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Oxidative Stress Response in an Anaerobic Hyperthermophilic Archaeon: Presence of a Functional Peroxiredoxin in *Pyrococcus horikoshii*

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Oxidative stress response in an anaerobic hyperthermophilic archaeon *Pyrococcus horikoshii* OT-3 was analyzed by two-dimensional gel electrophoresis. When *P. horikoshii* was grown on medium supplemented with air, a marked increase in the level of a 25-kDa protein was observed in comparison with cells grown under anaerobic conditions. The N-terminal amino acid sequence of the protein was determined to be VVIGEKFPEVEVKTTHGVIKLPDYF, which coincides with that of the putative alkyl hydroperoxide reductase that has been predicted in the genome database of *P. horikoshii*. The gene (PH1217) encoding the protein was cloned and expressed in *Escherichia coli*. The produced enzyme was a hyperthermostable peroxiredoxin whose activity was not lost after incubation at 90°C for 20 min. The enzyme catalyzes the reduction of cumene hydroperoxide and hydrogen peroxide using dithiothreitol as an electron donor. Northern blot analysis revealed that the transcription of the gene increased by the addition of exogenous oxygen and by the addition of an oxidative stress-inducing reagent, and reached maximum within 30 min. These results suggest that the peroxiredoxin plays an important role in the peroxide-scavenging system in an anaerobic archaeon *P. horikoshii*.

Key words: alkyl hydroperoxidase, archaea, hyperthermophile, oxidative stress, peroxiredoxin, *Pyrococcus horikoshii*.

Aerobic organisms utilize molecular oxygen as a terminal electron acceptor. As a result of the incomplete reduction of oxygen, reactive oxygen species (ROS) are formed (*1*). ROS are also generated by UV radiation and by autoxidation of materials such as thiols and flavins (*2*). Increased levels of ROS can lead to severe damage of membrane lipids, proteins and DNA (*3*, *4*). Thus, aerobic organisms have developed efficient mechanisms to eliminate ROS: superoxide anion is converted to hydrogen peroxide and O_2 by superoxide dismutase (*1*), and the accumulation of hydrogen peroxide is prevented by the action of catalases and peroxidases (*5*). On the other hand, in some obligate anaerobic bacteria, the presence of these antioxidative enzymes has also been reported (*6–9*). Recent studies have shown that these enzymes play an important role in the detoxification of ROS in anaerobic bacteria as in aerobic organisms, and might be related to oxygen tolerance of these organisms (*6*, *7*).

Peroxiredoxin (Prx), commonly referred to as alkyl hydroperoxidase (AhpC), comprises a newly discovered group of antioxidant enzymes that catalyze both the reduction of hydrogen peroxide to water and alkyl hydroperoxides to the corresponding alcohols (*10*). It has been identified in a variety of organisms from bacteria, including obligate anaerobic bacteria (*7*), to eukarya (*11*, *12*), and has been found to have similar functions. An electron donor is required for the catalytic turnover of the Prx reaction, and the type of electron-donor varies among organisms. Bacteria such as *E. coli*, *Salmonella typhimurium*, and *Bacillus subtilis* possess the alkyl hydroperoxide reductase F-protein (AhpF) as an electron-donor partner for Prx (*13–15*). In yeast and mammalian systems, the thioredoxin/thioredoxin reductase system (Fig. 1B) substitutes for AhpF (*11*, *16*). In *Amphibacillus xylanus*, it has been shown that NADH oxidase can also reduce Prx (*17*). On the other hand, Prx from *Mycobacterium tuberculosis* uses dithiothreitol (DTT) as an electron-donor and efficiently oxidizes DTT in the presence of substrate (*18*, *19*). Because AhpF is absent in *M. tuberculosis*, it has been proposed that the mycobacterial Prx might use small-molecule materials such as mycothiol as the electron-donor partner (*18*). Very recently, Prx from a strict aerobic hyperthermophilic archaeon, *Aeropyrum pernix*, was characterized (*20*). Interestingly, the enzyme utilizes a novel thioredoxin/thioredoxin reductase system in *A. pernix* (*21*) and was stated to be a thioredoxin peroxidase (TPx). A gene showing some homology to Prx has been found in anaerobic archaea such as *Pyrococcus horikoshii* (*22*) and *Methanococcus jannaschii* (*23*); however, the function and properties of the Prx from anaerobic archaea remain unclear.

