The Tetrameric Structure of *Haemophilus influenza* Hybrid Prx5 Reveals Interactions between Electron Donor and Acceptor Proteins*

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Cellular redox control is often mediated by oxidation and reduction of cysteine residues in the redox-sensitive proteins, where thioredoxin and glutaredoxin (Grx) play as electron donors for the oxidized proteins. Despite the importance of protein–protein interactions between the electron donor and acceptor proteins, there has been no structural information for the interaction of thioredoxin or Grx with natural target proteins. Here, we present the crystal structure of a novel *Haemophilus influenza* peroxiredoxin (Prx) hybrid Prx5 determined at 2.8-Å resolution. The structure reveals that hybrid Prx5 forms a tightly associated tetramer where active sites of Prx and Grx domains of different monomers interact with each other. The Prx-Grx interface comprises specific charge interactions surrounded by weak interactions, providing insight into the target recognition mechanism of Grx. The tetrameric structure also exhibits a flexible active site and alternative Prx-Grx interactions, which appear to facilitate the electron transfer from Grx to Prx domain. Differences of electron donor binding surfaces in Prx proteins revealed by an analysis based on the structural information explain the electron donor specificities of various Prx proteins.

Peroxiredoxin (Prx)\(^1\) is a family of proteins that degrade reactive oxygen species in cells (1–3). Members of the Prx family are implicated in the defense of cells against oxidative stress as well as in the regulation of important cellular processes such as transcription, apoptosis, and cellular signaling (1–3). Prx proteins can be divided into two subgroups, 2-Cys Prx (1) and 1-Cys Prx (4), depending on the number of conserved cysteines. 2-Cys Prx proteins have two conserved cysteines, one in the N-terminal domain and the other in the C-terminal domain. The N-terminal cysteine directly reacts with reactive oxygen species substrates, resulting in the formation of cysteine sulfenic acid. The C-terminal cysteine, which is in the vicinity of the N-terminal cysteine of the other monomer in the dimeric structure found in most 2-Cys Prx proteins, forms a disulfide bond with the N-terminal cysteine sulfenic acid (5, 6). The disulfide bond is reduced by thioredoxin (Trx) to regenerate enzyme activity (1). In comparison, 1-Cys Prx has only the N-terminal cysteine, and enzyme activity of 1-Cys Prx usually does not involve either intermolecular disulfide bond formation or thioredoxin reduction (4). However, some 1-Cys Prx proteins have an extra non-conserved cysteine that plays the role of the conserved C-terminal cysteine in 2-Cys Prx (7). For example, human Prx5, which is a 1-Cys Prx implicated in tumor necrosis factor a signaling (7) and apoptosis regulation (8), uses thioredoxin as the electron donor, and the disulfide bond in human Prx5 is formed between the N-terminal conserved cysteine and the extra non-conserved cysteine (7).

Crystal structures for 1-Cys Prx (hORF6 (9) and Prx5 (10)) and 2-Cys Prx (HPB23 (6), TpxB (11), TryP (12), and AhpC (13)) proteins were reported. These structures revealed that Prx proteins have a thioredoxin fold with several insertions between secondary structural elements of the core thioredoxin fold. All Prx proteins except for human Prx5 were found as tightly associated dimers (\(a_2\)) or decamers (\((a_2)_5\)) utilizing the dimer as a basic unit. 2-Cys Prx proteins exhibit the structural transition between the dimeric and decameric states depending on environmental status such as redox states, ionic strength, and pH (11, 13–15). For example, the *Salmonella typhimurium* AhpC, a 2-Cys Prx, strongly favors the decameric structure in the reduced state, whereas the oxidized enzyme exists as a mixture of lower order oligomeric assemblies (13). Crystal lattices of human Prx5 (10) do not show the same dimer found in other Prx proteins, although the possibility of dimerization was reported previously (7).

