Characterization of Novel Hexadecameric Thioredoxin Peroxidase from

*Aeropyrum pernix K1*

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The abbreviations used are: TPx, Thioredoxin peroxidase; Trx, Thioredoxin; TR, Thioredoxin reductase; Prx, Peroxiredoxin; ROS, reactive oxygen species; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HPLC, high-pressure liquid chromatography; MCO, metal catalyzed oxidation; TEM, transmission electron microscope.

ABSTRACT

A gene (APE2278) encoding the peroxiredoxin (Prx) homologous protein of yeast and human was identified in the genome database of the aerobic hyperthermophilic archaeon *Aeropyrum pernix*. We cloned the gene and produced the encoded protein in *E. coli* cells. The isolated recombinant protein showed peroxidase activity *in vitro* and used the thioredoxin system of *A. pernix* as an electron donor. These results indicate that the recombinant protein is in fact thioredoxin peroxidase (*ApTPx*) of *A. pernix*. Immunoblot analysis revealed that the expression of *ApTPx* was induced as a cellular adaptation in response to the addition of exogenous H$_2$O$_2$ and may exert an antioxidant activity *in vivo*. Analysis of the *ApTPx* oligomers by HPLC and electron microscopic studies showed that *ApTPx* exhibited the hexadecameric protein forming twofold toroid-shaped structure with outer and inner diameters of 14 and 6 nm, respectively. These results indicated that *ApTPx* is a novel...
hexadecameric protein, composed of two identical octamers. Although oligomerization of individual subunits does not take place through an intersubunit disulfide linkage involving Cys\(^{50}\) and Cys\(^{213}\), Cys\(^{50}\) is essential for the formation of the hexadecamer. Mutagenesis studies suggest that the sulphydryl group of Cys\(^{50}\) is the site of oxidation by peroxide and that oxidized Cys\(^{50}\) reacts with the sulphydryl group of Cys\(^{213}\) of another subunit to form an intermolecular disulfide bond. The resulting disulfide can then be reduced by thioredoxin. In support of this hypothesis, ApTPx mutants lacking either Cys\(^{50}\) or Cys\(^{213}\) showed no TPx activity, whereas the mutant lacking Cys\(^{207}\) had a TPx activity. This is the first report on the biochemical and structural features of a novel hexadecameric thioredoxin peroxidase from the archaea.

INTRODUCTION

Reactive oxygen species (ROS) are generated by the incomplete reduction of oxygen during respiration or by exposure to external factors such as light, radiation, redox-cycling drugs, and stimulated host phagocytes (1, 2). Increased levels of ROS, referred to as oxidative stress, can lead to damage to all major classes of biological macromolecules, including lipids, proteins and DNA (2, 3) in all aerobic organisms. Therefore, all aerobic organisms have evolved protection mechanisms against oxidative damage. As a result, most cells have developed antioxidative enzymes and also produce antioxidant molecules such as superoxide dismutases, catalases, peroxidases, thioredoxin, and glutathione, to protect themselves against ROS.

Peroxisiredoxin (Prx) forms a large family of newly discovered antioxidant enzymes that act as peroxidases, which reduce hydrogen peroxide and alkyl hydroperoxides to water or the corresponding alcohol, respectively (4). The family can be divided into two subgroups, 1-Cys and 2-Cys Prxs, based on the presence of one or two conserved cysteine residues. Recently, an atypical 2-Cys Prxs, which contains only the N-terminal conserved Cys and requires an additional Cys for their peroxidase activity, has been classified into the third group (5). The 2-Cys Prxs contain a strictly conserved Cys residue in the N-terminal region which forms the site of oxidation by peroxides and an additional conserved Cys residue in the C-terminal region which forms an intermolecular disulfide bond along with the N-terminal Cys residue of another subunit (6, 7). The disulfide bond in 2-Cys Prxs is reduced by thioredoxin (Trx), and these are referred to as thioredoxin peroxidases (TPx). Oxidized Trx, in turn, is reduced by thioredoxin reductase (TR), an NADPH-dependent flavoenzyme.

TPx that is able to reduce \(\text{H}_2\text{O}_2\) using electrons provided by Trx was first reported in yeast (8). In a variety of organisms from bacteria, eukarya, and archaea (9), more than 40 proteins have been found to have a similar function. The gene which has some homology to
TPx has been found in archaea such as *Thermoplasma volcanium* (10), *Thermotoga maritima* (11), *Thermoplasma acidophilum* (12), *Sulfolobus tokodaii* (13), and *Aeropyrum pernix* (14). In archaea, however, TPx has not been isolated and its functions and properties remain unclear.

