Vibrio cholerae Thiol Peroxidase-Glutaredoxin Fusion Is a 2-Cys TSA/AhpC Subfamily Acting as a Lipid Hydroperoxide Reductase*

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Recently, novel hybrid thiol peroxidase (TPx) proteins fused with a glutaredoxin (Grx) were found from some pathogenic bacteria, cyanobacteria, and anaerobic sulfur-oxidizing phototroph. The phylogenetic tree analysis that was constructed from the aligned sequences showed two major branches. Haemophilus influenzae TPx-Grx was grouped in one branch as a 1-Cys subfamily of the thiols-specific antioxidant protein/AhpC family. Most TPx-Grx proteins, including Vibrio cholerae TPx-Grx, were grouped in the 2-Cys subfamily. To explain the existence of two subgroups in novel hybrid TPx proteins, we have compared the kinetics given by V. cholerae TPx-Grx, H. influenzae TPx-Grx, their separated TPx domains, and a set of mutants devoid of the redox-active cysteines. The kinetic study described here demonstrates clearly that V. cholerae TPx-Grx is a 2-Cys TPx subfamily. For the first time, we also demonstrate the lipid peroxidase activity of V. cholerae TPx-Grx fusion and suggest the in vivo function of 2-Cys TPx-Grx fusion serving as a lipid peroxidase.

Aerobic organisms intrinsically encounter reactive oxygen species, such as hydrogen peroxide (H₂O₂), the superoxide anion radical (O₂⁻), and the hydroxyl radical (OH⁻) during some respiratory reduction of O₂ to water or following exposure to environmental factors (1, 2). Physiologically, bacterium is versatile and well adapted to its characteristic habitats. Bacteria can grow in the presence or absence of O₂. The respiratory electron transport chain is a major and continuous source of reactive oxygen species (ROS). The bacteria-infected host cells induce a defense response that results in an oxidative burst with the increased generation of ROS (3). ROS can also be formed by exposure of bacteria to redox-cycling chemicals present in the environment or by exposure to heavy metals (4). These endogenous or exogenous sources of ROS damage many biological molecules. For example, lipid hydroperoxides can be generated from the attack of ROS to the bacterial membrane. To alleviate the oxidative damage of these compounds, bacteria induces the synthesis of a variety of antioxidant defense enzymes, such as hydroperoxidases (catalases) I and II (gene products of katG and katE, respectively), that decompose H₂O₂ (5) and superoxide dismutases (manganese superoxide dismutase, sodA; iron superoxide dismutase, sodB; copper-zinc superoxide dismutase, sodC) that eliminate superoxide anion (6). Additional defenses in bacteria against alkyl and lipid hydroperoxides are suggested to be provided by alkyl hydroperoxide reductase (AhpC) (7), bacterioferritin-comigratory protein (BCP) (8), and periplasmic thiol peroxidase (p20) (9). AhpC and BCP are all bacterial members of the ubiquitous thiol peroxidase (TPx) (TSA/AhpC) family (7–11). For AhpC, reduction of peroxide is achieved by a specialized electron donor, AhpF (12), whereas BCP and p20 receive electrons from a reducing system composed of Trx and Trx reductase (11, 13). P20 has been characterized as a periplasmic protein (9) that has been reported to exist in Gram-negative bacteria such as Escherichia coli (9), whereas AhpC and BCP as cytoplasmic proteins have been found in all species of bacteria (14).

Rouhier et al. (15, 16) previously described the glutaredoxin (Grx)-dependent reduction of a plasmat phloem TPx. Recently, novel hybrid TPx proteins fused with a Grx domain were found from several pathogenic bacteria, including Haemophilus influenzae (17, 18), Neisseria meningitidis, Yersinia pestis, and Vibrio cholerae. In addition, an anaerobic sulfur-oxidizing phototroph, Chromatium gracile, and a cyanobacteria, Nostoc sp. (PCC 7120), also have the hybrid TPx (19). Most TPx-Grx fusions appear to have conserved two cysteines in their TPx domains. Only in two cases (H. influenzae and Actinobacillus actinomycetemcomitans) do TPx-Grx fusions have one conserved N-terminal cysteine. Previously, H. influenzae TPx-Grx, which is a prototype example of the 1-Cys hybrid TPx proteins, was characterized as a GSH-supported peroxidase (17). A more thorough investigation of the catalytic role under each of its three cysteine residues, including two conserved cysteines in the conserved CXXC motif of Grx domain and their roles in catalysis, is required for clarifying the TPx-Grx fusion serving as TPx family.