In the present study, the oxidative stress response of an anaerobic hyperthermophilic archaeon *P. horikoshii* was examined. As a result, we observed a significant

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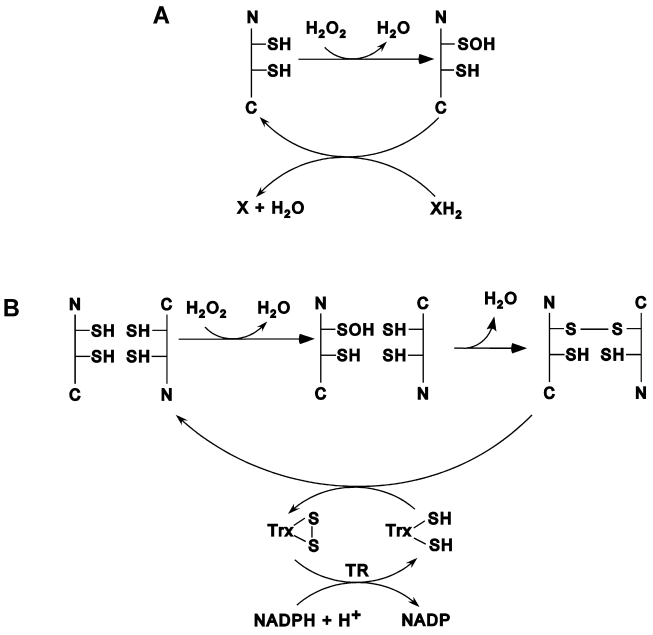

Fig. 1. **Reaction mechanisms of 1-Cys Prx (A) and TPx (2-Cys Prx) (B).** XH_2 denotes the unidentified electron donor for 1-Cys Prx. Trx and TR indicate thioredoxin and thioredoxin reductase, respectively.

increase in the level of a protein when the microorganism was cultivated under aerobic conditions as compared with the anaerobic control. The protein was identified as a Prx that catalyzes the reduction of alkyl hydroperoxide and hydrogen peroxide in the presence of DTT. We present here a functional Prx from an anaerobic archaeon. While this work was in progress, the same oxidative stress responsible protein was identified in *P. horikoshii* (*24*). However, analyses of the transcriptional response of the protein are still lacking. In this paper, we show the transcriptional regulation of the Prx by oxidative stress in *P. horikoshii*. In addition, we propose that the electron-donor partner of the enzyme might be different from that in *A. pernix* based on genome analysis.

MATERIALS AND METHODS

Materials—*E. coli* strain BL21 (DE3) codon plus RIL was obtained from Stratagene (La Jolla, CA). Plasmid DNA pET15b was obtained from Novagen, Inc. (Madison, WI). KOD DNA polymerase was obtained from Toyobo (Osaka). Cumene hydroperoxide and *t*-butyl hydroperoxide were purchased from nacalai tesque (Kyoto). DIG High Prime DNA Labeling and Detection Kit was purchased from Roche Diagnostics (Tokyo). All other chemicals were of reagent grade.

Organism and Growth Conditions—The hyperthermophilic archaeon *P. horikoshii* OT-3 (JCM 9975) was obtained from the Japanese Collection of Microorganisms (JCM), Wako, Saitama, Japan. The microorganism was cultured in medium containing 5 g of tryptone, 1 g of yeast extract, 25 g of NaCl, 1 g of cysteine-HCl, 1.3 g of $(NH_4)_2SO_4$, 0.28 g of KH_2PO_4, 0.25 g of $MgSO_4{\cdot}7H_2O$, 0.07 g of $CaCl_2{\cdot}2H_2O$, 0.02 g of $FeCl_3{\cdot}6H_2O$, 1.8 mg of $MnCl_2{\cdot}4H_2O$, 4.5 mg of $Na_2B_4O_7{\cdot}10H_2O$, 0.22 mg of $ZnSO_4{\cdot}7H_2O$, 0.05 mg of $CuCl_2{\cdot}2H_2O$, 0.03 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.03 mg of $VOSO_4{\cdot}2H_2O$, 0.01 mg of $CoSO_4{\cdot}7H_2O$, and 5 g of elemental sulfur per liter (pH 6.5, adjusted with 3 N NaOH). Dissolved oxygen was removed from the medium by an aspirator, and liquid paraffin was then layered on the surface of the medium to prevent its contact with air. Anaerobic conditions were achieved by flushing the medium with N_2 gas (99.9999%) introduced via butyl-rubber tubes from an EYELA GMU-1 gas flow controller (Tokyo) at a flow rate of about 50 ml/min and an N_2-gas cylinder. Oxidative stress was induced by replacing the N_2 gas with air under the same conditions. Air was introduced from a Hitachi BEBICON air compressor (Tokyo) equipped with a 30 liter air receiver. The seed culture (50 ml) was inoculated into a bottle (1 liter volume) filled with medium (950 ml), and the bottle was incubated at 90°C on a hot plate with stirring with a magnetic bar. After 18-h cultivation (early-stationary phase), aeration (50 ml/min) was started. The cells were collected by centrifugation (10,000 × *g* for 10 min) and washed twice with 3% NaCl solution. The washed cells were suspended in 50 mM Tris/HCl buffer (pH 7.0) containing 1 mM EDTA and 10 % glycerol.