*Aeropyrum pernix* is a strict aerobic hyperthermophilic archaea (15). Therefore, a better understanding of the mechanism by which it protects itself against ROS would be of interest. A themostable superoxide dismutase was found in the genomic sequence of *A. pernix*, and characterized (16). However, additional enzymes related to oxidative damage have not been reported. The *A. pernix* genome contains an open reading frame (ORF, APE2278) that codes for a protein that is homologous to yeast Prx1p (17). The Thioredoxin and thioredoxin reductase system in *A. pernix* has been characterized previously (18). In this paper, we report on the cloning of the gene (APE2278) and the characterization of the product in order to clarify the role of the protein and it catalytic mechanism.

**EXPERIMENTAL PROCEDURES**

*Construction and Expression of the Recombinant Protein*

Chromosomal DNA of *A. pernix* K1 was prepared as described by Sako et al. (15). The gene (APE2278) of *A. pernix* was amplified by PCR using the chromosomal DNA as
template, and two primers TP1: 5′-CCGGGCAGCATTCCGCTGATCGGA-3′ (forward); and TP2: 5′-ACTAGATCTTTAGTGATTGTGTTGCGGC-3′ (reverse), which were designed based on an open reading frame coding for a protein of 250 amino acids. Amplification by PCR was carried out at 94 °C for 30 sec, 55 °C for 2 sec, 74 °C for 30 sec for 30 cycles using KOD DNA polymerase (Toyobo, Osaka, Japan). The plasmid pET-3d was then digested with Nco I, treated with T4 DNA polymerase to fill in the cohesive ends and, finally, digested with Bam HI again. The amplified PCR products were digested with Bgl II (the Bgl II site in primer TP2 is underlined) and inserted into the pET-3d vector. Site-directed mutagenesis was performed by an overlap extension method using PCR (19). The nucleotide sequences of the inserted genes were confirmed using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The transformant E. coli BL21(DE3)-pLysS cells harboring the expression plasmid were grown to an OD600 of approximately 0.4 and the recombinant protein was then expressed with the induction of 0.5 mM IPTG for 4 h at 37 °C.

Purification of the Recombinant Proteins

E. coli cells containing the expressed recombinant enzyme were centrifuged and frozen at 70 °C. The cells, after thawing, were then disrupted by sonication in buffer A (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The suspension of disrupted cells was centrifuged at 27,000 × g for 30 min and the supernatant fraction was heat-treated at 85°C for 30 min followed by recentrifugation. The supernatant was loaded on a HiTrap Q column (Amersham Biosciences, Piscataway, NJ, USA) equilibrated in buffer A and the bound protein was eluted with a linear gradient of NaCl (0 to 1.0 M in the same buffer). The protein solution was concentrated using a centricron 10 filter from Amicon (Millipore, Bedford, MA, USA) and dialyzed against buffer B (50 mM sodium phosphate, pH 7.0, 150 mM NaCl). The dialyzed solution was loaded on a HiPrep Sephacryl S-200 HR 26/60 column (Amersham Biosciences) and eluted with buffer B. The preparation of thioredoxin (ApTrx) and thioredoxin reductase (ApTR) from A. pernix were carried out as described previously (18). The purity of the recombinant protein was determined by means of 0.1-12% SDS-PAGE. Protein concentrations were determined using a Bio-Rad protein assay system (Hercules, CA, USA) with bovine serum albumin as the standard.

Assay of Peroxidase Activity

The peroxidase reaction was initiated by the addition of 0.2 mM H2O2 into 0.6 ml of a reaction mixture containing 0.25 mM NADPH, 0.12 μM of ApTR, 0.45 μM of ApTrx, 2 μM of ApTPx, and 50 mM potassium phosphate, pH 7.0, and then incubated at 80 °C. At appropriate reaction times, 50 μl of the reaction mixture was added to 0.95 ml of trichloroacetic acid solution (10%, w/v) to stop the reaction. The precipitated proteins were removed by centrifugation, and the peroxidase activity was determined from the amount of peroxide remaining. The peroxide content was determined by measurement of the purple-colored ferrithiocyanate complex [0.2 ml of 10 mM Fe(NH4)2(SO4)2 and 0.1 ml of 2.5 N KSCN were added to a 1 ml aliquot of supernatant], using H2O2 as a standard (20). The amount of the ferrithiocyanate complex present was determined by measurement of the absorbance at 480 nm.

