Dimer-Oligomer Interconversion of Wild-type and Mutant Rat 2-Cys Peroxiredoxin

DISULFIDE FORMATION AT DIMER-DIMER INTERFACES IS NOT ESSENTIAL FOR DECAMERIZATION

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Rat heme-binding protein 23 (HBP23)/peroxiredoxin (Prx I) belongs to the 2-Cys peroxiredoxin type I family and exhibits peroxidase activity coupled with reduced thioredoxin (Trx) as an electron donor. We analyzed the dimer-oligomer interconversion of wild-type and mutant HBP23/Prx I by gel filtration and found that the C52S and C173S mutants existed mostly as decamers, whereas the wild type was a mixture of various forms, favoring the decamer at higher protein concentration and lower ionic salt concentration than the wild type was. The C83S mutant was predominantly dimeric, in agreement with a previous crystallographic analysis (Hirotsu, S., Abe, Y., Okada, K., Nagahara, N., Hori, H., Nishino, T., and Hakoshima, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12333–12338). X-ray diffraction analysis of the decameric C52S mutant revealed a toroidal structure (diameter, ~130 Å; inside diameter, ~55 Å; thickness, ~45 Å). In contrast to human Prx I, which was recently reported to exist predominantly as the decamer with Cys83-Cys83 disulfide bonds at all dimer-dimer interfaces, rat HBP23/Prx I has a Cys83-Cys83 disulfide bond at only one dimer-dimer interface (S–S separation of ~2.1 Å), whereas the interactions at the other interfaces (mean S–S separation of 3.6 Å) appear to involve hydrophobic and van der Waals forces. This finding is consistent with gel filtration analyses showing that the protein readily interconverts between dimer and oligomeric forms. The C83S mutant exhibited similar peroxidase activity to the wild type, which is exclusively dimeric, in the Trx/Trx reductase system. At higher concentrations, where the protein was mostly decameric, less efficient attack of reduced Trx was observed in a [14C]iodoacetamide incorporation experiment. We suggest that the dimer-decamer interconversion may have a regulatory role.

Peroxiredoxin (Prx) family proteins are found in all biological kingdoms from bacteria to animals and humans. Among six isotypes of Prx in mammals, four (Prx I-IV) contain two conserved cysteine residues, Cys52 and Cys173 (numbering is that of Prx I) in their sequences, and they are referred to as 2-Cys Prx. Rat HBP23, heme-binding protein 23, is a major cytosolic 2-Cys Prx, type I (Refs. 1–6 and see Ref. 7 for review).

Prxs are not only involved in the defense system against oxidative stress but also in the regulation of important cellular processes such as transcription, apoptosis, and cellular signaling (4–14). They interact not only with thiol proteins to express peroxidase activity but also with other proteins, such as the non-receptor tyrosine kinase c-Abl, the transcription factor c-Myc, and the macrophage migration inhibitory factor MIF, as well as forming stable oligomers with other Prx proteins (15–18). The functions of Prx are modulated in many different ways, including C-terminal truncation, phosphorylation, and peroxidation, as well as by the sulfiredoxin system (19–23).

The 2-Cys Prxs exhibit thioredoxin (Trx)-dependent peroxidase activity, reducing hydrogen peroxide, peroxinitrite, and organic peroxides to water, nitrite, and the corresponding alcohols, respectively. During the reaction cycle, the active site Cys52 of the homodimeric form (the minimal catalytic unit) is oxidized to an intermediate sulfenic acid (Cys52-SOH) and regenerated by Trx with the assistance of Cys173 from another subunit. We previously reported the crystal structure of the oxidized dimeric form of the rat HBP23/Prx I C83S mutant (PDB entry 1QQ2), in which the active site Cys52 forms a disulfide bridge with Cys173 from another subunit by C-terminal trapping and the corresponding alcohols, respectively. During the reaction cycle, the active site Cys52 of the homodimeric form (the minimal catalytic unit) is oxidized to an intermediate sulfenic acid (Cys52-SOH) and regenerated by Trx with the assistance of Cys173 from another subunit. We previously reported the crystal structure of the oxidized dimeric form of the rat HBP23/Prx I C83S mutant (PDB entry 1QQ2), in which the active site Cys52 forms a disulfide bridge with Cys173 from another subunit by C-terminal trapping and the corresponding alcohols, respectively.

The abbreviations used are: Prx, peroxiredoxin; Trx, thioredoxin; HBP23, heme-binding protein 23; DTT, dithiothreitol; IAA, iodoacetamide; MIF, migration inhibitory factor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PDB, Protein Data Bank.
distance from Cys\textsuperscript{52} to Cys\textsuperscript{173} is \(~13\) Å. In the oxidized dimeric structure, however, Cys\textsuperscript{52} is separated from Arg\textsuperscript{28} by about 7.4 Å \((24)\). On the basis of biological and structural findings with Trx, it has been suggested that a mixed disulfide bond between Trx Cys\textsuperscript{33} and Prx Cys\textsuperscript{73}, formed during the catalysis, is attacked by Trx Cys\textsuperscript{36}, generating reduced Prx and oxidized Trx \((\text{numbering in Escherichia coli, Refs. } 29–33)\). Trx, which is a small protein \((\text{molecular mass } \approx 12 \text{ kDa})\), plays an important role in cellular processes via redox regulation by undergoing reversible oxidation/reduction reaction of two cysteine residues, Cys\textsuperscript{33} and Cys\textsuperscript{36}. The 2-Cys Prxs are abundantly expressed in mammalian cells, and their content is estimated to be \(>20\)-fold higher than that of Trx \((1, 23, 34)\). These findings suggest that there is some modulating system for the interaction between the two proteins.