Polyacrylamide Gel Electrophoresis—The cells were disrupted by sonication, the cell debris was removed by centrifugation (15,000 × *g*, 10 min), and the supernatant solution was used as the crude extract. SDS–polyacrylamide gel electrophoresis (PAGE, 12.5% acrylamide slab gel, 1mm thick) was performed by the procedure of Laemmli (*25*). For two-dimensional gel electrophoresis, 38 μg of total protein (crude extract) was mixed with rehydration solution (final concentration: 8 M urea, 2% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}, 20 mM DTT, a trace amount of bromophenol blue, and 0.5% IPG buffer (pHs 4 to 7; Amersham Bioscience, Piscataway, NJ)). The solution (125 μl) was loaded onto Immobiline DryStrips (pH 4 to 7, 7 cm; Amersham Bioscience) and rehydration was performed according to the recommendation of the manufacturer (Amersham Bioscience). Proteins were focused using an IPGphor isoelectric-focusing apparatus (Amersham Bioscience) in the following steps: step 1, 12 h at 50 V (step-n-hold); step 2, 1 h at 500 V (gradient); step 3, 1 h at 1,000 V (gradient); and step 4, 2 h at 8,000 V (gradient). After isoelectric focusing, the strips were soaked in 50 mM Tris/HCl buffer (pH 6.8) containing 8 M urea, 30% glycerol, 2% SDS, and 65 mM DTT for 10 min, and then soaked in 50 mM Tris/HCl buffer (pH 8.8) containing 8 M urea, 30% glycerol, 2% SDS, and 0.25% iodoacetamide for 10 min. The strips were then placed onto a 12.5% acrylamide gel, and the proteins were separated in the second dimension by SDS-PAGE. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

N-terminal Amino Acid Sequencing—After two-dimensional gel electrophoresis, the proteins were electroblotted onto a polyvinylidene difluoride membrane, stained with Ponceau S, and destained. A protein spot was excised and subjected to automated Edman degradation using a Shimadzu Model PPSQ-10 protein sequencer.

Cloning and Expression of the Gene Encoding Prx and Purification of the Recombinant Enzyme—The following set of oligonucleotide primers was used to amplify the Prx gene (PH1217) fragment by PCR: the first primer (5′-

TCTCCATATGGTAGTGATTGGAGAAAAGTT-3′) introduced a unique *Nde*I restriction site overlapping the 5′ initiation codon, and the second primer (5′-ATATGGATCCTCATTCAAGCTTCTTATAGC-3′) introduced a unique *Bam*HI restriction site proximal to the 3′ end of the termination codon. The chromosomal *P. horikoshii* DNA was isolated as described before (*26*) and used as the template. The amplified 0.7 kb fragment was digested with *Nde*I and *Bam*HI and ligated with the expression vector pET15b linearized with *Nde*I and *Bam*HI to generate pHPRX. *E. coli* strain BL21 (DE3) codon plus RIL was transformed with pHPRX. The transformants were cultivated at 37°C in 500 ml of medium containing 6 g of tryptone, 12 g of yeast extract, 2.5 ml of glycerol, 6.25 g of K_2HPO_4, 1.9 g of KH_2PO_4, and 50 μg/ml of ampicillin until the optical density at 600 nm reached 0.6. Induction was carried out by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to the medium, and the cultivation was continued for 3 h. Cells were harvested by centrifugation, suspended in 20 mM sodium phosphate buffer (pH 7.4) containing 10% glycerol, and disrupted by ultrasonication. The crude extract was heated at 90°C for 20 min, and the denatured protein was then removed by centrifugation (15,000 × *g* for 15 min). NaCl was added to the enzyme solution to 0.5 M. The enzyme solution was loaded on a Ni-charged Hi Trap Chelating HP (50 ml; Pharmacia) equilibrated with buffer A (20 mM sodium phosphate buffer, pH 7.4, containing 10% glycerol and 0.5 M NaCl). After the column was washed with buffer A containing 50 mM imidazole, the protein was eluted with 300 ml of buffer A containing 0.5 M imidazole. The active fractions were pooled, dialyzed against 20 mM Tris/HCl buffer (pH 7.2) containing 10% glycerol and 1 mM EDTA, and used as the purified enzyme preparation. For enzyme purification, the entire operation was done at room temperature (~25°C).