Glutamine synthetase Protection Assay
TPx antioxidant activity was assayed by monitoring its ability to inhibit the thiol/Fe$^{3+}$/O$_2$-mediated inactivation of *E. coli* glutamine synthetase (GS), as described by Kim et al. (21). Protection assays were performed in 25 µl reaction mixtures containing 50 mM HEPES-NaOH (pH 7.4), 5 µg of GS, 3 µM FeCl$_3$, 10 mM DTT, and various concentrations of *Ap*TPx. After 10 min at 37 °C, the remaining GS activity was measured by the addition of 2 ml γ-glutamyltransferase assay mixture, as described previously (21). The reaction was terminated by the addition of 1 ml of stop mixture and the absorbance resulting from the γ-glutamylhydroxamate-Fe$^{3+}$ complex was measured at 540 nm.

**Analysis of the Molecular Weight with HPLC**

The molecular weight of wild-type and mutant *Ap*TPx was determined by HPLC analysis. Purified wild-type and mutant *Ap*TPx were applied to a gel filtration column (G3000SW, Tosoh Co., Tokyo, Japan) on a Tosoh HPLC system (Tosoh Co., Tokyo, Japan). The column was equilibrated and eluted at a flow rate of 0.6 ml/min with 50 mM Na-PO$_4$ (pH 7.0) containing 150 mM NaCl, and the elution was monitored at 280 nm.

**Electron Microscopy**

The purified wild type and mutant *Ap*TPx were diluted with 50 mM Tris-HCl (pH 7.5) to final protein concentrations of 50 µg/ml. A drop of sample (10 µl) was applied onto a carbon-coated grid glow-discharged just before sample addition. The samples were negatively stained with 1% (w/v) sodium phosphotungstic acid (Nanoprobes) for one minute, and then excess liquid was removed with blotting paper. The grids were examined with a Hitachi H-9000 transmission electron microscope (TEM) operating at 100kV.

**Sedimentation Equilibrium**

Sedimentation equilibrium measurements of *Ap*TPx were performed using a Beckman XL-A (Beckman Coulter, Inc., Fullerton, CA) analytical ultracentrifuge. The protein solution was dialyzed extensively against 50 mM sodium phosphate buffer (pH 7.0). Two different concentrations (0.3 and 0.6 mg/ml) of sample (100 µl) were loaded into the sample channels of double-sector, 12-mm centerpieces and 100 µl of buffer into the corresponding reference channels. The centrifugation was conducted in an AnTi-60 rotor at 10,000 rpm for 25 h at 20 °C. Radial absorbance scans were collected in the continuous scan mode at either 280 or 250 nm at 4 min intervals with two replicates and a step size of 0.005 cm. Apparent point-average weight-average molecular weight values ($M_{w,app}$) were calculated from plots of ln[$c(r)$] versus $r^2$ plots (22), where $c(r)$ is the concentration of protein (g/L) at radial position $r$.

**Immunoblot Analysis**

BALB/C mice were immunized with 50 µg of *Ap*TPx in complete Freund adjuvant (Wako, Tokyo, Japan) and then, at weekly intervals, with incomplete (in Freund adjuvant) until anti-*Ap*TPx antibodies were detected in the sera. The antisera were examined for specificity by an enzyme-linked immunosorbent assay (ELISA), and confirmed that anti-*Ap*TPx detected *Ap*TPx. The cells from 10-h (exponential phase) cell cultures of *Aeropyrum pernix* were resuspended in lysis buffer [50 mM Tris-HCl pH 7.5, 15 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100] and disrupted by sonication for 1 min at 4 °C. The suspension of disrupted
cells was centrifuged at 40,000 × g for 30 min at 4 °C, and the supernatant was heated at 95 °C for 5 min. Samples were subjected to electrophoresis in 0.1% SDS-12% polyacrylamide gels (SDS-PAGE) and electro-blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-rad). Blots were blocked using 5% semi-skimmed milk and incubated with the mouse anti-ApTPx serum at 1:1000 dilution and with anti-mouse polyvalent immunoglobulins conjugated to alkaline phosphatase diluted 1:3000 (Sigma, St. Louis, MO, USA) with intermediate washes in phosphate-buffered saline/0.1% Tween. The bands were visualized by adding nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Bio-Rad, Hercules, CA, USA).

RESULTS

Identification of APE2278 Gene

In the Aeropyrum pernix K1 genome database (http://www.bio.nite.go.jp:8080/dogan/Top), we identified an ORF (accession No. APE2278) encoding thioredoxin peroxidase homologue.