It seems reasonable to suppose that the interaction between Prx and Trx is in part regulated through their quaternary structures. Oligomers larger than the dimer have been observed in solution for 2-Cys Prxs from bacteria, plants, and humans \((27, 28, 35–39)\). Redox state, protein concentration, pH, and ionic strength are reported to influence the oligomeric state but differ in their effects upon different 2-Cys Prxs. Furthermore, the state of amino acid residues at the dimer-dimer interface also influences oligomerization and peroxidase activity \((20, 28, 39)\). Further, human Prx I and Prx II have distinct quaternary structures; Prx I was recently reported to exist predominantly as the decamer with a Cys\textsuperscript{83} - Cys\textsuperscript{83} disulfide bridge at the dimer-dimer interface, whereas Prx II exists as a dimer, because the corresponding Cys residue is replaced with Thr in Prx II \((40)\). Based on this feature, it was proposed that the function of Prx I is distinct from that of Prx II.

Here, to address the relationship between the oligomeric properties and peroxidase activity of mammalian 2-Cys Prxs, we prepared HBP23/Prx I variants by using site-directed mutagenesis, and we also performed an x-ray structure analysis of decameric Prx I. In contrast to the recently reported result for human Prx I \((40)\), our crystal structure of the decameric rat C52S mutant \((\text{Protein Data Bank (PDB) entry 2Z9S})\) showed that only one out of the five dimer-dimer interfaces involved a disulfide bridge from Cys\textsuperscript{83} to Cys\textsuperscript{83} of the adjacent dimer, indicating that stabilization of the decamer structure is not due to Cys\textsuperscript{83} - Cys\textsuperscript{83} disulfide bridges at the dimer-dimer interfaces, but rather, is predominantly due to hydrophobic interaction and structural fitting, as is seen in the decameric structures of bacterial \((26–28)\) and human \((25)\) Prx II.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Proteins**—The coding region of HBP23/Prx I was amplified by PCR using the forward primer, 5\textsuperscript{′}-AGC CAT ATG TCT TCA GGA AAT GCA A-3\textsuperscript{′}, and reverse primer, 5\textsuperscript{′}-TCG GAT CCT CAC TTC TGC TTA GAG AAA TAC TC-3\textsuperscript{′}. The PCR product was digested with NdeI/BamHI and cloned into the corresponding site of pET3c vector. This expression vector was named pET3c-HBP23. Mutants of HBP23/Prx I were prepared at Cys\textsuperscript{52}, Cys\textsuperscript{83}, Cys\textsuperscript{73}, Arg\textsuperscript{128}, and Arg\textsuperscript{151}, which were replaced with Ser, Lys, Ala, Glu, and Asp, respectively, using a QuikChange\textsuperscript{TM} site-directed mutagenesis kit \((\text{Stratagene})\). All of the site-directed mutations were confirmed by DNA sequencing. The E. coli strain BL21/DE3 was transformed with pET3c-HBP23 for overexpression of HBP23/Prx I and cultured as described previously \((41)\). Each HBP23/Prx I recombinant protein was purified by the methods described previously \((41)\).

The Trx gene of E. coli \((\text{E-TrxA})\) was prepared from pTrx plasmid \((\text{Invitrogen})\). The Ndel/SalI DNA fragment from pTrx was cloned into pET30a or pET28a and designated pET30-ETRxA or pET28-HisETxA, respectively. pET30-ETRxA plasmid is the expression vector for the wild-type Trx, and pET28-HisETxA is the expression vector for modified Trx containing a His tag sequence \((\text{six histidine residues})\) at the N-terminal region. Site-directed mutation of E-TrxA, changing Cys\textsuperscript{33} or Cys\textsuperscript{36} to Ser, was carried out using the QuikChange\textsuperscript{TM} site-directed mutagenesis kit \((\text{Stratagene})\). All of the variant genes for Trx and His-tagged Trx were confirmed by DNA sequencing. The E. coli strain BL21/DE3 was transformed with pET30-ETRxA or pET28-HisETxA, cultured in LB medium containing 50 \(\mu\)g/ml kanamycin at 37 °C to an absorbance of 0.7 at 600 nm, and induced with 0.1 mm isopropyl-1-thio-\(\beta\)-d-galactopyranoside for 4 h before being harvested by centrifugation. The cell pellets were suspended in 50 mm Tris-HCl, pH 8.0, containing 0.1 mm NaCl and 1 mm EDTA \((\text{buffer A})\) and disrupted by sonication. The soluble fraction recovered by centrifugation was treated at 65 °C for 5 min. After centrifugation, the supernatant was dialyzed against 10 mm Tris-HCl, pH 8.0, and applied to a DE-52 ion-exchange column \((2.5 \times 5.0 \text{ cm})\). Proteins were eluted with a gradient of 0–0.3 mm NaCl. His-tagged Trx was purified on a Ni\textsuperscript{2+} -nitrilotriacetic acid column according to the procedures recommended by the manufacturer.