Assay of Enzyme Activity by the Ferrithiocyanate Method—Prx activity was assayed spectrophotometrically by measuring the removal of the peroxide substrate from the reaction mixture as described before (*18*). Peroxides form a complex with ferrithiocyanate with an absorption maximum of 480 nm. The reaction system comprised, unless specified otherwise, 50 mM citrate/NaOH buffer, pH 4.8, 1 mM EDTA, 2 mM cumene hydroperoxide, 10 mM DTT, and the enzyme preparation (0.65–1.0 mg) in a total volume of 1.0 ml. The reaction was started by the addition of DTT, and the mixture was incubated at 50°C. The reaction was terminated by adding an equal amount of 40% trichloroacetic acid. After removal of the precipitated protein by centrifugation, 0.65 ml of H_2O, 0.2 ml of 10 mM ferrous ammonium sulfate, and subsequently 0.1 ml of 2.5 M sodium thiocyanate were added to a 50 μl aliquot of the supernatant. Absorption of the red ferrithiocyanate complex was measured at 480 nm and was compared with cumene hydroperoxide standards. Protein was determined by the method of Bradford (*27*) with bovine serum albumin as the standard.

DTT-Oxidation Assay—The rate of DTT oxidation catalyzed by Prx in the presence of the peroxide substrate was measured by monitoring the change in absorbance at 310 nm due to the formation of the DTT disulfide as described previously (*19*). The buffer and water used for the assay were pretreated with Chelex (BioRad) as recommended by the supplier. The reaction system comprised, unless specified otherwise, 50 mM citrate/NaOH buffer, pH 4.8, 1 mM EDTA, 2 mM cumene hydroperoxide, 10 mM DTT, and the enzyme preparation (0–2.0 mg) in a total volume of 1.0 ml. The reaction was started by the addition of cumene hydroperoxide and the mixture was incubated at 50°C. The initial rate of DTT oxidation was obtained by calculating the slope over the first 15 s after addition and mixing of the peroxide. The initial rates were corrected for the background oxidation of DTT by peroxides in the absence of enzyme.

Thermal Stability, pH Stability and pH Optimum—To determine thermostability, the enzyme in 20 mM Tris/HCl buffer (pH 7.2) containing 10% glycerol and 1mM EDTA was incubated at different temperatures for 20 min, and the residual activity was determined by the DTT oxidation assay. To determine pH stability, the enzyme in buffers of various pH was incubated at 50°C for 20 min, and the remaining activities were then assayed by the same method. The following buffers (50 mM) were used: citrate/NaOH buffer, potassium phosphate buffer, Hepes/NaOH buffer, Tris/HCl buffer, glycine/NaOH buffer, and potassium phosphate buffer for pH ranges of 3.0–5.5, 5.5–7.0, 7.0–8.0, 8.0–9.0, 9.0–11.0, and 11.0–12.0, respectively. The optimal pH of the enzyme was determined by performing the assay at 50°C using citrate/NaOH buffer (50 mM) for pH ranges of 3.5–6.5.

Isolation of Total RNA and Northern Analysis—Cells were cultivated and harvested under the same conditions as described above, and RNA was isolated using the Acid guanidinium-Phenol-Chloroform method (*28*). For Northern blot analysis, 50 μg of total RNA was separated in a formaldehyde–1% agarose gel (15 × 13 cm) and subsequently transferred to a Hybond-N^+ membrane by capillary blotting (*26*). A 0.7-kb *Nde*I–*Bam*HI restriction fragment of DNA from pHPRX was used as the gene-specific probe for Prx. DIG labeling and detection were performed according to the protocols supplied by the manufacturer.

RESULTS AND DISCUSSION

Oxidative Stress Response in P. horikoshii—To investigate the oxidative stress response in the anaerobic hyperthermophilic archaeon *P. horikoshii*, we cultivated the organism under aerobic conditions. Flushing the medium with air at a flow rate of about 50 ml/min/liter-medium did not inhibit the growth of the organism. About 1 g (wet weight) of cells was obtained after cultivation under both aerobic and anaerobic conditions. SDS-PAGE analysis revealed a significant increase in the level of a protein in cells cultivated under aerobic conditions as compared with the anaerobic control (Fig. 2A). The protein had a molecular mass of about 25 kDa. Considerable amounts of the protein were still observed in cells grown on medium supplemented with N_2 gas (99.9999%) via silicone tubes, which are known to be permeable to only minor amounts of air. This indicates that the protein was induced in response to the presence of trace amounts of oxygen. The protein profile was further examined by two-dimensional gel electrophoresis (Fig. 2B). The increase in the amount of the 25-kDa protein was the most significant alteration in the profile under aerobic conditions.