The ORF APE2278 encodes a protein of 250 amino acids with a predicted molecular mass of 28702 Da and pI 6.79 that has been classified as member of the one-conserved cysteine peroxiredoxin family (1-Cys Prx) (4, 9, 17, 23) (Fig. 1). The deduced amino acid sequence shows a 38% identity with the yeast Prx1p, a 35% identity with the human 1-Cys
Prx, and a 31% identity with the yeast Tsa2p. The consensus sequence surrounding the conserved cysteine (Cys\textsuperscript{50}) of APE2278 protein, which corresponds to Cys\textsuperscript{47} of human 1-Cys Prx, is PVCTTE and differs from the corresponding consensus sequence, FVCPTE and PGCSKT, of 2-Cys Prx (5) and mammalian Prx V (24), respectively. Its C-terminal region is larger than those of Prx enzymes and contains two Cys residues at positions 207 and 213 in addition to the conserved Cys\textsuperscript{50} (Fig. 1). The homology sequences surrounding Cys\textsuperscript{207} and Cys\textsuperscript{213} were not observed in that of yeast Tsa2p (2-Cys Prx enzyme) (Fig. 1).

**Thioredoxin Peroxidase Activity of APE2278 Protein**

This ORF (APE2278) was amplified by PCR from a *A. pernix* genomic DNA, cloned, and sequenced to confirm the sequences in the database. The gene was expressed in *E. coli* cells, and the recombinant protein was purified to homogeneity as described under “Experimental Procedures.” The question of whether the reducing equivalents required for the presumed peroxidase activity of APE2278 protein could be provided by the Trx system (Trx, TR, and NADPH) were examined. The Thioredoxin peroxidase (TPx) activity of the recombinant APE2278 protein was examined by time-dependent removal of H\textsubscript{2}O\textsubscript{2} in the presence of the Trx system identified previously in *A. pernix* (18). The rate of H\textsubscript{2}O\textsubscript{2} consumption at 80 °C was measured by monitoring the decrease in \(A_{480}\). The APE2278 protein showed peroxide reductase activity in the presence of the Trx system (Fig. 2); all four components (APE2278 protein, Trx, TR, and NADPH) were required for the reduction of H\textsubscript{2}O\textsubscript{2}, which was negligible in the absence of any of one them. This protein, linked to the Trx system, displays peroxidase activity with a temperature optimum of 85 to 95 °C, which is in the temperature range for the growth of *A. pernix* (15). These results indicate that the APE2278 protein is a functional thioredoxin peroxidase (*ApTPx*) of *A. pernix*.

**Structure and Role of Cysteine Residues in ApTPx**

*ApTPx* contains three cysteines at positions 50, 207, and 213. To examine the catalytic role of the Cys residues, we constructed three C50S, C207S, and C213S mutant enzymes in which cysteines at positions 50, 207, and 213, respectively, were replaced by serine. For the combinations of all the three cysteines, C50/207S, C50/213S and C207/213S mutants were also constructed. The wild-type and mutant *ApTPx* proteins were expressed in *E. coli* cells and purified from the soluble fraction of the bacterial cells with the method described in experimental procedures.

A recent study reported that yeast Prx1p and human 1-Cys Prx forms a dimeric structure (17, 25). In order to elucidate the oligomeric structure of *ApTPx*, we examine the molecular weight of *ApTPx* proteins by SDS-PAGE using a DTT-free gel (PhastGel, Amersham Biosciences) after heating at 95 °C for 5 min in the presence or absence of 5 mM DTT. Under reducing conditions, protein with a molecular sizes (29 kDa) corresponding to the monomeric form (Fig. 3A) was detected for the wild-type, as well as the other mutant proteins. Under nonreducing conditions, however, the wild-type and C207S mutant showed two bands, corresponding to a monomeric and a dimeric form, while the other mutants appeared as one band, corresponding to the monomeric form (Fig. 3B). Similar patterns of distribution between monomeric and dimeric forms were also observed by SDS-PAGE after treatment with H\textsubscript{2}O\textsubscript{2} (not shown). These results indicate that the wild-type and C207S mutant is present as a mixture of monomers and dimers which are linked by an intersubunit
disulfide linkage (Cys$^{50} \rightarrow$ Cys$^{213}$).