Recently, novel hybrid Prx proteins with a glutaredoxin (Grx) domain fused in them were found from several pathogenic bacteria including *Haemophilus influenza* (GenBank\(^\text{TM}\) accession number P44758) (16), *Neisseria meningitidis* (GenBank\(^\text{TM}\) accession number CAB94403), and *Vibrio cholerae* (GenBank\(^\text{TM}\) accession number AE004330). An anaerobic sulfur-oxidizing phototroph, *Chromatium gracile* also has the hybrid Prx (17). The Prx and Grx domains of these hybrid proteins have sequence homology with human Prx5 and *Escherichia coli* Grx3, respectively (16, 18). The fusion of Grx domain in these hybrid Prx5 proteins suggests that Grx domain is likely to act as the electron donor for Prx domain of these proteins (18). *H. influenza* hybrid Prx5, which is a prototype example of these hybrid Prx proteins, was first found.
Crystal Structure of Hybrid Prx5

RESULTS AND DISCUSSION

Overall Structure—The refined structure of hyPrx5 reveals two discrete domains, peroxiredoxin (residues 3–162) and glutaredoxin (residues 171–241) domains with a connecting loop (residues 163–170) (Fig. 1a). No direct interactions are found between Prx and Grx domains in the monomeric structure. There are two monomers of hyPrx5 in the asymmetric unit of crystals, and the two monomers have different domain arrangements enabled by the flexible linker between Prx and Grx.

EXPERIMENTAL PROCEDURES

Crystallization and Structure Determination—HyPrx5 was crystallized at room temperature by the vapor diffusion method. For the crystallization, 2 μl of reservoir solution (1.6 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M Tris-HCl (pH 8.0)) and 2 μl of protein solution (10 mg/ml) were mixed and equilibrated against the reservoir. Tetragonal crystals appeared within 2 or 3 days and grew to their full size (1.002 g/ml), where r is the universal gas constant, T is the absolute temperature, ρ is the solvent density (1.002 g/ml), and ω is the angular velocity of the rotor. The final model contains residues 3–238 in molecule A, residues 3–241 in molecule B, and 4 sulfate ions. The R-value of the final model is 23.9% (Rfree = 28.3%) at 2.8 Å resolution. The figures were prepared by using the programs GRASP (27), RIBBONS (28), MOLESCRIPT (29), and BOBSCRIPT (30).

Crystallization and Structure Determination—HyPrx5 is a hybrid peroxiredoxin–glutaredoxin protein that consists of a two-domain structure. The first domain is a peroxiredoxin domain, residues 3–162, and the second domain is a glutaredoxin domain, residues 171–241. The two domains are connected by a flexible linker. The overall structure of hyPrx5 provides a framework for understanding the mechanism of target recognition by Grx as well as activities of the Prx-Grx hybrid proteins.

Table I

| Data collection statistics | Remote (A3) |
|---------------------------|-------------|
| Wavelength (Å)            | 0.9791      |
| Highest resolution (Å)    | 2.8 (2.95–2.80) |
| Unique reflections (total)| 13,143 (209,213) |
| Completeness (%)          | 98.5 (100.0) |
| Rmerge (%)                | 8.5 (26.4) |
| I/σ(I)                    | 7.9 (23.4) |

Refinement statistics

| Resolution range (Å)   | 99.2.8 |
| Number of reflections | 15,821 |
| Number of atoms (protein/non protein) | 3,718/20 |
| Rcryst | 23.9 |
| Rmerge | 28.3 |
| Root mean square deviations | Bond lengths (Å) | 0.008 |
| Bond angles (°)         | 1.4 |
| Improper (°)            | 0.83 |
| Dihedrals (°)          | 24.2 |

The values in parentheses (completeness and Rmerge) are for the highest resolution bins. The completeness of the highest resolution bin is higher than the overall completeness. The phenomenon that is often observed in protein structure determination is due to the beam-stop shadow, the rejection of overflowed reflections, and the crystal orientation.

from a sequence search of the bacterium’s genome and originally named as H10572 (16). There are three cysteines in H. influenza hybrid Prx5 that comprise a total of 241 residues. The first cysteine, Cys-49, corresponds to the N-terminal cysteine that is absolutely conserved throughout all Prx proteins. The second and third (Cys-180 and Cys-183) are the two cysteines in the conserved CXXC motif of Grx domain. There are no non-conserved cysteines in H. influenza hybrid Prx5.