The Trx reductase \((\text{TrxR})\) gene was prepared from genomic DNA of E. coli strain JM109 by PCR \((\text{DNA Data Bank in Japan/ European Molecular Biology Laboratory (DDBJ/EMBL accession number J03762})\). The primers used for PCR were as follows: forward primer, 5\textsuperscript{′}-GGG ATC CCA TGG CAG CAC CAA ACA C-3\textsuperscript{′}, and reverse primer, 5\textsuperscript{′}-ACC CAT AGT CGC ATG GTG TC-3\textsuperscript{′}. The PCR fragment was cloned into the Smal site of pUC119 vector, and the cloned gene was sequenced. The direction of the TrxR gene was the same as that of the lacZ gene of pUC119, and the plasmid was designated pUC-TrxR. The E. coli strain JM109 was transformed with pUC-TrxR, and the transformant constitutively overproduced the Trx reductase protein. To prepare Trx reductase, the transformant was cultured in LB medium containing 50 \(\mu\)g/ml ampicillin at 37 °C for 12–18 h. The cell pellets were suspended in buffer A and disrupted by sonication. After centrifugation, the supernatant was fractionated with ammonium sulfate \((40–80\% \text{ saturation})\). After dialysis against 20 mm Tris-HCl, pH 8.0, the fraction was applied to a DE-52 ion-exchange column \((2.5 \times 5.0 \text{ cm})\), and proteins were eluted with a gradient of 0–0.4 mm NaCl. Fractions containing the protein were dialyzed against 20 mm Tris-HCl, pH 8.0, and applied to a 2’,5’-ADP-Sepharose affinity column \((0.6 \times 7.5 \text{ cm})\). Trx reductase was eluted with 20 mm Tris-HCl, pH 8.0, containing 0.1 mm NaCl and 10 mm NADP. Examination of Coomassie Blue-stained bands after gel electrophoresis indicated that each isolated recombinant protein was \(>90\%\) pure.

Oligomerization of Rat Peroxiredoxin I
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The concentration of purified proteins, HBP23/PrxI, Trx, and Trx reductase, was determined with a Coomassie Blue protein assay reagent kit (Pierce). Bovine serum albumin was used as a standard.

**Determination of Peroxidase Activity**—The peroxidase activity was measured with two systems, the Trx- and DTT-based systems (42, 43). The peroxidase activity in the presence of Trx was assayed in 0.7 ml of reaction mixture containing 50 mM HEPES-NaOH (pH 7.0), 0.5 mM EDTA, 60 μg of Trx, 2 μg of Trx reductase, 0.1 mM H2O2, 150 μM NADPH, and the appropriate amount of HBP23/PrxI at 25 °C. Reaction was initiated by the addition of H2O2, and consumption of NADPH was monitored spectrophotometrically at 340 nm. The peroxidase activity in the presence of DTT was assayed as described previously, with a slight modification. The reaction mixture (0.7 ml) contained 150 mM potassium phosphate buffer, pH 7.4, 50 mM DTT, 30 mM t-butyl hydroperoxide and the appropriate amount of HBP23/PrxI at 25 °C. The activity was measured spectrophotometrically at 310 nm.

**Size-exclusion Chromatography**—Size-exclusion chromatography was performed at room temperature using the fast protein liquid chromatography system (Amersham Biosciences) with a Sephacryl S-200 column. Ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and myoglobin (17 kDa) were used as molecular mass standard markers for calibration of the column. Elution of liver extract or molecular mass standards was monitored at 280 nm. Absorbance at 280 nm was measured with a Hitachi U-3200 spectrophotometer to monitor elution of protein from the column. Protein-containing fractions of liver extracts were subjected to SDS-PAGE (15% gel) according to the method of Laemmli (44). Antibody against HBP23/PrxI was prepared as described previously (45).

**[14C]IAA Labeling of Sulphydryl Groups of HBP23/Prx I in the Presence of Trx for Determination of Reductive Reaction of Cysteine Disulfide Bond by Reduced Trx**—HBP23/PrxI wild type and the C83S mutant were incubated for 30 min on ice with sufficient iodoacetamide (IAA) to alkylate free cysteine residues and then passed through a NAD™ column (Amersham Biosciences) equilibrated with 0.1 M Tris-HCl, pH 8.0. The reaction mixture (0.2 ml), containing the indicated amounts of the treated HBP23/PrxI and reduced Trx in 0.1 M Tris-HCl, pH 8.0, with 4 μl of 4 mM IAA, 0.2 μCi of [14C]IAA, was incubated for various periods on ice. The reaction was initiated by the addition of reduced Trx. At the indicated times, an aliquot (20 μl) was removed, the reaction was immediately stopped by adding 50 mM cysteine, and the mixture was separated on 15% SDS-PAGE gel. The incorporation of [14C]IAA into protein was estimated by using a BAS 2000 instrument.