The molecular weight of wild-type and mutant ApTPx was determined using gel filtration on a TSK-GEL G3000SW column. The wild-type ApTPx showed three peaks (about 490, 240, and 30 kDa), one of which corresponded to the hexadecameric form of ApTPx along with others corresponding to the octameric and monomeric forms, unlike the dimeric homologues from eukaryotes (Fig. 4A). To analyze the oligomer assembly of ApTPx, we examined wild-type and mutant proteins with electron microscopy. Micrographs of negatively stained wild-type ApTPx showed that this protein exhibited toroid-shaped particles with an outer diameter of 14 nm, inside diameter of 6 nm, and thickness of 5 nm (Fig. 5A, C). The majority of the particles have approximately the same diameter. In the side views, appears as single (Fig. 5D) or paired (Fig. 5E) toroid-shaped particles, corresponding to octameric or hexadecameric form, respectively. These observations imply that the three dimensional structure of ApTPx is mainly consist from toroid-shaped octameric form and its twofold toroid-shaped form. The octameric form of the quaternary structure was dissociated into monomers in the presence of 10 mM DTT (Fig. 4B), suggesting that the octameric form is sensitive to DTT and that the intersubunit disulfide linkage is responsible for the octameric form in the protein. The Cys$^{50}$ mutated proteins (C50S and C50/207S) were fully converted into an octameric form, and the C50/213S mutant showed only a peak corresponding to the monomeric form (Fig. 4C, E). These data indicate that the Cys$^{50}$ residue is critical for the production of the hexadecameric form of ApTPx and that mutations at both Cys$^{50}$ and Cys$^{213}$ have a profound effect on the dissociation of the octameric form. The C207S and C213S mutants showed a peak corresponding to the hexadecameric form, whereas the C207/213S mutant was converted to an octameric form (Fig. 4C, D). The C207/213S mutant also forms a toroid-shaped structure (Fig. 5B). The size and shapes of the top view are similar to those of the wild-type. However, the side view has only one toroid-shaped particle, which reflects the octameric subunit arrangement. These results were consistent with the data from gel filtration experiments. In the case of the C50S, C207S, C213S and C50/207S mutants, DTT treatment had no effect on their quaternary structure, whereas the octameric form of C207/213S mutant dissociated into monomers in the presence of 10 mM DTT. These results indicate that the hexadecameric form is insensitive to DTT but a Cys$^{50} \rightarrow$ Cys$^{50}$ intersubunit disulfide linkage appears to be reduced by DTT. No toroid-shaped particles were observed in micrographs of the C50S and C50/207S mutants, suggesting that Cys$^{50}$ plays an essential role to form the toroid-shaped structure in the oligomerization of ApTPx. The molecular weight of the C50S mutant was also confirmed by sedimentation equilibrium data from the analytical ultracentrifuge. Plots of apparent weight-average molecular weight, $M_w\text{ app}$, versus concentration of protein, $c(r)$, for two different loading concentrations of protein indicate that the C50S mutant exists as an octamer in its native state ($M_w\text{ app}$, 242947) (Fig. 6).

**Peroxidase Activity of Cys Mutants of ApTPx**

In the presence of an electron donor such as DTT, Fe$^{3+}$ catalyzes the reduction of O$_2$ to H$_2$O$_2$, which is further converted to hydroxyl radicals (OH•) by the Fenton reaction (26). The DTT mediated metal catalyzed oxidation (MCO) system therefore inflicts damage to various enzymes, including glutamine synthetase, and this damage can be prevented by an enzyme...
that catalyzes the elimination of H$_2$O$_2$. Yeast and mammalian TPx enzymes protect glutamine synthetase from inactivation induced by the MCO system; oxidized TPx can be reduced by DTT (8). ApTPx can also protect glutamine synthetase against damage by the MCO system. To evaluate the peroxidase activity of ApTPx mutants, we used this glutamine synthetase protection assay. The C207S mutant showed protection activity although it was less effective than the wild-type, whereas the mutants lacking Cys$^{50}$ (C50S, C50/207S and C50/213S) failed to show any activity (Fig. 7A). Similarly, the C213S mutant was also inactive. Interestingly, the double mutant C207/213S retained a protection activity similar to the wild-type, thus acting as a revertant of the C213S mutation. These results indicate that Cys$^{50}$ and Cys$^{213}$ are required for the enzymatic reaction to proceed, that an efficient reaction involves all the three cysteines, and that Cys$^{50}$SOH can be converted back to Cys$^{50}$SH by DTT. We also compared the peroxidase activities of the wild-type and mutants by directly monitoring the decrease in H$_2$O$_2$ concentration in the presence of the Trx system (Fig. 7B). Consistent with the results of the glutamine synthetase protection assay, C207S was found to be partially active in the Trx-dependent assay, and no activity was detected for the other mutants. However, in contrast to the results obtained with the glutamine synthetase protection assay, C207/213S was inactive. These data suggest that Cys$^{50}$ is essential for both Trx- and DTT-dependent peroxidase activities of ApTPx, and that Cys$^{213}$ is essential for Trx-dependent activity but not for DTT-dependent activity.

The kinetic parameters for the wild-type and C207S mutant were determined by measuring the initial rates of NADPH oxidation in the presence of various concentrations of H$_2$O$_2$ (8). Lineweaver-Burk plots (not shown) revealed that the $K_m$ values for H$_2$O$_2$ of wild-type and C207S mutant were 72 ± 10 and 209 ± 32 µM, respectively, and that the $V_{max}$ at 37 °C was 0.65 ± 0.04 or 0.71 ± 0.09 µmol/min/mg of protein for the wild-type and C207S mutant, respectively.