We determined the crystal structure of H. influenza hybrid Prx5 (hyPrx5) to understand the functional role of fusion between Prx and electron donor proteins as well as electron donor specificity. The tetrameric structure of hyPrx5 reveals detailed information on the conformation of catalytic sites that account for the glutathione-dependent peroxidase activity of hyPrx5. The tetramerization is mediated by interconnecting Prx and Grx domains of the protein, suggesting that the fusion is essential for the tetramerization. Most importantly, Prx and Grx domains of different monomers form significant interaction with each other to allow the intermolecular electron transfer between the two domains. The Prx-Grx interaction found in the hyPrx5 tetramer reveals the atomic-level features of the Grx surface used for the interaction with an intact protein target. Thus, the tetrameric structure of hyPrx5 provides a framework in understanding the mechanism of target recognition by Grx as well as activities of the Prx-Grx hybrid proteins.
domains (Fig. 1b). The dimer in the asymmetric unit associates with another dimer generated by a crystallographic 2-fold symmetry operation of the first dimer, resulting in a tightly associated tetramer (Fig. 1, c and d). The tetramer has a donut shape with the outer and inner diameters of about 85 and 18 Å, respectively (Fig. 1c). When viewed from the side, the tetramer appears as a cylinder of 65 Å height (Fig. 1d).

Prx domain of hyPrx5 has a typical thioredoxin fold, which comprises the central β-sheet with four strands (β3, β4, β8, and β9) and three helices (α2, α4, and α5) flanking the sheet (Figs. 1a and 2). In addition to the thioredoxin fold, Prx domain of hyPrx5 has several insertions, (i) N-terminal two antiparallel β strands (β1 and β2) followed by a short α-helix (α1), (ii) helix α3 and strand β5 between strand β4 and helix α4, (iii) two short antiparallel β-strands (strands β6 and β7) between helix α4 and strand β8. A search for homologous structures by using the DALI server (32) confirmed that the structure of Prx domain of hyPrx5 is similar to that of other Prx proteins. Of these, the structure of human Prx5 (10) is most similar to that of the hyPrx5 Prx domain. A total of 151 of 160 Ca atoms in the hyPrx5 Prx domain can be aligned with the corresponding atoms of human Prx5 with an root mean square deviation of 1.50 Å. The structure of the hyPrx5 Grx domain is very similar to that of Grx3 (33), comprising a central β sheet with four strands (β10, β11, β12, and β13) flanked by three helices (α6, α7, and α8) (Figs. 1a and 2). The root mean square value of Ca-carbon alignment between the hyPrx5 Grx domain and Grx3 is 1.32 Å for 66 of 69 residues in the hyPrx5 Grx domain. **Tetramerization Association**—The tetramerization of hyPrx5 is achieved mainly by two strong subunit contacts, Prx-Prx (monomers A-B and C-D) and Grx-Grx (monomers A-D and B-C) contacts (Fig. 1, c and d). In addition to these contacts, the Prx-Grx interaction, which has important implications in the peroxidase mechanism of hyPrx5, is also involved in the tetramerization (see below). To verify the tetramerization of hyPrx5 in solution we determined the molecular weight of hyPrx5 by analytical ultracentrifugation (Fig. 3). In oxidized and reduced conditions, the molecular masses were estimated.
to be 117.0 and 115.1 kDa, respectively. These values are close to the expected tetrameric molecular mass of hyPrx5 (~108 kDa), indicating that hyPrx5 forms a tetramer in solution and the oligomeric state is not dependent on redox conditions. Dynamic light scattering and gel filtration experiments showed similar results (data not shown). The redox-independent tetramerization of hyPrx5 should be important for its biological function since the electron transfer reaction between the Prx and Grx domains occur in the tetrameric interface, and the tetramerization maintains the optimal geometry between the Prx and Grx domains fused by a flexible linker (see below).

