999). Stimulation of peroxidase activity by decamerization related to ionic strength: ahpC protein from *Amphibacillus xylanus*. J. Biochem. *126*, 313-319.
Lee, G.J., Roseman, A.M., Saibil, H.R., and Vierling, E. (1997). A small heat shock protein stably binds heat-denatured model substrates and can maintain a substrate in a folding-competent state. EMBO J. *16*, 659-671.
Lee, J.R., Lee, S.S., Jang, H.H., Lee, Y.M., Park, J.H., Park, S.-C., Moon, J.C., Park, S.K., Kim, S.Y., Lee, S.Y., et al. (2009). Heat-shock dependent oligomeric status alters the function of a plant-specific thioredoxin-like protein, AtTDX. Proc. Natl. Acad. Sci. USA *106*, 5978-5983.
Moon, J.C., Hah, Y.S., Kim, W.Y., Jung, B.G., Jang, H.H., Lee, J.R., Kim, S.Y., Lee, Y.M., Jeon, M.K., Kim, C.W., et al. (2005). Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H_2O_2-induced cell death, J. Biol. Chem. *280*, 28775-28784.
Nooren, I.M.A., and Thornton, J.M. (2003a). Structural characterisation and functional significance of transient protein-protein interactions. J. Mol. Biol. *325*, 991-1018.
Nooren, I.M.A., and Thornton, J.M. (2003b). Diversity of protein-protein interactions. EMBO J. *22*, 3486-3492.
Papp, E., Nardai, G., Söti, C., and Csermely, P. (2003). Molecular chaperones, stress proteins and redox homeostasis. BioFactors *17*, 249-257.
Park, S.G., Cha, M.K., Jeong, W., and Kim, I.H. (2000). Distinct physiological functions of thiol peroxidase isoenzymes in *Saccharomyces cerevisiae*. J. Biol. Chem. *275*, 5723-5732.
Schröder, E., Littlechild, J.A., Lebedev, A.A., Errington, N., Vagin, A.A., and Isupov, M.N. (2000). Crystal structure of decameric 2-Cys peroxiredoxin from human erythrocytes at 1.7 Å resolution. Structure *8*, 605-615.
Wood, Z.A., Schroder, E., Robin, H.J., and Poole, L.B. (2003). Structure, mechanism and regulation of peroxiredoxins. Trends Biochem. Sci. *28*, 32-40.