Research over the past decades has led to the characterization of a new family of peroxidases, collectively called peroxiredoxins, TSA/AhpC family (20) or TPx family (8, 9, 21, 34). TPx proteins can be divided into two subgroups, 2-Cys TPx (22) and 1-Cys TPx (23, 35), depending on the number of conserved cysteines. The N-terminal Cys acts as the primary catalytic site for reduction of peroxides. In 2-Cys TPx, the C-terminal Cys forms an intermolecular (24) or intramolecular (25) disulfide bond with the N-terminal cysteine. In comparison, 1-Cys TPx has only the N-terminal cysteine, and the enzyme activity usually does not involve intermolecular disulfide bond formation (23, 35).

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**Vibrio cholerae TPx-Grx as a Lipid Hydroperoxidase**

In this report, *V. cholerae* TPx-Grx, which is a prototype example of the 2-Cys hybrid TPx proteins, was first characterized. We expanded the knowledge of the chimeric enzyme by demonstrating that in contrast to HI (*H. influenzae*) TPx-Grx, VC (*V. cholerae*) TPx-Grx is a 2-Cys TWp subfamily forming an intramolecular disulfide bond between two cysteines in the TPx domain. By studies with the separate regions of the TPx and Grx domains of VC fusion are underlined. The redox-active cysteines (Cys-54, -79, -185, and -188) within the VC TPx domain, has a preferential capability to reduce lipid hydroperoxide such as linoleic hydroperoxide as a substrate, we demonstrate that in contrast to HI (*H. influenzae*) TPx-Grx, HI TPx-Grx (27). The DNA sequences corresponding to VC TPx-Grx, and their N-terminal domain designated as the TPx domain were obtained by PCR from the corresponding genomic DNA using the forward primer (5'-GGATCC ATC CATAG AGG AAC ACA ATG TTA TTA TCTAA-3') for VC TPx-Grx and the N-terminal domain, 5'-GGATCC ATC CATAG TCT AGT ATG GAA GAA AAA AAGG-3' for HI TPx-Grx and the TPx domain) containing an NdeI (underlined) site and the initiation codon (boldface) and the reverse primer (5'-CGG GATATC TTA TTTG AGAT GTA GAC TTC TAAGG-3' for VC TPx-Grx, 5'-CGG GATATC TTA GCC AGT GTA GAC TTC TAAGG-3' for VC TPx domain, glutamyltransferase assay mixture was added. After incubation at 37 °C for 10 min, the remaining activity of GS was determined by measuring the absorbance at 540 nm.

**Determination of Peroxidase Activity of TPx—Peroxidase reaction was performed in 50 μl of a reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.5 mM DTT or GSH, varying concentrations of TPx, and 50–700 μM peroxides at 37 °C. The residual amount of peroxide was determined by FOX assay (26). Peroxidase reaction was started by the addition of 0.5 mM DTT. The reaction mixture was added to 1 ml of FOX reagent and then incubated at room temperature for 30 min. The remaining amount of peroxide was monitored by measuring the absorbance at 560 nm. Linoleic acid hydroperoxide (LAOOH) was generated by incubating 100 μM linoleic acid with 10 μg/ml soybean lipoxidase in 100 mM Tris-HCl, pH 7.4, at room temperature for 30 min. The concentration of LAOOH was determined spectrophotometrically using an extinction coefficient of 234 nm of 25,000 M⁻¹cm⁻¹.**