Expression of ApTPx in A. pernix Cells

To quantitatively analyze ApTPx expression in A. pernix cells, a polyclonal mouse antiserum specific for the ApTPx protein was raised against recombinant ApTPx protein. Anti-ApTPx antisera was obtained and used in a western blot analysis for cytoplasmic extracts from exponential and stationary growth phases (Fig. 8A). Signals for 29 kDa corresponding to ApTPx were constantly detected in both the exponential and stationary phases. Furthermore, the signal intensity of the stationary phase was increased by 1.9-fold over that of the exponential phase, as evidenced by densitometric analysis (Gel-Pro Analyzer 4.0, MediaCybernetics, MD, USA) (data not shown). This suggests that the expression of ApTPx is paralleled by the cellular oxygen consumption levels. We also analyzed whether ApTPx expression is elevated in cells that are exposed to peroxide. Growing cells at the exponential phase were exposed to different concentrations of H$_2$O$_2$ for 1 h. The expression of ApTPx showed a maximal induction (11-fold) between 0.1 and 0.25 mM H$_2$O$_2$ (Fig. 8B). Subsequently the levels of ApTPx decreased even below that of the control because of toxicity (26 and 15% survival at 0.75 and 1.0 mM H$_2$O$_2$, respectively) at higher concentrations of H$_2$O$_2$. 

DISCUSSION

The Prx family can be divided into two subgroups as follows: 2-Cys Prx proteins, which contain Cys residues at both the N- and C-terminal regions, and 1-Cys Prx proteins, which contain only one Cys at the N-terminal region (23). 1-Cys Prxs is able to reduce peroxides using DTT as electron donor, but the physiological electron donor for 1-Cys Prxs is currently unknown. The 2-Cys Prxs reduce peroxides with electrons provided by the thioredoxin system (Trx/Trx reductase/NADPH) and are referred to as thioredoxin peroxidases (TPx). Recently, however, it was reported that yeast contains a 1-Cys peroxiredoxin (Prx1p) with TPx activity (17). Thioredoxin-dependent peroxidase would be expected to be present in A. pernix cells because a thioredoxin system (ApTrx and ApTR) in A. pernix has already identified and characterized (18). The protein coded by the ORF APE2278 in the A. pernix genome showed a higher homology to the 1-Cys Prx than the 2-Cys Prx. The issue of whether it could use electrons donated by the thioredoxin system, and whether the recombinant protein (ApTPx) had TPx activity, despite of its higher sequence similarity with 1-Cys Prx was examined.

The crystal structure of the human 1-Cys Prx showed that the protein forms a dimeric structure (25). In the human 1-Cys Prx, most of the amino acids important for dimerization (e.g. Phe-43, Thr-44, Pro-45, Val-46, Thr-48, Thr-49, Glu-50, Tyr-149, Pro-191, Tyr-217, and Leu-218) are conserved in ApTPx (Fig. 1). The recombinant ApTPx was expected to be present as a dimeric form in the natural state, similar to its homologues from other eukaryotes. Interestingly, from the gel filtration and electron microscopy experiments, however, ApTPx was found to exist in a novel hexadecameric structure, possibly in equilibrium with a small amount of the octamer. Electron microscopy revealed clearly that ApTPx is organized in twofold toroid-shaped particles (Fig. 5). The toroid-shaped decameric structures of Prx enzymes have been identified in human TPx-B and Salmonella typhimurium.
AhpC, based on their crystal structures (27-29). However, no evidence for the hexadecamerization of this type of enzyme has been reported for members of the Prx family to date. Since the hexadecameric structure of the ApTPx was not disrupted by the addition of a reductant such as DTT (Fig. 4B), it does not appear that the oligomeric structure is maintained by disulfide bonds at the surface of the molecule. In contrast, the octamers of the ApTPx were dissociated into monomers under reducing conditions, implying the presence of an intersubunit disulfide linkage. The above results indicate that the oligomeric structure is related to intermolecular disulfide bonds and the hexadecameric structure requires additional factors. The fact that the C207S and C213S mutants form the hexadecameric structure but C50S and C50/207S mutants do not indicates that the Cys\textsuperscript{50} residue is critical for its structure. However, the fact that the hexadecameric form is not sensitive to DTT indicates that the hexadecameric form may be stabilized, not only by an intersubunit disulfide linkage, but also by non-covalent bond. The result that the double mutation C207/213S did not form the hexadecameric structure suggest that a little conformational change around Cys\textsuperscript{50} may be induced by this mutation. Furthermore, octamer formation was inhibited when both Cys\textsuperscript{50} and Cys\textsuperscript{213} were replaced with Ser, indicating that one of their cysteine residues is required for octamer formation. The result that no more than twofold toroid-shaped particles was observed by electron microscopy, suggests that the buried interface between the two toroid-shaped particles is different from the outer side of it.