**X-ray Crystallography of HBP23/Prx I**—The C52S mutant was crystallized by the hanging drop method at 20 °C by mixing 2 μl of the protein solution (5 mg/ml in 5 mM sodium acetate, pH 5.0, 2 mM DTT, 1 mM CHAPS) with 2 μl of reservoir solution (0.17 mM ammonium acetate, 20% glycerol, 25% polyethylene glycol 4000, 84 mM sodium acetate, pH 5.0). Plate crystals appeared within 2 or 3 days and grew to their full size in 2 weeks. The crystals were soaked with a precipitant solution containing 30% glycerol and then plunged into liquid nitrogen. The data set was collected at the BL-5 beamline of the Photon Factory (Tsukuba, Japan) using a Quantum 315 area detector. The data set was obtained at 2.9 Å resolution. The data were processed and scaled with HKL2000. Molecular replacement was performed with EPMR using the coordinates of the crystal structure of human decameric 2-Cys Prx II purified from erythrocytes (Protein Data Bank entry 1QMV; Ref. 22 and see also Ref. 46). The model was refined with CNS (47). The final R-factor and free R-factor were 20.5 and 28.0%, respectively.

**RESULTS AND DISCUSSION**

**Molecular Mass of HBP23/Prx I**—Although we previously determined the crystal structure of the C83S mutant of rat liver HBP23/Prx I as a dimer (PDB entry 1QQ2), oligomerized forms other than the dimer are observed in solution for many 2-Cys Prxs from bacteria, plants, and humans (27, 28, 35–39) (supplemental Fig. 1). To examine the molecular species of HBP23/PrxI in rat liver, the homogenate was subjected to size-exclusion chromatography using a Sephacryl S-200 column pre-equilibrated with 50 mM phosphate buffer, pH 7.4, containing 0.1 M NaCl (Fig. 1). SDS-PAGE and Western blotting showed that protein was eluted in at least two different positions. Comparison with molecular mass standards showed that HBP23/PrxI in the 20% homogenate was predominantly eluted at the position of molecular mass 44 kDa, but HBP23/PrxI in the 33% homogenate was also eluted at the position of molecular mass 230 kDa, thus being present as both dimer and decamer. HBP23/PrxI seems to form dimeric and decameric structures depending on the protein concentration or ionic strength, similarly to other 2-Cys Prxs. Therefore, we further studied the quaternary structure of the recombinant HBP23/PrxI by means of chromatographic analysis under various conditions of protein concentration/ionic strength, as well as in the absence or presence of DTT.

When 0.5 mg of freshly purified wild-type protein (1 ml) was applied to a Sephacryl S-200 column at 0.2 M NaCl, the elution profile was more complex in the absence of DTT than in its presence (Fig. 2). In the absence of DTT, the wild-type protein tended to form the decamer at high protein concentration, but the decamer tended to dissociate into tetramer or dimer as the solution was diluted (Fig. 2, A and C) or when the ionic strength was increased (Fig. 2D), whereas in the presence of DTT, the decamer was the only form under all the conditions studied (Fig. 2B). These results suggested that Cys83 may not be involved in decamer formation through disulfide bond formation, although it is located at the dimer-dimer interface in the decameric structure (see below). The mutants C52S and C173S, which cannot form an intermolecular disulfide bridge in the vicinity of the active site under oxidative conditions, were eluted only as decamers under all conditions studied (Fig. 2, A and B), which is consistent with the observation that reduced, DTT-treated decameric HBP23/PrxI is rather stable. The mutant C83S was eluted at the position corresponding to the dimer in the absence of DTT. Upon the addition of DTT, however, the dimer reverted to the monomer rather than forming the
decamer, suggesting that the replacement of the sulfur atom with oxygen influences the dimer-dimer interaction. The double mutants, C52S/C83S and C83S/C173S, were eluted at positions corresponding to both dimer and monomer. However, in both cases, the monomer content was increased by the addition of DTT, probably because disulfide bridge formation between Cys$^{173}$ and Cys$^{173}$ or between Cys$^{52}$ and Cys$^{52}$ became less favorable (Fig. 2, A and B).

Crystal Structure of Decameric HBP23/Prx I—To elucidate in detail the structural features of HBP23/Prx I, the C52S mutant was successfully crystallized, and its structure was determined (PDB entry 2Z9S) (supplemental table). The final model was compared with that of the oxidized dimeric C83S mutant. The diffraction data were collected to 2.9 Å resolution. The crystal belongs to the P2$_1$ space group with one molecule in the asymmetric unit. The structure was determined by molecular replacement techniques and subjected to molecular dynamics refinement. The model has good overall stereochemistry, with 82.9% of all residues in the most favorable region of the Ramachandran plot. Residues in disallowed regions are defined in 2Fo−Fc electron density maps.