**Cloning of TPx-Grx Fusions and Their Separated TPx Domains—**

Basic cloning protocols used were described by Sambrook and Russell (27). The DNA sequences corresponding to VC TPx-Grx, HI TPx-Grx, and their N-terminal domain designated as the TPx domain were obtained by PCR from the corresponding genomic DNA using the forward primer (5'-GGATCC ATC CATAG AGG AAC ACA ATG TTA TTA TCTAA-3') for VC TPx-Grx and the N-terminal domain, 5'-GGATCC ATC CATAG TCT AGT ATG GAA GAA AAA AAGG-3' for HI TPx-Grx and the TPx domain) containing an NdeI (underlined) site and the initiation codon (boldface) and the reverse primer (5'-CGG GATATC TTA TTTG AGAT GTA GAC TTC TAAGG-3' for VC TPx-Grx, 5'-CGG GATATC TTA GCC AGT GTA GAC TTC TAAGG-3' for VC TPx domain,
some pathogenic bacteria, cyanobacteria, and anaerobic sulfur-oxidizing photrophs (see Fig. 1). A BLAST search with the deduced amino acid sequence of HI TPx-Grx identified the highly homologous TPx-Grx fusion proteins. To elucidate the diversity of TPx-Grx fusions, HI TPx-Grx and the other homologous proteins were included in this phylogenetic analysis. The phylogenetic tree analysis (Fig. 1) that was constructed from aligned sequences showed two major branches. HI TPx-Grx and A. actinomycetemcomitans (AA) TPx-Grx were grouped together in one branch representing the 1-Cys subfamily of the TPx (TSA/AhpC, peroxiredoxin) family (17). Other TPx-Grx proteins were present in the other branch containing a group of TPx-Grx having an additional C-terminal cysteine within their TPx domains, suggesting that they may belong to the 2-Cys subfamily. When we compared the positions of the C-terminal Cys (C2) of the VC TPx-Grx-containing subunit, the C2 was very well aligned, indicating that the C2 is likely to be the C-terminal Cys of the 2-Cys subfamily. Previously, *H. influenzae* hybrid TPx, which was suggested to be a prototype example of these hybrid TPx proteins, was characterized as a prokaryotic GSHis-supported peroxidase. It was also suggested that based on the study on HI fusion, TPx-Grx fusions could be classified as a 1-Cys subfamily of the TPx family (1-Cys TPx) devoid of C-terminal cysteine. There are three cysteines in *H. influenzae*.
enzymes in Tpx. The first cysteine, Cys-49, corresponds to the
N-terminal cysteine that is absolutely conserved throughout all
Tpx proteins. The second and third (Cys-180 and -183) are the
two cysteines in the conserved CXXC motif of the Grx domain.
There are no non-conserved (i.e. C-terminal) cysteines in
H. influenza hybrid Tpx. In comparison, the VC fusion as the
representative form of another branch of Tpx-Grx proteins
contains the C-terminal cysteine, Cys-79. Therefore, it is inter-
esting to investigate whether or not the C-terminal cysteine
involves inter- or intramolecular disulfide bond formation as a
catalytic process, because all Tpx fusions except for two cases
(HI and AA fusions) seem to have the C terminal cysteine.

**Physical Characteristics of VC Tpx and VC Tpx-Grx Fu-
sion—Recombinant fusion proteins (VC, VC C1S, VC C2S, VC
C1S/C2S, VC C3S, VC C4S, VC C3S/C4S, HI, HI C3S, and Hi
C4S) and the separated Tpx domains were purified to homoge-
nity. Members of the Tpx family can be divided into two
subgroups, such as one-cysteine and two-cysteine groups ac-
cording to the number of conserved cysteines within the protein
(29). The 2-Cys-containing proteins exist as a homodimer via
an intermolecular or intramolecular disulfide bond. Previously,
we suggested that E. coli p20 (9, 21, 34) and yeast nuclear thiol
peroxidase (30, 31) are 2-Cys Tpx and exist as a monomer in
which the N-terminal Cys is bonded to the C-terminal Cys via
intramolecular disulfide linkage. Pauwels et al. (17) and Kim
et al. (18) demonstrated that HI Tpx-Grx as the dimer
linked via an intermolecular disulfide bond between 1-Cys of
the Tpx domains. In contrast to HI Tpx-Grx, VC Tpx-Grx exists
as the monomer form in non-reducing SDS-PAGE gel (Fig. 2A,
lane 6). Single mutation of C1 or C2 (see Fig. 1) resulted in
conversion of the monomer to the dimer in non-reducing gel,
although a significant amount of the C1S mutant remained as
the monomer form (Fig. 2A, lanes 7 and 8). The double
mutation of C1 and C2 completely led to the monomer form (Fig. 2A,
lane 9). Analysis of the non-reducing SDS-PAGE gel for the
separated Tpx domain and its C1 and C2 and the double
mutant (Fig. 2B) gave the same conclusion as the experiment
using the full-sized proteins. In addition, it is worth noting that
compared with C2S more than half the C1S remained as the
monomer form in the non-reducing gel, because mutation of C2
has a much stronger dimerization effect compared with muta-
tion of C1, leading us to speculate that free C1 cysteine of C2S
is exposed to the outside but free C2 cysteine of C1S resides in
the interior of the protein. The steric hindrance during the
formation of an intermolecular disulfide bond between the in-
terior C2 cysteines could provide the reason why the lack of C1
cysteine did not completely destroy the ability to form a dimer.
The almost complete dimeric conversion of HI Tpx-Grx or its
separated Tpx domain as a mimic for corresponding C2S de-
derived from VC Tpx-Grx in the non-reducing gel (Fig. 2C) also
suggests the outside localization of the C1 cysteines. Taken
together, these data demonstrate that in contrast to HI
TPx-Grx, two cysteines (C1 and C2) within the VC TPx domain are linked with intramolecular disulfide bond. Based on this observation, we suggest the possibility that VC TPx-Grx is a so-called atypical 2-Cys subgroup member that forms an intramolecular disulfide as an intermediate (32). Our suggestion is also supported by observation of the existence of two major branches in TPx-Grx homologous fusions. These results, together with the analysis of the phylogenic tree shown in Fig. 1, suggest that VC TPx-Grx belongs to the 2-Cys subfamily of the TPx family but HI TPx-Grx to the 1-Cys subfamily.