Site-directed mutagenesis studies indicate that all three cysteine residues in the ApTPx are important for its activity. The Cys\textsuperscript{50} corresponds to a residue that is highly conserved in the Prx proteins, assumes an essential role in catalysis, and the Cys\textsuperscript{213} is also essential for activity. Mutant ApTPx proteins laking either Cys\textsuperscript{50} or Cys\textsuperscript{213} had no activity. In the absence of Cys\textsuperscript{207} and Cys\textsuperscript{213}, the enzyme is active in the presence of DTT but not in the presence of the Trx system. Furthermore, under oxidation conditions by H\textsubscript{2}O\textsubscript{2}, C50S and C213S mutants failed to form a dimer linked by disulfide bonds, whereas the wild-type and C207S mutant readily formed a dimer. From the proposed catalytic mechanism of 2-Cys Prx (8), we suggest the following mechanism of ApTPx shown in Fig. 9. In the process of the reaction, Cys\textsuperscript{50} is the primary site of oxidation by H\textsubscript{2}O\textsubscript{2}, and the oxidized Cys\textsuperscript{50}S forms an intermolecular disulfide bond that is subsequently reduced by Trx. The proposed reaction intermediate with the intermolecular Cys\textsuperscript{50}Cys\textsuperscript{213} disulfide bonds is also consistent with SDS-PAGE results. Thus, despite the higher sequence similarity of ApTPx with 1-Cys Prx, this protein seems to be functionally closer to 2-Cys TPx than to 1-Cys TPx. In human 1-Cys Prx (23), the Cys\textsuperscript{47} is the site of oxidation by H\textsubscript{2}O\textsubscript{2}, but the resulting Cys\textsuperscript{47}SOH cannot form a disulfide bond because there is no other Cys\textsuperscript{SH} nearby. A thiol such as DTT can support the regeneration of the enzyme \textit{in vitro}. In this respect, the catalytic mechanism of 1-Cys Prx resembles that of the C207/213S mutant, which is active in the presence of DTT. However, the C213S mutant has no peroxidase activity. It is possible that the Cys\textsuperscript{50}Cys\textsuperscript{207} disulfide bond is occurred in region inaccessible to the solvent because the activity of the protein is intricately related to conformational changes, intersubunit disulfide bond formation and oligomerization. A similar phenomena was observed by Chauhan et al. (30). The activity of C207S showed a lower efficiency in comparison to the wild-type. The kinetic parameters for wild-type and C207S indicate that
Cys^{207} is related to substrate binding.

The novel structural and catalytic characterization of the Trx-dependent peroxidase in aerobic hyperthermophilic archaea reported here show that ApTPx is dependent on a functional NADPH/TR/Trx system to reduce H_{2}O_{2} \textit{in vitro}. Furthermore, ApTPx forms a hexadecamer, composed of two identical octamers and the mechanism of its action appears to be identical to 2-Cys Prx. In yeast, induction of an antioxidant protein synthesis by thiol-dependent mixed-function oxidation (MFO) systems was first demonstrated by Kim et al. (31). Since Prx1p has been reported to be induced as a cellular adaptation in response to the presence of exogenous H_{2}O_{2} and to play an important role in protection against oxidative stress (17). We found that ApTPx is induced as a cellular adaptation against external oxidative stress, similar to the report of yeast Prx1p. The expression of ApTPx in A. pernix cells under oxidizing conditions also reflects the importance of this peroxidase in cellular protection against external peroxide. In aerobic archaea, the presence of a thioredoxin peroxidase/thioredoxin system suggests that these may play an important protective role against oxidative damages caused by peroxides and may reflect the ancestral features of the antioxidant defense system. The novel oligomeric structure may represent tactic for getting multifunctional antioxidant system and achieving hyperthermostability of the enzyme.

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FIGURE LEGENDS

Fig. 1. Alignment of the amino acid sequence of A. pernix TPx and other known Prx proteins. The alignment was obtained using clustal W (version 1.7). The conserved cysteine residue is boxed, and the positions of Cys207 and Cys213 of A. pernix TPx (closed circle) are indicated. Asterisks indicate amino acids conserved among A. pernix TPx, yeast Prx1p, human 1-Cys Prx, and yeast Tsa2p.
Fig. 2. Thioredoxin peroxidase activity of recombinant \textit{Ap}TPx. Peroxidase activity was measured using ferrithiocyanate at 85 °C as described under “Experimental Procedures.” The peroxidase reaction was carried out in a 0.5 ml reaction volume containing different mixtures of \textit{Ap}TR, \textit{Ap}Trx, \textit{Ap}TPx, and NADPH, as indicated. The nonenzymatic removal of H_2O_2 by heat was also performed in parallel. Experiments were done in triplicate. Error bars indicate the S.E.