The C52S mutant was crystallized as a toroidal decamer (Fig. 3) very similar to the reported structures of other Prx species (25–28, 36). The toroid has a diameter of $\sim$130 Å, an inside diameter of $\sim$55 Å, and a thickness of $\sim$45 Å. A decamer is composed of five dimers, forming an ($\alpha$)$_5$ complex. The chain trace of each monomer is quite clear and continuous from amino acid residues 3–198. We observed clear densities of the C-terminal loops (residues 176–198), whereas this was not the case in the C83S mutant dimer, probably because of dislocation (24). The extra C-terminal fold of the C52S mutant contains a loop region (residue 176–187) and a short helix (residue 188–197).

Only weak interactions appear to exist between dimers. The dimer-dimer interfaces involve mainly van der Waals contacts of hydrophobic residues, including Thr$^{46}$, Phe$^{81}$, Phe$^{82}$, Ala$^{85}$, and Trp$^{87}$. All 10 Cys$^{83}$ residues are located at the interfaces. Among them, eight are in pairs without any electron density between SG atoms (Cys$^{83}$ of chains B and D, and G, E and J, and H and I). The distances between the SG atoms of these four cysteine pairs are obtained as 3.4–3.6 Å, which are too far for a disulfide bond but
consistent with van der Waals contacts between the sulfur atoms. However, one Cys83 pair (Cys83 of chains A and C) has clear electron density between the SG atoms, with a separation of ∼2.1 Å, which is consistent with a disulfide bond (Fig. 4). This disulfide bond is buried in the protein and is not exposed to the solvent, suggesting that it may not easily dissociate even in the presence of DTT. The structural changes accompanying the disulfide formation appear to be restricted. In the chain A and C pair, Cα of Cys83 and Ala84 move ∼0.7 Å toward their counterparts of the monomer pair, but this structural change does not extend to the rest of the protein. The root mean square deviations of the traces of chains A and B are 0.37 Å, whereas the root mean square deviation of chains A and C is 0.41 Å.

Fig. 5A compares the dimer structures of the C52S and C83S mutants. The core structures are very similar to each other, but some differences can be seen. The most significant difference between the two mutants is the position of the two cysteines (or corresponding Ser52 residue) in the active site. In the C83S mutant, Cys52 and Cys173 form a disulfide bond that is exposed to the solvent (24). On the other hand, Ser52 is positioned in the N terminus of helix α2 and is located in a hydrophobic pocket in the C52S mutant. The distance between the α-carbons of Ser52 and Cys173 is ∼13 Å, suggesting that this structure is in a catalytically inactive form since the two cysteine residues would be too far apart to form a disulfide bond efficiently in the catalytic cycle. This structural change in the active site extends to the dimer-dimer interface region. Formation of the disulfide bridge between Cys52 and Cys173 causes partial unfolding of the α2 helix, and Thr54 moves ∼7 Å. This change causes positional changes of residues Trp87 and Phe48, which are located at the dimer-dimer interface (Fig. 5B). The new locations of these residues may result in a steric clash with Ala86 from the adjacent monomer.

**Influence of Oligomerization on the Peroxidase Activity**—The molecular mechanism of the reduction of target proteins by reduced Trx is well understood in the *E. coli* system (29–32). *E. coli* Trx Cys33 forms a mixed disulfide intermediate with the target protein during the reduction process, and then attack of Cys36 results in release from the reduced protein. In the case of rat HBP23/Prx I, it was suggested that Cys173 is also a target sulfur for reduced Trx since Cys173 is located close to the surface of the molecule in the crystal structure of the oxidized dimer (PDB code 1QQ2). To confirm this, the interaction between HBP23/Prx I and Trx was examined by Ni2+ chromatography with a His-tagged *E. coli* Trx variant as the bait. Mutant HBP23/Prx I was applied to a Ni2+ column with the mutant His-tagged Trx C36S, and the fractions eluted with DTT were subjected to complex formation analysis and then resolved by SDS-PAGE (Fig. 6). The wild type and the HBP23/Prx I mutants C83S and C52S/C83S each formed a stable complex with the Trx mutant C36S. However, the HBP23/Prx I mutant C83S/C173S was not trapped on the column (Fig. 6, panel c). It should be noted that none of the HBP23/Prx I mutants studied was trapped by a Ni2+ column with the His-tagged Trx mutant C33S. In parallel, it was found that none of the HBP23/Prx I mutants studied showed peroxidase activity in the reaction system containing the Trx mutant C33S, instead of the wild-type Trx (not shown). These results clearly demonstrate that HBP23/Prx I Cys173 is indeed the target sulfur of reduced Trx. The reduced Cys52 residue is likely attacked by hydrogen peroxide, and then a water molecule is released by heterolytic cleavage. Finally, another water molecule is released by attack of the sulfur base of Cys173 to form Cys52·Cys173 disulfide-bridge.