Comparison of Redox-active Sites—HI TPx-Grx has two redox-active sites; one in the TPx domain (Cys-49) is used to react with peroxides as substrates, and the other in the Grx domain (Cys-180 and Cys-183 in the CXXC motif) reduces the oxidized active site cysteine in the TPx domain to regenerate peroxidase activity of the enzyme (17, 18). In comparison, VC TPx-Grx appears to have two redox-active disulfides (i.e. C1–C2 and C3–C4) in the oxidized state. To investigate whether or not the C2 (Cys-79) is involved in the catalytic cycle of the reduction reaction, we compared the reaction patterns given by HI TPx-Grx, VC TPx-Grx, their separated TPx domains, and their C1, C2, C3, and C4 mutated fusions. The most striking feature in the comparison studies is the Grx domain-deleted effect on their antioxidative activities. Antioxidant activities were determined by measuring the activity to protect the inactivation of E. coli GS by a thiol metal-catalyzed oxidation system (DTT or GSH/Feo+/O2) (25). In the case of VC TPx-Grx, deletion of the Grx domain gave a dramatic increase in antioxidant activity, whereas deletion of the Grx domain in HI TPx-Grx resulted in a great reduction of the antioxidative activity (Fig. 3, A and B). The ~10-fold increase (VC TPx-Grx) or decrease (HI TPx-Grx) of the activity by the deletions strongly suggests that the structure of VC fusion is quite different from that of HI fusion. In both HI and VC fusions, GSH-supported activities were much higher than DTT-supported reactions, suggesting the GSH-dependent Grx-fused characteristics as described by Frederik et al. (17). However, in sharp contrast to the case of the TPx domain of HI fusion, replacement of GSH with DTT in the reaction of the TPx domain of VC fusion dramatically increased antioxidant activity (~6-fold) (Fig. 3B). All members of the 2-Cys TPx subfamily have a high preference toward a redox-active dithiol-containing reducing agent such as DTT over mono-thiol-containing reductant (i.e. GSH) as the electron donor (30). The DTT selectivity of the 2-Cys TPx subfamily has been thought to be caused by the direct involvement of dithiol within DTT in the catalytic cycle (30). Therefore, in sharp contrast to the case of the HI TPx domain, the high preference of the VC TPx domain to DTT over GSH as a reducing reagent can be taken as evidence that the TPx domain is a 2-Cys subfamily of the TPx family. Fig. 3C compares the antioxidative activity given by various thiol peroxidases. E. coli p20 is an atypical 2-Cys subfamily (9), and AhpC is a typical 2-Cys subfamily (12). A typical subfamily links the N-terminal cysteine to the C-terminal cysteine via an intermolecular disulfide bond. Analysis of the DTT-supported activities indicates that the VC TPx domain and p20 (atypical 2-Cys subfamily) have considerably higher activities than those of the HI TPx domain and AhpC, suggesting the catalytic efficiency of the atypical 2-Cys subfamily caused by an intramolecular disulfide bond. Collectively, these data lead us to suggest that VC fusion is an atypical 2-Cys subfamily, whereas HI fusion is a 1-Cys subfamily.