The Prx-Prx contact in hyPrx5 is completely different from the dimeric interface in other Prx proteins (6, 9–12). The dimeric contact between two Prx domains of hyPrx5 is formed by the face perpendicular to the direction of strands in the central sheet, whereas that of other Prx proteins makes use of the face parallel to the direction of strands. The Prx-Prx contact of hyPrx5 involves residues in loops β3-α2 (residues 45–47), β4-α3 (residues 81–82), β5-α4 (residues 101–102), and β6-β7 (residues 118–121). In addition, residues Trp-22, Asp-79, Ala-85, and Arg-123, whose side chains are near the loop, regions play an important role in the dimerization. The interface, which buries 10.5% (826 Å²) of the total surface area of Prx domain, is highly complementary and involves mainly hydrophobic interactions with a salt bridge (Arg-A123–Asp-B79). The same dimeric association is found also in the structure of human Prx5 that is highly homologous to Prx domain of hyPrx5. Even though the report (10) describing the structure of human Prx5 did not mention dimeric contact, the crystal lattice assembled by using the reported structure shows the same dimeric association. Conservation of the dimeric contact in the two homologous proteins indicates that the dimeric
association may play an important role in the function of human Prx5 as in hyPrx5. Previous biochemical studies also suggested the dimerization of human Prx5 in solution (7).

The Grx-Grx interface of hyPrx5 is formed by donating helix α7 of each monomer to the dimeric interface, resulting in a five-layered αβαβα sandwich structure. Helix α7 of the first monomer is placed above the central β sheet of the monomer and interacts with the central β sheet of the next monomer. There are also strong interactions between the two helices. The Grx-Grx interface, which is predominantly made of hydrophobic interactions, includes residues in helix α7 (residues 208–210 and 212–214) and loop β11-α7 (residues 205–207). The interface buries 716 Å² that corresponds to 17.0% of the total surface area of Grx domain. Helix α7 is conserved in other Grx proteins, suggesting a possibility of dimerization in other Grx proteins, too. Consistent with the possibility, an NMR study of Grx3 showed that the protein had monomer/dimer equilibrium in its intrinsic geometry for both peroxidase and reduction reactions, increasing the antioxidant enzyme efficiency by forming optimal geometry for both peroxidase and reduction reactions.

Active Sites—HyPrx5 has two reductive-active sites; one in the Prx domain (Cys-49) is used to directly react with reactive oxygen species substrates, and the other in the Grx domain (Cys-180 and Cys-183 in the CXXC motif) reduces the oxidized active site cysteine in Prx domain to regenerate peroxidase activity of the enzyme. The most striking thing in the tetrameric structure of hyPrx5 is that active sites of the Prx and Grx domains from different monomers come close to each other in the tetrameric state (Figs. 1 a and 4a), whereas the two active sites in the same monomer are placed distantly. The Ca distance between Cys-A49 of monomer A and Cys-D180 of monomer D is 15.08 Å, and there is no intervening structure between the two residues. Although the distance is not close enough for forming a disulfide bond, we propose that the local conformation change (see below) could easily bring the two cysteines into the proximity. For example, the Ca distance between the disulfide bond partner cysteines in reduced state of human Prx5 is 13.84 Å, and a local conformation change is thought to enable the disulfide bond formation (10). In comparison, the distance between Cys-A49 and Cys-A180 of the same monomer A is 32.08 Å, which is too far for a disulfide bond formation without a major change in the tetrameric association.