The finding in this study that the distance between Ser52 and Cys173 in the crystal structure of the C52S mutant (PDB code 2Z9S) is as large as 13 Å suggests that the structure of mutant C52S is catalytically inactive. However, the results of the gel filtration study indicate that even wild-type oxidized enzyme can form the decamer under certain conditions, such as high protein concentration. Thus, as proposed previously (24–28), the decameric
structure of the oxidized wild-type enzyme may have a different conformation from that determined in this study. As it was of interest to examine the relationship between decamerization of the wild-type HBP/Prx I and peroxidase activity, we compared the reductive half-reaction by reduced Trx at high concentrations of wild-type enzyme (mainly in decameric form) and the C83S mutant (exclusively in dimeric form). It should be noted, however, that the determination of activity using the standard Trx reductase/Trx system is not practically easy since large amounts of Trx reductase/Trx are necessary to ensure that the rate-limiting step of the overall reaction is the Prx peroxidase reaction under conditions of high Prx concentration where the main species of Prx is the decamer. It is likely that the Trx-dependent peroxidase activity of HBP23/Prx I is influenced by Trx binding efficiency. It is noteworthy that the C83S mutant showed similar activity to that of the wild type in the Trx system under diluted conditions (Table 1). To estimate the influence of the quaternary structure of HBP23/Prx I, the apparent reduction rate was estimated by mixing the wild-type HBP/Prx I with a higher concentration of reduced Trx under ice cooling followed by incorporation of $^{14}$CIAA, as described under “Experimental Procedures.” After mixing a high concentration of HBP/Prx I with reduced Trx, significant incorporation of $^{14}$CIAA was observed, as shown in Fig. 7, whereas only a small amount of $^{14}$CIAA was incorporated into HBP/Prx I alone; ~7% of free cysteine residues of HBP23/Prx I was modified by $^{14}$CIAA after incubation for 1 h on ice (data not shown). These results indicated that HBP23/Prx I is oxidized even in decameric form, and the active site disulfide was reduced by reduced Trx followed by incorporation of $^{14}$CIAA. The efficiency of time-dependent incorporation of $^{14}$CIAA into the mutant C83S, however, was 1.4-fold higher than that into the wild type during 8–24 min (13 nmol of Trx and 11 nmol of HBP23/Prx I), and then the incorporation reached a plateau level because of consumption of reduced Trx because of simultaneous competitive incorporation of $^{14}$CIAA into reduced Trx. The incorporation of radioactivity into Trx was confirmed to be 1.3 times greater with the wild type than with mutant C83S after separation of Trx by means of SDS-PAGE. A similar value, 1.4-fold, was obtained when 35 nmol of Trx and 26 nmol of HBP23/Prx I were used (data not shown). These results suggest that decamerization of HBP23/Prx I decreases the binding efficiency of Trx.

**Influence of the Mutation on Peroxidase Activity**—To clarify the role of amino acid residues in the active site of HBP23/Prx I, the activity of variants was examined in two ways, i.e. toward H$_2$O$_2$ in the presence of the Trx system, NADPH, Trx, and Trx reductase and toward t-butyl hydroperoxide in the presence of DTT. The former was determined by measuring the consumption of NADPH in terms of decrease of absorption at 340 nm, and the latter was determined by estimating the amount of oxidized DTT, as described under “Experimental Procedures.” For HBP23/Prx I, the system-composed *E. coli* Trx and Trx reductase were found to be effective (supplemental Fig. 2), so the following analysis was done with *E. coli* Trx and Trx reductase. The activity of all mutants determined in the Trx system was not significantly
different from that in the DTT system except in the case of mutation at position 173 (Table 1). As expected, the C52S mutant exhibited no activity in either of the assay systems. The C173S mutant was fully active using DTT as an electron transfer partner, although it was inactive in the Trx system. This is consistent with the above conclusion that Cys\(^{173}\) plays an essential role in the interaction with Trx as the immediate electron donor. It should be noted that C83S exhibited activity similar to that of the wild type in the Trx system, and it is present exclusively as the dimer, as determined with size-exclusion chromatography. In addition, the double mutant C83S/C173S exhibited the highest DTT-dependent activity among the variants studied, like the plant enzyme (39), in accordance with the idea that DTT can act in place of Cys\(^{173}\) and that Cys\(^{52}\) is involved in normal peroxidase activity. The substitution of the arginine residues at positions 128 and 151 dramatically affected the activity. When either of these was replaced with a lysine, alanine, or glutamate residue in the C83S mutant, the activity decreased to 2–7% of that of the wild type in both systems. Thus, Arg\(^{128}\) appears to be important for the reactivity of the active site Cys\(^{52}\), as discussed previously (24–28). Substitution to lysine at these positions did not restore the activity, probably due to its lower strength as a base. Substitution to alanine or glutamine at these residues does not allow hydrogen bond formation with Cys\(^{52}\).