To confirm the deletion effect described above, we constructed a VC TPx-Grx mutant devoid of the CXXC motif within the Grx domain and compared the antioxidant activity given by the resulting mutant (VC C3S/C4S) with the wild protein in the presence of DTT or GSH. Overall analysis of the data shown in Fig. 4 indicated that replacement of Cys-185 (C3) and Cys-188 (C4) with respective serine within the Grx domain increased the activity (panel B) compared with the activity of the wild protein (panel A). Also, we observed that replacement of GSH with DTT as a reducing equivalent resulted in a ~3-fold increase in antioxidant activity compared with that in the absence of GSH. These data supported the great activity given by the VC TPx domain compared with the activity of the VC fusion and the DTT replacement effects of the VC TPx domain shown in Fig. 3. For the purpose of making a HI TPx-Grx mimic using VC TPx-Grx, we replaced Cys-79 (C2) with serine because there is no C-terminal cysteine (C2) in HI fusion. In addition, to determine the primary site for the reaction, we point-mutated Cys-54 (C1) to serine and comparatively measured the antioxidative activity given by the resulting VC C1S. Fig. 4A shows that deletion of C1 in VC fusion resulted in a complete loss of the activity regardless of the type of reducing equivalent, which demonstrates that C1 acts as a primary site of the catalysis. VC C2S, a mimic of HI fusion in terms of absence of C-terminal cysteine, did not show activity comparable with HI fusion. This result excluded the possibility that the superior activity of HI...
fusion is not due to the absence of C-terminal cysteine within the TPx domain. We also observed the great reduction of C2S-catalyzed activity in the presence of DTT when compared with the DTT-supported activity of VC fusion, which is taken as evidence for the existence of an intramolecular disulfide bond between C1 and C2 within the TPx domain.

Taken together, the data presented here demonstrate that VC fusion is an atypical 2-Cys subfamily of the TPx family, whereas HI fusion is a 1-Cys subfamily as described previously (17). Also, based on a significant difference in kinetic properties between VC and HI fusions, we suggest that the two proteins are structurally different enzymes.

Comparison of Peroxidase Activities—Previous results have indicated that the VC TPx domain itself is well designed for antioxidative activity in terms of ability to prevent GS inactivation by the MCO system compared with the HI TPx domain, whereas the catalytic superiority of the VC TPx domain as the fused form was very poor. To address this kinetic property, we comparatively determined the antioxidative activities of VC TPx, VC fusion, HI TPx, and HI fusion in terms of ability to remove various peroxides. Fig. 5 showed that the VC TPx domain has superior activity to remove various hydroperoxides such as \( \text{H}_2\text{O}_2 \), t-butyl hydroperoxide (t-BOOH), cumene hydroperoxide (COOH), and LAOOH compared with the HI TPx domain, which is consistent with the results shown in Fig. 3. However, the pattern of peroxidase activity of the VC fusion toward the hydroperoxides appeared to be more complex (Fig. 6). Compared with the peroxidase activities given by HI fusion, the peroxidase activity of VC fusion toward small-sized \( \text{H}_2\text{O}_2 \) is higher, whereas the activity of bulky t-BOOH or COOH is much lower. This led us to speculate that the lower activity toward t-BOOH or COOH resulted from steric hindrance to access to the active site because of the large size of the peroxides. Interestingly, peroxidase activity of VC fusion toward LAOOH, a bulky fatty acid hydroperoxide, is the same as the activity of HI fusion toward LAOOH. This effect suggests that VC fusion may be designed to remove the hydroperoxide linked to long chain alkyl groups, such as the alkyl groups of membrane lipids.