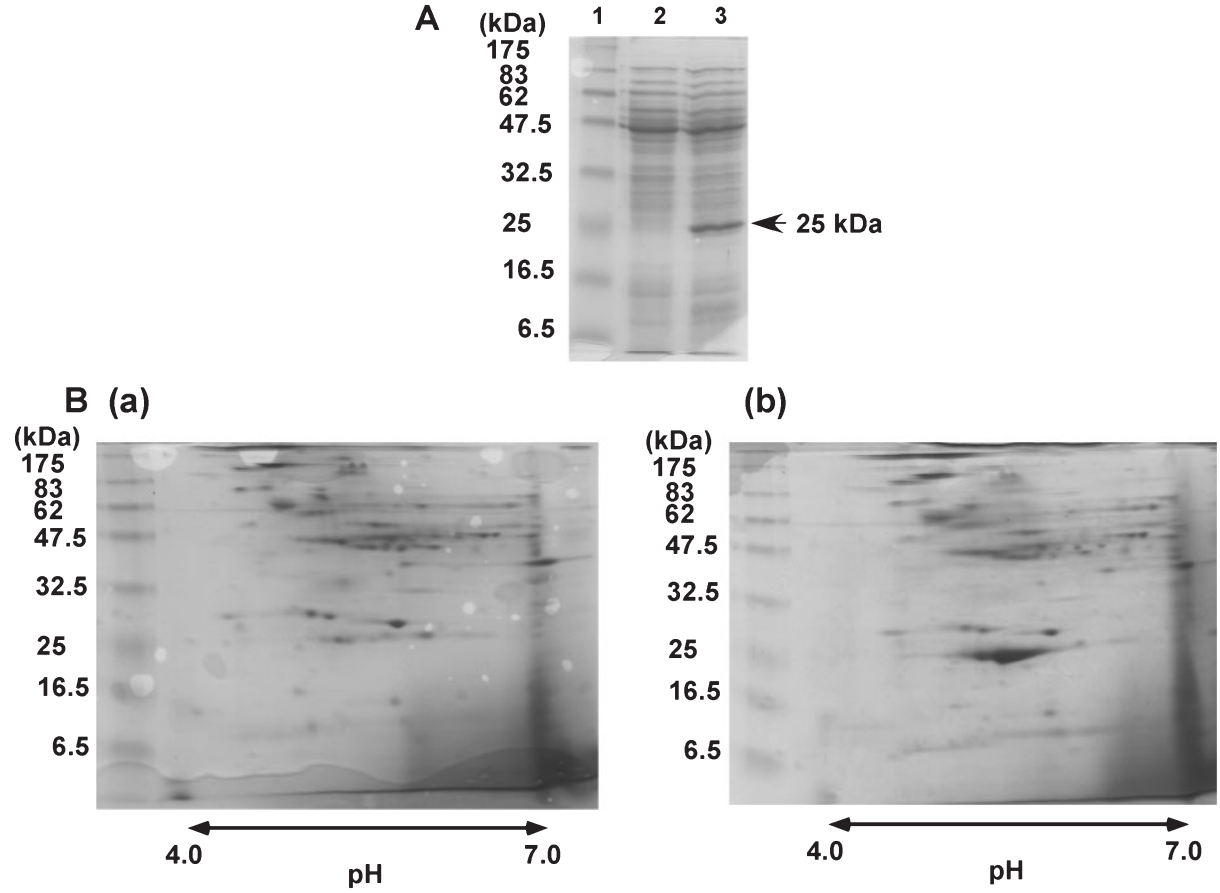

Fig. 2. **Polyacrylamide gel electrophoresis of *P. horikoshii* extract.** (A) One-dimensional SDS-PAGE. Lanes: 1, molecular mass standards; 2, crude extract prepared from cells cultivated under anaerobic conditions; and 3, crude extract prepared from cells cultivated under aerobic conditions. (B) Two-dimensional gel electrophoresis. (a) crude extract prepared from cells cultivated under anaerobic conditions; and (b) crude extract prepared from cells cultivated under aerobic conditions. Molecular mass standards are shown on the left.

This suggests that the induction of the protein is a major response of *P. horikoshii* to oxygen exposure.

Identification of the Gene Encoding the 25-kDa Protein—The complete sequence of the genome of *P. horikoshii* has been reported by Kawarabayasi *et al.* (*22*). The N-terminal amino acid sequence of the 25-kDa protein was determined to be VVIGEKFPEVEVKTTHGVIKLPDYF, which coincides with the N-terminal amino acid sequence of the putative alkyl hydroperoxide reductase, except for the N-terminal methionine, predicted in the genome of *P. horikoshii*. The N-terminal methionine might be removed by processing in *P. horikoshii* cells as described by Kashima *et al.* (*24*). PH1217 (651 bp, positions 1096033–1096683 in the entire genome) has been assigned as the gene encoding the protein (a protein of 216 amino acids with a molecular weight of 24,756 and a pI of 5.53). The molecular weight and pI value coincided with those estimated from the two-dimensional gel electrophoresis (Fig. 2B).

Purification and Characteristics of the Recombinant Enzyme—*E. coli* BL21 (DE3) codon plus RIL cells transformed with the expression vector pHPRX produced hyperthermostable Prx, the activity of which was not lost upon incubation at 90°C for 20 min. The enzyme was purified to homogeneity from the *E. coli* cell extract. The molecular mass of the recombinant protein was determined to be 26 kDa by SDS-PAGE, which corresponds to the expected size. About 54 mg of purified enzyme was obtained from 500 ml of *E. coli* culture.