CONCLUSIONS

The present study of rat HBP23/Prx I demonstrates that the redox state of the active site cysteine residues and small conformational changes of the amino acid residues located at the dimer-dimer interface have an important influence on the quaternary structure of HBP23/Prx I. Although the Cys\(^{83}\) residue, which is replaced with other residues in other Prx species, is located at the dimer-dimer interface, only one out of five cysteine pairs in the decamer was found to be disulfide-bonded on the basis of x-ray diffraction analysis. This result was also confirmed by other diffraction data sets and is in contrast to a recent report on human Prx I, which has a very similar amino acid sequence to rat HBP23/Prx I; both rat and human Prx I contain conserved Cys\(^{83}\) residues. It was reported that human Prx I is mainly in a decamer form having disulfide bridges at the dimer-dimer interfaces, and it was therefore suggested that the main function of Prx I might be to work as a chaperone, rather than as a peroxidase (40). Our present crystallographic analysis of the rat Prx I decamer indicated that only one dimer-dimer interface involves a disulfide bond, and the others involve weak interactions, such as hydrophobic interaction and van der Waals contacts (including the sulfur atom of Cys\(^{83}\)). This is consistent with the results of gel filtration experiments showing that
The decameric form of the wild-type enzyme dissociates into dimer and probably tetramer forms under conditions of low protein concentration or high ionic strength solvent. The addition of DTT to the wild-type enzyme favored the decameric form rather than dimeric form, possibly due to reduction of the disulfide bond in the active site. The fact that only one of the cysteine pairs was disulfide-bonded suggests that the decamer might modulate not only the functions of 2-Cys Prx but also the regulation mechanism based on protein-protein interaction. Phosphorylation, peroxidation, and sulfiredoxin (19–23) have been reported, including C-terminal truncation, one of the regulatory systems for the functions of 2-Cys Prx. The effect of assembly on the peroxidase activity and the binding efficiency with the direct electron donor protein seems to vary from species to species in the Prx family (20, 28, 35, 38). The dimeric form of Salmonella typhimurium 2-Cys Prx exhibited lower binding efficiency with AhpF, a direct electron donor, and lower peroxidase activity than the decameric form (28). However, the binding efficiency of reduced Trx with dimeric HBP23/Prx I was rather higher than that of the decamer, possibly because of a higher collision probability. It was also reported that the high molecular weight 2-Cys Prxs of human or yeast formed by phosphorylation show lower peroxidase activity (20). Thus, it is possible that oligomer formation is one of the regulatory systems for the functions of 2-Cys Prx. Various other modulation systems for the peroxidase activity of 2-Cys Prx have been reported, including C-terminal truncation, phosphorylation, peroxidation, and sulfiredoxin (19–23). The regulation mechanism based on protein-protein interaction may modulate not only the functions of 2-Cys Prx but also the function of Trx.

The interaction between HBP23/Prx I and Trx appears to be regulated by redox state and by the quaternary structure of HBP23/Prx I, as described above. Trx, which is functionally involved in a variety of cellular processes as a potent endogenous reducing reagent, is estimated to be present at a concen-
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FIGURE 8. Proposed functional role of oligomerization of HBP23/Prx I. The present study shows that the dimer-decamer interconversion of HBP23/Prx I is influenced by protein concentration, ionic strength, and redox state. For efficient binding to Trx and other molecules and for translocation into the nucleus, the dimeric form is preferred to the decameric form.