Oxidized Grx proteins are reduced by reduced glutathione (GSH) via the Grx-SG mixed disulfide intermediate. The structure of E. coli Grx3 in complex with GSH (Grx3-SG) (33) showed that GSH binds to a concave surface near the CXXC motif. In the complex structure, the main chain atoms of the Cys residue in GSH form hydrogen bonds with those of Val-52 in Grx3 (corresponding to Val-220 in hyPrx5 (Fig. 2)), resulting in an antiparallel intermolecular β-bridge (33). When we superimposed the structure of the hyPrx5 Grx domain with that of Grx3 in complex with GSH, the GSH-interacting region of Grx3 was very well aligned with the corresponding region of the hyPrx5 Grx domain (data not shown), indicating that GSH is likely to bind to the hyPrx5 Grx domain involving the formation of intermolecular β-bridge as it does to Grx3. In the similar superposition carried out with the tetrameric hyPrx5, we found that the location of GSH bound to Grx3 corresponds to the intermolecular cleft in the hyPrx5 tetramer between Grx domain of molecule D and Prx domain of molecule A (Fig. 4a), suggesting that GSH is able to reduce the oxidized Grx of hyPrx5 without affecting the tetrameric geometry. Thus, the tetramerization of hyPrx5, which is possible by the fusion of Prx and Grx domains, seems to play an important role in increasing the antioxidant enzyme efficiency by forming optimal geometry for both peroxidase and reduction reactions, which is necessary for the pathogenic bacterium H. influenzae in...
defending itself against the antimicrobial defense system of host phagocytes.

Two crystallographically independent molecules in the asymmetric unit of hyPrx5 crystals exhibit different environments for the reactive cysteine (Cys-49) that also affect the Prx-Grx interface in the tetrameric structure (see below). In one hyPrx5 molecule (monomer A), the reactive cysteine is located at the start of helix a2 as in other Prx proteins (3). The positively charged side chain of Arg-126 makes good interaction with the sulfur atom of Cys-49 (distance, 3.04 Å) (Fig. 4, b and c), which stabilizes the thiolate form of Cys-49 as in other Prx proteins (3). In addition, the sulfur atom forms a hydrogen bond with the side chain of Thr-46 (distance, 3.09 Å) (Fig. 4, b and c) that is also strongly implicated in the stabilization of the thiolate form (3). These strong charge and hydrogen bond interactions of the active site sulfur atom should contribute to increasing nucleophilic reactivity of the sulfur atom of hyPrx5.

In the other hyPrx5 molecule (monomer B) of the asymmetric unit, the region of active site cysteine (Cys-49) is unwound from helix a2, and the active site Cys-49 becomes a part of loop β3-α2. The loop makes no significant interactions with other parts of the tetramer, indicating that the region may be flexible in solution. The unwound structure would have an important role in the formation of the mixed disulfide bond formation.

Because of the unwinding, the Ca distance between Cys-B49 and Cys-C180 becomes 11.63 Å, indicating that the conformation change and flexibility of the redox loop containing Cys-B49 may bring the cysteine into the distance needed for the disulfide bond formation with Cys-C180. Consistent with the observation in the hyPrx5 structure, the helix unwinding near the active site cysteine of 2-Cys Prx proteins was proposed earlier for the mechanism of the disulfide bond formation between two distant cysteines in TpxB (11). The flexibility of the redox active loop in the redox-dependent transcription factor, OxyR, also plays a role in the disulfide bond formation between two distant cysteines (35).

**Prx-Grx Interaction**—In the hyPrx5 tetramer, the surface near the CXXC motif of Grx domain, which is at one end of the central β sheet, interacts with the active site region of Prx domain (Fig. 5, a and b). Because of different domain organizations of two monomers in the asymmetric unit of hyPrx5 crystals (Fig. 1b), the tetramer has two different types of Prx-Grx association. The interaction surface of Grx domain, which comprises two prominent positive-charged regions (Fig. 5b), forms charge interactions with the Prx domain. Although the Prx interaction surface of Grx domain is the same in both interactions, there are two different Grx interaction surfaces of Prx domain (Fig. 5a). In monomers A(Prx)-D(Grx) interaction (equivalent to monomers C(Prx)-B(Grx) interaction), Arg-D212 and Lys-D177 form charge interactions with the Asp-A154–Asp-A156 patch and Asp-A148, whereas in monomers B(Prx)-C(Grx) interaction (equivalent to monomers D(Prx)-A(Grx) interaction) the same residues of Grx domain (Arg-C212 and Lys-C177) interact with the Asp-B89–Glu-B90 patch and Glu-B59. In A(Prx)-D(Grx) interactions, Ile-D208 that protrudes from the surface fits into a shallow pocket made by Phe-A150. The A(Prx)-D(Grx) and B(Prx)-C(Grx) interactions bury 255 and 222 Å$^2$ of intermolecular surfaces, respectively. Interestingly, the two different interaction surfaces are correlated with conformation of the redox active site of Prx domain. That is, in A(Prx)-D(Grx) interaction, the Prx active site (monomer A) is well arranged for the catalytic activity (Fig. 4, b and c), whereas in the B(Prx)-C(Grx) interaction, the Prx active site (monomer B) is unwound and flexible (see "Active Sites"), suggesting that A(Prx)-D(Grx) interaction may be the form of the initial peroxidase reaction, and B(Prx)-C(Grx) interaction may be used for the reduction of oxidized Prx domain by the Grx domain of the neighboring monomer. Thus, the Prx-Grx interaction in the hyPrx5 tetramer is likely to alternate between the two states in a coordinate fashion during the peroxidase and reduction reactions, maintaining overall geometry of the tetramer. The possible flip-flop mechanism of hyPrx5 indicates that hyPrx5