Because no homologues of AhpF or thioredoxin appear to be encoded in the *P. horikoshii* genome, we chose DTT for use in the activity assay of the Prx as described for the *M. tuberculosis* enzyme (*18*). In the DTT-oxidation assay, the formation of DTT disulfide is analyzed directly. The oxidation of DTT in the presence of cumene hydroperoxide was catalyzed by the *P. horikoshii* Prx. We also measured DTT oxidation in the absence of substrate or enzyme as control experiments. DTT oxidation was not observed at 50°C when the substrate or enzyme was absent. In addition, in the final purification procedure of HiTrap chelating HP, we confirmed that the DTT oxidation activity depends on the protein elution, indicating that DTT oxidation is caused by the peroxiredoxin activity. A linear dependence was observed for the oxidation of DTT as a function of Prx concentration (Fig. 3). However, an increase in the background absorbance at 310 nm was observed in the absence of enzyme at temperatures over 50°C because of substrate instability. Thus we assayed the enzyme activity at 50°C in all experiments. To confirm the enzyme activity, a ferrithiocyanate assay was

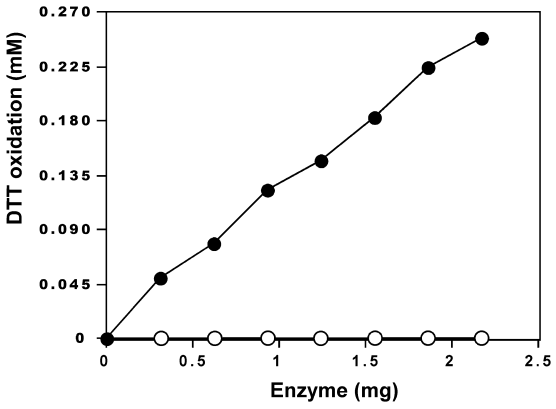

Fig. 3. **DTT oxidation as a function of enzyme concentration.** The oxidation of DTT in the presence of cumene hydroperoxide (solid circles) and in the absence of substrate (open circles) was measured by monitoring the change in absorbance at 310 nm due to the formation of DTT disulfide.

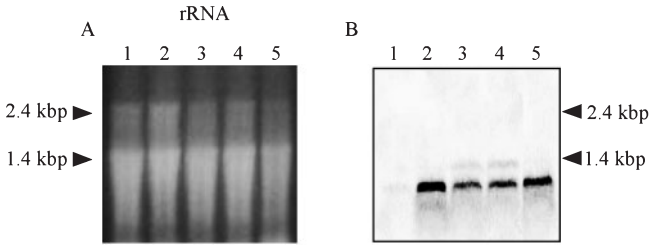

Fig. 4. **Northern blot analysis of total RNA extracted from *P. horikoshii* cells grown under anaerobic and aerobic conditions.** (A) shows ribosomal RNA stained with ethidium bromide, demonstrating that an equal amount (50 μg) of total cellular RNA was loaded in each lane. (B) Northern blot analysis with the gene-specific probe for Prx. Total RNA was prepared from cells grown under anaerobic conditions (Lane 1), and those in which oxidative stress was induced by aeration for 0.5, 1.0 and 2.0 h (Lanes 2–4, respectively) and by the addition of H_2O_2 for 0.5 h (Lane 5). Size standards are shown on both sides.

performed to measure the decrease in substrate concentration, as described in "MATERIALS AND METHODS." Upon incubation of the enzyme with cumene hydroperoxide and DTT, a continuous decrease in the amount of cumene hydroperoxide was observed (data not shown). These results indicate that the *P. horikoshii* Prx catalyzes the reduction of cumene hydroperoxide using DTT as an electron-transfer partner as reported for the *M. tuberculosis* enzyme (*18*). When cumene hydroperoxide was used as a substrate, the specific activity of the purified Prx was estimated to be 0.16 μmol/min/mg at 50°C based on the DTT oxidation assay. The optimum pH of the reaction was approximately pH 4.8. The enzyme also showed a hydrogen peroxide reducing activity, and the specific activity was calculated to be 0.098 μmol/min/mg under the same conditions. The enzyme retained full activity on heating at 90°C for 20 min, and 75% of the activity remained after 20 min at 100°C. The enzyme is extremely stable over a wide range of pH; upon heating at 50°C for 20 min, the enzyme did not lose activity at any pH in the range of 4.0 to 11.0.