REFERENCES

1. Iwahara, S., Satoh, H., Song, D. X., Webb, J., Burlingame, A. L., Nagae, Y., and Muller-Eberhard, U. (1995) Biochemistry 34, 13398–13406
2. Immenschuh, S., Stritzke, J., Iwahara, S., and Ramadori, G. (1999) Hepatology 30, 118–127
3. Matsunoto, A., Okado, A., Fuji, T., Fuji, J., Egashira, M., Niikawa, N., and Taniguchi, N. (1999) FEBS Lett. 443, 246–250
4. Immenschuh, S., Baumgart-Vogt, E., Tan, M., Iwahara, S., Ramadori, G., and Fehimi, H. D. (2003) J. Histochem. Cytochem. 51, 1621–1631
5. Kato, S., Kato, M., Abe, Y., Matsumura, T., Nishino, T., Aoki, M., Itayama, Y., Asayama, K., Arawa, A., Hirano, A., and Ohama, E. (2005) Acta Neuropathol. 110, 101–112
6. Kato, M., Kato, S., Abe, Y., Nishino, T., Ohama, E., Aoki, M., and Itayama, Y. (2006) Histol. Histopathol. 21, 729–742
7. Hofmann, B., Hecht, H. J., and Flohe, L. (2002) Biol. Chem. 383, 347–364
8. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) EMBO J. 10, 2247–2258
9. Klebic, T., Kinter, A., Poli, G., Anderson, M. E., Meister, A., and Fauci, A. S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 986–990
10. Montemartini, M., Kalisz, H. M., Hecht, H. J., Steinitz, P., and Flohe, L. (1999) Eur. J. Biochem. 264, 516–524
11. Andoh, T., Kiuie, C. C., and Chock, P. B. (2003) J. Biol. Chem. 278, 8038–8050
12. Uwayama, J., Hirayama, A., Yanagawa, T., Warabi, E., Sugimoto, R., Itoh, K., Yamamoto, M., Yoshida, H., Koyama, A., and Ishii, T. (2006) Biochem. Biophys. Res. Commun. 339, 226–231
13. Chen, S. T., and Van Etten, R. A. (1997) Genes Dev. 11, 2456–2467
14. Jung, H., Kim, T., Chae, H. Z., Kim, K. T., and Ha, H., (2001) J. Biol. Chem. 276, 15504–15510
15. Mu, Z. M., Yin, X. Y., and Prochownik, E. V. (2002) J. Biol. Chem. 277, 43175–43184
16. Egler, R. A., Fernandes, E., Rothermundt, K., Sereika, S., de Souza-Pinto, N., Jaruga, P., Dizdaroglu, M., and Prochownik, E. V. (2005) Oncogene 24, 8038–8050
17. Koo, K. H., Lee, S., Jeong, S. Y., Kim, E. T., Kim, H. J., Kim, K., Song, K., and Chae, H. Z. (2002) Arch. Biochem. Biophys. 397, 312–318
18. Jang, H. H., Kim, S. Y., Park, S. K., Jeon, H. S., Lee, Y. M., Jung, J. H., Lee, S. Y., Chae, H. B., Jung, Y. J., Lee, K. O., Lim, C. O., Chung, W. S., Bak, J. D., Yun, D. J., Cho, M. J., and Lee, S. Y. (2006) FEBS Lett. 580, 351–355
19. Bitez, B., Labarre, J., and Toledano, M. B. (2003) Nature 425, 980–984
20. Jeong, W., Park, S. J., Chang, T. S., Lee, D. Y., and Rhee, S. G. (2006) J. Biol. Chem. 281, 14400–14407
21. Georgiou, G., and Mapil, L. (2003) Science 300, 592–594
22. Hirotsu, S., Abe, Y., Okada, K., Nagahara, N., Horii, H., Nishino, T., and Hakoishi, T., (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12333–12338
23. Schröder, E., Littlechild, J. A., Lebedev, A. A., Errington, N., Vagin, A. A., and Isupov, M. N. (2000) Structure (Lond.) 8, 605–615
24. Alphesy, M. S., Bond, C. S., Tetaud, E., Fairlamb, A. H., and Hunter, W. N. (2000) J. Mol. Biol. 300, 903–916
25. Wood, Z. A., Poole, L. B., Hantgan, R. R., and Karplus, P. A. (2002) Biochemistry 41, 5493–5504
26. Parsonage, D., Youngblood, D. S., Sarma, G. N., Wood, Z. A., Karplus P. A., and Poole, L. B. (2005) Biochemistry 44, 10583–10592
27. Holmgen, A., Söeck Eklund, H., and Bra Prokson, T., and Warren, G. L., derberg, B. O., Eklund, H., and Brändén, C. I. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2305–2309
28. Katti, S. K., LeMaster, D. M., and Eklund, H. (1990) J. Mol. Biol. 212, 167–184
29. Kallis, G. B., and Holmgen, A. (1980) J. Biol. Chem. 255, 10261–10265
30. Nakamura, H., De Rosa, S., Roederer, M., and Matsumura, T. (2007) J. Biol. Chem. 282, 1561–1569
31. Lillig, C. H., and Holmgen, A. (2007) Antioxid. Redox Signal. 9, 25–47
32. Nakanuma, H., De Rosa, S., Roederer, M., Anderson, M. T., Dub, J. G., Yodoi, J., Holmgen, A., Herzenberg, L. A., and Herzenberg, L. A. (1996) Int. Immunol. 8, 603–611
33. Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999) J. Biol. Chem. 274, 313–319
34. Kitano, K., Kita, A., Nakahshima, T., Niimura, Y., and Miki, K. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 644–647
35. Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15504–15510
36. Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15504–15510
37. Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15504–15510
38. Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15504–15510
39. Kitano, K., Niimura, Y., Nishiyama, Y., and Miki, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15504–15510
40. Lee, W., Choi, K. S., Riddell, J., Ip, C., Ghosh, D., Park, J. H., and Park, Y. M. (2006) J. Biol. Chem. 281, 8038–8050
41. Lee, W., Choi, K. S., Riddell, J., Ip, C., Ghosh, D., Park, J. H., and Park, Y. M. (2006) J. Biol. Chem. 281, 8038–8050
41. Hirotsu, S., Abe, Y., Nagahara, N., Hori, H., Nishino, T., Okada, K., and Hakaoshima, T. (1999) J. Struct. Biol. 126, 80–83
42. Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) J. Biol. Chem. 269, 27670–27678
43. Iyer, K. S., and Klee, W. A. (1973) J. Biol. Chem. 248, 707–710
44. Laemmli, U. K., and Favre, M. (1973) J. Mol. Biol. 80, 575–599
45. Abe, Y., and Okazaki, T. (1987) Arch. Biochem. Biophys. 253, 241–248
46. Kissinger, C. R., Gehlhaar, D. K., and Fogel, D. B. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, 484–491
47. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, 905–921