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**Fig. 3. Analytical ultracentrifugation.** The oxidized (a) and reduced (b) hyPrx5 samples were centrifuged at 8000 rpm with successive measurements of absorbance (Abs) at 280 nm until equilibrium. The absorbance data were fit with curves for molecular masses of 117.0 and 115.1 kDa for the oxidized and reduced samples, respectively. Interestingly, the two different interaction surfaces are correlated with conformation of the redox active site of Prx domain. That is, in A(Prx)-D(Grx) interaction, the Prx active site (monomer A) is well arranged for the catalytic activity (Fig. 4, b and c), whereas in the B(Prx)-C(Grx) interaction, the Prx active site (monomer B) is unwound and flexible (see "Active Sites"), suggesting that A(Prx)-D(Grx) interaction may be the form of the initial peroxidase reaction, and B(Prx)-C(Grx) interaction may be used for the reduction of oxidized Prx domain by the Grx domain of the neighboring monomer. Thus, the Prx-Grx interaction in the hyPrx5 tetramer is likely to alternate between the two states in a coordinate fashion during the peroxidase and reduction reactions, maintaining overall geometry of the tetramer. The possible flip-flop mechanism of hyPrx5 indicates that hyPrx5
may be a new example of multimeric enzymes with half-of-the-sites reactivity, where only half of multiple active sites are catalytically active at a time (36).

Although Prx domain of hyPrx5 accepts electrons from Grx domain, human Prx5 with strong homology with hyPrx5 uses only Trx for the electron donor (7). Examination of the molecular surface of human Prx5 provides an explanation for the specificity of electron donors (Fig. 5c). The surface of human Prx5 equivalent to the Grx interaction surface of the hyPrx5 Prx domain is mostly hydrophobic, and there are no negatively charged surface patches for the interaction with Grx. The hydrophobic surface is likely to interact favorably with the hydrophobic surface of Trx (Fig. 5d) that is located at the equivalent place on the Prx interaction surface of the hyPrx5 Grx domain.
The tetrameric structure of hyPrx5 reveals for the first time the interaction of Grx with its natural target protein (the hyPrx5 Prx domain). In the interaction between Prx and Grx domains of hyPrx5, the major interaction force is derived from two charge interactions. Other parts of the interaction are made of weak hydrogen bonds and van der Waals interactions, indicating that the interaction between Grx and Prx domains is less specific than the usual protein-protein interactions. Previously, the complex structure of human Trx with peptide fragments of target proteins, NFXB (38) and Ref-1 (39), revealed that the target peptides bind a crescent-shaped groove near the reactive cysteine of Trx (Fig. 5d). The peptide interaction surface corresponds to the upper part of the Prx interaction surface of the hyPrx5 Grx domain. However, the peptide interaction could not predict the complete surface of Trx for the interaction with three-dimensional target proteins. In contrast, the Prx interaction surface of the hyPrx5 Grx domain provides information on the surface needed to interact with an intact protein target. The nature of Prx-Grx interaction involving weak interactions together with the pivotal charge interactions seems to allow Grx to interact with diverse target proteins having variations of the surface.

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