Expression of the Prx Gene in P. horikoshii—To investigate the expression of the Prx gene, Northern blot analysis using a gene-specific probe was performed using cells grown under aerobic and anaerobic conditions. A hybridizing band with the expected size of 0.7 kb was obtained when RNA was extracted from cells grown on medium supplemented with air, but not from cells grown under anaerobic conditions (Fig. 4, lane 1). Furthermore, we analyzed the time dependency of gene transcription. A remarkable increase in the transcription of the gene was observed within 30 min after the start of aeration (Fig. 4, lane 2). A similar increase was observed following the addition of H_2O_2 (Fig. 4, lane 5). This shows that the peroxiredoxin from *P. horikoshii* is induced not only by aeration, but also by peroxide. The size of the mRNA signal indicates that the gene is transcribed as monocistronic mRNA. These results show that Prx is induced at the transcriptional level by exogenous oxygen or peroxide.

Analysis Based on Amino Acid Sequence—In general, the mammalian Prx family can be divided into three groups referred to as 2-Cys, atypical 2-Cys, and 1-Cys Prxs (*12*, *16*, *29*). The 2-Cys Prxs contain Cys residues in both the N- and C-terminal regions, whereas 1-Cys Prxs contain only one Cys in the N-terminal region (*11*). The atypical 2-Cys Prxs contain the conserved N-terminal Cys as well as two additional Cys residues, neither of which corresponds to the conserved C-terminal Cys of other members of the Prx family (*29*). In the 2-Cys Prxs, the conserved Cys residue in the N-terminal region is the site oxidized by peroxide (Fig. 1B). The additional conserved Cys residue in the C-terminal region forms an intermolecular disulfide bond with the N-terminal Cys residue of another subunit (*30*, *31*). The disulfide bond is then reduced by thioredoxin and the oxidized thioredoxin is subsequently reduced by thioredoxin reductase (thioredoxin/thioredoxin reductase system). Thus, 2-Cys Prxs are referred to as TPx. The atypical 2-Cys Prxs form an intramolecular disulfide as a reaction intermediate. Thioredoxin has been found to be able to support the peroxidase activity of the human atypical 2-Cys Prx (*29*). The 1-Cys Prxs form neither an intermolecular nor intramolecular disulfide intermediate, unlike 2-Cys and atypical 2-Cys Prxs (*32*). The physiological electron donor for 1-Cys Prxs is currently unknown (Fig. 1A). The consensus sequence surrounding the conserved Cys of 1-Cys Prxs is PVCTTE, and differs from the corresponding consensus sequences of FVCPTE for 2-Cys Prxs and PGCSKT for atypical 2-Cys Prxs (*12*, *29*). The amino acid sequence homology among the three groups is relatively low (<20% identity).

In bacteria, the type of electron-donor partner varies among species. *E. coli*, *S. typhimurium*, and *B. subtilis* utilize AhpF protein as an electron-donor partner for Prx (*13–15*). NADH oxidase is known to be able to reduce Prx in *Am. xylanus* (*17*). Prx from *M. tuberculosis* can use DTT as an electron-donor (*18*, *19*). There is no apparent AhpF gene in *M. tuberculosis*, and the *M. tuberculosis* Prx does not utilize the thioredoxin/thioredoxin reductase system as a redox partner (*19*). Thus, it has been proposed that the mycobacterial Prx might use small-molecule materials, such as mycothiol, to complete the enzymatic cycle (*18*). Although all of the Prxs from these bacteria share the consensus sequence of FVCPTE con-

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P.horikoshii     1 -----VVIGEKFPEVEVKTTHGVIKLPDYFTKQGKWFILFSHPADFTPVCTTEFYGMQKR 55
A.pernix         1 MPGSIPLIGERFPEMEVTTDHGVIKLPDHYVSQGKWFVLFSHPADFTPVCTTEFVSFARR 60
human_1-Cys      1 MPG-GLLLGDVAPNFEANTTVGRIRFHDFLGDS--WGILFSHPRDFTPVCTTELGRAAKL 57
                           *   *  *  *  * *   *       *  ***** *********

P.horikoshii    56 VEEFRKLGVEPIGLSVDQVFSHIKWIEWIKDN----LSVEIDFPVIADDRGELAEKLGMI 111
A.pernix        61 YEDFQRLGVDLIGLSVDSVFSHIKWKEWIERH----IGVRIPFPIIADPQGTVARRLGLL 116
human_1-Cys     58 APEFAKRNVKLIALSIDSVEDHLAWSKDINAYNCEEPTEKLPFPIIDDRNRELAILLGML 117
                      *    *  * ** * *  *  *   *             ** * *     *  **

P.horikoshii   112 PSG------ATITARAVFVVDDKGIIRAIVYYPAEVGRDWDEILRLVKALKISTEKGVAL 165
A.pernix       117 HAES-----ATHTVRGVFIVDARGVIRTMLYYPMELGRLVDEILRIVKALKLGDSLKRAV 171
human_1-Cys    118 DPAEKDEKGMPVTARVVFVFGPDKKLKLSILYPATTGRNFDEILRVVISLQLTAEKRVAT 177
                          * * **             **   **  ***** *  *        *