Considering the steric effect of VC fusion toward bulky hydroperoxide as the substrate, the severe reduction of antioxidant activity of VC fusion to prevent GS inactivation by the MCO system, even though the VC TPx domain itself has great catalytic activity, might be explained simply in terms of the steric hindrance given by the fused Grx domain. However, this explanation is not enough, because double deletion of C3 and C4 within the Grx domain of VC fusion without deletion of the Grx domain increases the GS-protecting activity comparable with that of the TPx domain itself. Therefore, it is worth investigating the peroxide activity given by the C3 or/and C4 mutants toward bulky hydroperoxides such as t-BOOH and LAOOH. In the case of HI fusion, deletion of C3 or C4, as expected on the basis of the Grx-linked peroxidase activity (17), resulted in a complete loss of peroxidase activity toward t-BOOH (Fig. 7A), whereas the deletion of C4 within VC fusion dramatically increased peroxidase activity toward t-BOOH compared with that of the wild protein (Fig. 7B). The reduction of peroxidase activity of VC C4S or VC C3S/C4S can be explained in terms of the catalytic function of C3 in the Grx reaction. The basal activity shown by the double mutant is thought to be the activity given by the TPx domain itself without support by the Grx reaction. Fig. 7C shows the lipid peroxidase activities exerted by a set of VC C3 and C4 mutants. The results with LAOOH showed a similar pattern with that of
the activities toward t-BOOH, but there is some difference in that the basal activity toward LAOOH is much higher compared with the basal activity toward t-BOOH. Together with the double deletion effect on the GS-protecting activity (Fig. 4), these kinetic results led us to suggest that reduction of redox-active C3–C4 disulfide within VC fusion may have induced the conformational change. As a result of the structural change, the active site within the TPx domain might be exposed. Similarly the binding of LAOOH on the active site of VC fusion may induce the structural change. However, this speculation remains to be proved by structural studies. In addition, we suggest the possibility that the structure of HI fusion in the oxidized state is already an "opened form" as the result of the catalytic cycle to form the intermolecular disulfide bond between C1 and C3. Recent crystal structure of the tetrameric structure of VI fusion supports our suggestion (18). Based on the observation that the distance between C1 and C3 of the same monomer is 32.08 Å, which is too far for disulfide bond formation in the tetrameric association, they suggested an intermolecular disulfide bond between C1 and C3 during the catalytic cycle (18). In comparison, the structure of VC fusion maintains a "closed form" through a direct exchange of reducing equivalent between two redox-active disulfides (i.e. C1–C2 and C3–C4).

Lipid Peroxide Activity of VC TPx-Grx Fusion—V. cholerae TPx-Grx fusion has been demonstrated to act as a lipid hydroperoxide peroxidase. To our knowledge, this was the first demonstration that TPx-Grx fusion is a lipid peroxidase. To confirm lipid hydroperoxide peroxidase activity, we investigated the substrate-dependent activity of VC fusion. Peroxidase activity as a function of LAOOH concentration deviated from normal Michaelis-Menten kinetics, giving a sigmoidal pattern (Fig. 8). The apparent K_m value was estimated to be 34.52 ± 5.966 μM. The sigmoidal kinetics supports our suggestion that binding of LAOOH may cause the structural change. Together with the substrate preference of VC TPx fusion toward a lipid hydroperoxide, the low K_m value indicates that VC TPx-Grx fusion acts as a lipid hydroperoxide peroxidase.

The phylogenetic tree analysis indicates there are two types of subgroups in TPx-Grx homologous proteins. To explain the existence of two subgroups, we have compared the kinetics given by VC TPx-Grx (the subfamily having two cysteines in the TPx domain) and HI TPx-Grx (the subfamily having one cysteine in the TPx domain) fusions. The study described here demonstrates clearly that VC TPx is a 2-Cys subfamily of the TPx family. For the first time, we also demonstrate the lipid peroxidase activity of VC TPx-Grx fusion and suggest the in vivo function of the 2-Cys TPx-Grx fusion serving as a lipid peroxidase. The higher preference for a LAOOH suggests that the VC TPx-Grx fusion may be designed to remove hydroperoxide linked to the membrane lipid. In conclusion, our comparative characterisation of two subgroups revealed by phylogenetic tree analysis provides a basis for physiological function of a new type of TPx-Grx fusion subfamily. Not only is VC TPx-Grx the first lipid hydroperoxide-specific thiol peroxidase, it is also, to our knowledge, the first TPx-Grx fusion having two redox-active sulfides as an atypical 2-Cys TPx subfamily (32). Structural studies with TPx-Grx fusion will provide new insights into the conformational change and the interaction between its two domains.

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