Fig. 3. Analysis of wild-type and mutant \textit{Ap}TPx proteins by reducing or nonreducing SDS-PAGE. The purified proteins were mixed with the same volume of reducing sample buffer [125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 5 mM DTT] (\textit{A}), or nonreducing sample buffer (reducing sample buffer without 5 mM DTT) (\textit{B}), following by a 5-min incubation at 25 °C. After heat treatment at 95 °C for 5 min, the samples were subjected to SDS-PAGE on PhastGel (Amersham Biosciences), and the gel was stained with Coomassie Blue. Lanes 1-7, recombinant wild-type, C50S, C207S, C213S, C50/207S, C50/213S, C207/213S \textit{Ap}TPx proteins, respectively.

Fig. 4. HPLC gel filtration analysis of wild-type \textit{Ap}TPx and its cysteine mutants. Purified wild-type and mutant \textit{Ap}TPx were applied to a TSK-gel G3000SW column (Tosoh Co., Tokyo, Japan) on a Tosoh HPLC system (Tosoh Co., Tokyo, Japan). The proteins were eluted at a flow rate of 0.6 ml/min with 50 mM Na-PO_4 (pH 7.0) containing 150 mM NaCl, except for (\textit{B}). Elution was monitored by measurement of A_280. (\textit{A}) Chromatogram of wild-type \textit{Ap}TPx; (\textit{B}) Wild-type \textit{Ap}TPx in 50 mM Na-PO_4 (pH 7.0) containing 150 mM NaCl and 10 mM DTT; (\textit{C}) C50S, C50/207S or C207/213S; (\textit{D}) C207S or C213S; (\textit{E}) C50/213S. The column was calibrated with molecular mass standards from Amersham Biosciences: ferritin (440 kDa), catalase (232 kDa), albumin (67 kDa), chymotrypsinogen A (25 kDa). The peak positions of molecular mass standards are indicated as arrow.

Fig. 5. Electron micrographs of the wild-type and mutant \textit{Ap}TPx. \textit{A}, wild-type. \textit{B}, C207/213S mutant. The toroid structure in the top views (\textit{C}). Single (\textit{D}) and paired (\textit{E}) toroid structures in the side views. Electron microscopy was carried out as described under “Experimental Procedures” and repeated more than two times for each protein. The distributions of particles obtained were found to be reproducible. The \textit{Bar} presents a distance of 20 nm.

Fig. 6. Sedimentation equilibrium data for the C50S mutant \textit{Ap}TPx. Apparent weight average molecular weight values (M_{w,app}) were calculated from absorbance values measured from the equilibrium distribution of the protein in the ultracentrifuge cells. Data obtained at two separate concentrations, 0.3 (\textit{TM}) and 0.6 mg/ml (\textit{L}).

Fig. 7. Effects of the replacement of Cys^{50}, Cys^{207}, or Cys^{213} of \textit{Ap}TPx with serine on peroxidase activity. \textit{A}, GS protection activity of \textit{Ap}TPx and its mutants. \textit{Ap}TPx proteins were assayed for their ability to protect glutamine synthetase from inactivation as described under “Experimental Procedures.” The extent of protection is expressed as a percentage of the inactivation value in the absence of \textit{Ap}TPx. \textit{B}, Time-dependent removal of H_2O_2 by \textit{Ap}TPx and its mutants. At the indicated times, the remaining concentration of H_2O_2 was measured.
using ferrithiocyanate as described under “Experimental Procedures.” Wild-type (ε), C50S (p), C207S (●), C213S (q), C50/207S (s), C50/213S (TM), C207/213S (r), and negative control (£). Data represent duplicate experiments.

Fig. 8. **Expression of ApTPx in A. pernix cells.** A, Lanes 1 and 2 were loaded with 50 µg of cytoplasmic proteins extracted from 8 h (mid-exponential phase) and 18 h (stationary phase) cell cultures of A. pernix, respectively, and analyzed by Western blot with anti-ApTPx antibodies. B, Cells in the exponential phase were exposed to different H2O2 concentrations for 1 h. Each lane was loaded with 20 µg of cytoplasmic proteins extracted from cells.

Fig. 9. **Proposed reaction mechanism for ApTPx.** Closed circles indicate the N-terminus.
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