P.horikoshii   166 PHKWPNNELIGDKVIVPPASTIE-EKKQREEAKAKGEIECYDWWFCYKKLE--------- 215
A.pernix       172 PADWPNNEIIGEGLIVPPPTTED-QARARMES---GQYRCLDWWFCWDTPASRDDVEEAR 227
human_1-Cys    178 PVDWKD----GDSVMVLPTIPEEEAKKLFPKGVF-----------------TKELPSGK 215
                   *  *      *    * *

P.horikoshii   216 ------------------------  215
A.pernix       228 RYLRRAAEKPAKLLYEEARTHLH  250
human_1-Cys    216 KYLRYTPQP-------------  224
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Fig. 5. **Alignment of the amino acid sequences of *P. horikoshii* Prx, *A. pernix* TPx, and human 1-Cys Prx.** The alignment performed using ClustalW (version 1.7). Asterisks represent residues conserved among the three enzymes. The conserved cysteine residues are boxed. The consensus sequence conserved in the 1-Cys Prx group is underlined. P.horikoshii, *P. horikoshii* Prx; A.pernix, *A. pernix* TPx; and human 1-Cys, human 1-Cys Prx.

served in the 2-Cys Prx group, it appears that these bacteria contain no common electron-transfer partner for Prx.

Recently, Jeon and Ishikawa (*21*) found a unique thioredoxin in *A. pernix* and characterized it. The protein size is about three times that of the normal thioredoxin. It was revealed that the protein and *A. pernix* thioredoxin reductase can act as a thioredoxin/thioredoxin reductase system (*21*). The *A. pernix* Prx has been shown to utilize this newly identified thioredoxin/thioredoxin reductase system and has been stated to be a TPx (*20*). Figure 5 shows the amino acid alignment of *P. horikoshii* Prx, *A. pernix* TPx and human 1-Cys Prx. The deduced amino acid sequence of the *P. horikoshii* Prx shows 57% identity with that of the *A. pernix* enzyme, although the C-terminal region of the *P. horikoshii* Prx is thirty-two amino acids shorter than that of the *A. pernix* enzyme. Both the *P. horikoshii* Prx and *A. pernix* TPx show relatively high homology with the human 1-Cys Prx (40% and 35%, respectively), and share the consensus sequence PVCTTE conserved in the 1-Cys Prx group (Fig. 5). Therefore, they can be classified as members of the 1-Cys Prx group. On the other hand, the *A. pernix* TPx contains two Cys residues (Cys^{207} and Cys^{213}) that have not been observed in 1-Cys Prx in addition to the conserved Cys^{50} (Fig. 5). Jeon and Ishikawa (*20*) have demonstrated that the Cys^{213} residue forms an intermolecular disulfide bond with the Cys^{50} residue of another subunit. The disulfide bond can be reduced by *A. pernix* thioredoxin. Thus, the *A. pernix* TPx was proposed to be functionally closer to a 2-Cys Prx than to a 1-Cys Prx despite the higher sequence similarity with 1-Cys Prx (*20*). The sequence surrounding Cys^{207} and Cys^{213} in the *A. pernix* TPx is also conserved in the *P. horikoshii* Prx (Fig. 5). We screened thioredoxin homologues in the genome database of *P. horikoshii*, however, neither a homologue of *A. pernix*-type thioredoxin nor other types of thioredoxin were found in *P. horikoshii*. These results indicate that the electron-donor partner for the *P. horikoshii* Prx might be different from that for *A. pernix*. Recently, the presence of NADH oxidase in *P. furiosus* has been reported (*33*), and an enzyme homologue is also present in *P. horikoshii*. NADH oxidase may play a role in the reduction of the Prx as is seen in the case of *Am. xylanus* (*17*), although the possible presence of unidentified proteins with a thioredoxin-type function can not be excluded.

While *A. pernix* is a strict aerobic hyperthermohilic archaeon, which was isolated from a coastal solfataric thermal vent (*34*), *P. horikoshii* is a strict anaerobic hyperthermohilic archaeon isolated from a thermal vent in the Okinawa Trench (*35*). It is likely that the organism encounters high levels of oxygen from the cool high-pressure water surrounding the vent. Recent studies have shown that these anaerobic organisms may have evolved an antioxidant defense system that is independent of the SOD- and catalase-based system of the aerobic world (*36*). In the present study, oxidative stress response in an anaerobic archaeon was studied. As a result, a significant induction of Prx in *P. horikoshii* in response to oxygen exposure was observed. Although the physiological electron donor for the *P. horikoshii* Prx remains unclear, our results strongly suggest that the Prx may play an important role in the peroxide-scavenging systems in *P. horikoshii*. The anaerobic hyperthermophilic archaea are considered to be phylogenetically ancient organisms.

Thus, the *P. horikoshii* Prx may be involved in an antioxidant defense system that developed in the early stages of evolution.

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