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Alkyl Hydroperoxide Reductase Dependent on Thioredoxin-Like Protein from *Pyrococcus horikoshii*

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***Pyrococcus horikoshii* is an obligate anaerobic hyperthermophilic archaeon. In *P. horikoshii* cells, a hydroperoxide reductase homologue ORF (PH1217) was found to be induced by oxygen. The recombinant protein, which was expressed in *E. coli* under aerobic conditions, exhibited no activity. However, the recombinant protein prepared under semi-anaerobic conditions exhibited alkyl hydroperoxide reductase activity. Furthermore, it was clarified that it was coupled with the thioredoxin-like system in *P. horikoshii*. Western blot analysis revealed that the protein was induced by oxygen and hydrogen peroxide. This protein seems to be sensitive to oxygen but forms a thioredoxin-dependent system to eliminate reactive oxygen species in *P. horikoshii*.**

Key words: alkyl hydroperoxide reductase, hyperthermophilic archaea, oxidative stress, *Pyrococcus horikoshii*, thioredoxin system.

Organisms have developed various mechanisms that have the ability to eliminate many forms of physiological and chemical stress from their environments, such as reactive oxygen species (ROS), temperature, pH, and osmotic pressure. In particular, oxidative stress caused by ROS has to be eliminated in all living creatures on earth, on which oxygen exists abundantly. Although, the elimination ability varies between aerobic and anaerobic organisms, a mechanism for such elimination is essential for all organisms, because ROS, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and alkyl hydroperoxide, are produced in cells if there is oxygen in the environment. ROS are cellular oxidizing substances that can injure DNA, proteins, and membrane lipids. Therefore, organisms have mechanisms involving some proteins such as superoxide dismutase (SOD) (*1–3*), superoxide reductase (SOR) (*4*), catalase (*5*), peroxiredoxin (*6*), thioredoxin- and glutathione-systems (*7–12*), and damage-repairing enzymes to eliminate ROS (*13–14*) for the protection of their cells. Alkyl hydroperoxide reductase (AhpC) has been reported in aerobic and anaerobic bacteria such as *Salmonella typhimurium* (*15*), *Clostridium pasteurianum* (*16*), *Mycobacterium tuberculosis* (*17*), *Hericobacter pylori* (*18*), and *E. coli* (*19*), in conjugation with alkyl hydroperoxide reductase subunit F (AhpF) (*20–22*) or thioredoxin-like systems (*16*, *18*). This enzyme eliminates damaging substances, such as hydrogen peroxide and alkyl hydroperoxide. Not only for aerobes but also for anaerobic microorganisms, in particular, the detoxification of peroxide is necessary for the systems that eliminate ROS.

Pyrococcus species are obligate anaerobic and hyperthermophilic archaea. The detoxification mechanisms for ROS have been reported to be SOR-rubredoxin systems in *P. furiosus* (*4*, *23–24*). It has been expected that an AhpC-like enzyme plays a critical role in the elimination of hydrogen peroxide that is produced through reduction of the superoxide anion by SOR. However, an enzyme for the elimination of peroxide has not been reported in hyperthermophilic archaea. In this paper, we report that a protein is induced by oxygen and may be involved in the peroxide detoxification system in *P. horikoshii* cells.

We investigated the response of intracellular proteins in *P. horikoshii* cells to oxygen-stimulation to clarify the resistance systems against ROS. *P. horikoshii* cells were cultured in medium containing, 25 g of polypeptone, 5 g of yeast extract, 25 g of NaCl, 0.75 g of $CaCl_2$, 1 mg of resazurin, 5 mg of citric acid, 100 ml of solution A [35 g of $MgSO_4{\cdot}7H_2O$, 27.5 g of $MgCl_2{\cdot}6H_2O$, 3.2 g of KCl, 0.5 g of NaBr, 0.15 g of H_3BO_3, 75 mg of $SrCl_2{\cdot}6H_2O$, 1 mg of KI, 5 g of KH_2PO_4 and 20 mg of $(NH_4)_2Ni(SO_4)_2{\cdot}6H_2O$/liter], and 10 ml of solution B [1.5 g of nitrilotriacetic acid, 3 g of $MgSO_4{\cdot}7H_2O$, 0.5 g of $MnSO_4{\cdot}xH_2O$, 1 g of NaCl, 0.1 g of $FeSO_4{\cdot}7H_2O$, 0.1 g of $CoSO_4{\cdot}7H_2O$, 0.1 g of $CaCl_2{\cdot}2H_2O$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 10 mg of $CuSO_4{\cdot}5H_2O$, 10 mg of $AlK(SO_4)_2$, 10 mg of H_3BO_3, 10 mg of $Na_2MoO_4{\cdot}2H_2O$, 10 mg of $Na_2WO_4{\cdot}2H_2O$, and 2 mg of Na_2SeO_3/liter] per liter,. The pH was adjusted to a final 6.8. The medium was saturated with nitrogen and then sterilized by autoclaving. Sterile sulfur (3.0 g/liter) and 2.5 ml of a 20% Na_2S solution were added to the medium after cooling to 90°C. The culture was grown in a 1-liter bottle at 95°C under anaerobic conditions. Cell growth was monitored as the turbidity at 600 nm. At the early logarithmic phase (9 h), 20 ml of air was injected into the culture bottles, followed by shaking. A further 60 min incubation at 95°C was performed, and then the cells were harvested by centrifugation after cooling down to 4°C. The cell paste was resuspended in a 2.5% NaCl solution, and then re-centrifuged. The pellet was homogenized in 20 mM Tris-HCl (pH 8.0) by ultrasonication, and then centrifuged. The proteins in the supernatant were precipitated by ammonium sulfate precipitation (90% saturation), and then dialyzed against 5 mM Tris-HCl (pH 8.0). The proteins (0.5 mg) were sep-

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P. horikoshii     1 ----------------MVVIGEKFPEVEVKTTH-GVI-KLPDYFTKQGKWFIL-FSHPAD    41
M. tuberculosis   1 MPLLTIGDQFPAYQLTALIGGDLSKVD--AKQPG-DYFTTITSDEHPGKWRVVF-FWPKD    56
H. pylori         1 -------------MLVTKLAPDFKAPAVLGNNEVDEHFELSK---NLGKNGAILFFWPKD    44
S. typhimurium    1 ----------------MSLINTKIKPFKNQAFKNGEFIEVTEKDTE-GRWSVF-FFYPAD    42
                                                                 *..    .. * *

P. horikoshii    42 FTPVCTTEFYGMQKRVEEFRKLGVEPIGLSVDQVFSHIKWIEWIKDNLSVEIDFPVIADD   101
M. tuberculosis  57 FTFVCPTEIAAFSKLNDEFEDRDAQILGVSIDSEFAHFQWRAQHN---DLKTLPFPMLSD   113
H. pylori        45 FTFVCPTEIIAFDKRVKDFQEKGFNVIGVSIDSEQVHFAWKNTPVEKGGIGQVTFPMVAD   104
S. typhimurium   43 FTFVCPTELGDVADHYEELQKLGVDVYSVSTDTHFTHKAWHSSSET-IAK-IKYAMIGDP   100
                    **.**.**      .   ..   .    ..* *  . *  *                .

P. horikoshii   102 RGELAEKLGMIPSGATITARAVFVVDDKGIIRAIVYYPAEVGRDWDEILRLVKALKISTE   161
M. tuberculosis 114 IKRELSQAAGVLNADGVADRVTFIVDPNNEIQFVSATAGSVGRNVDEVLRVLDALQSD--   171
H. pylori       105 ITKSISRDYDVLFEEAIALRGAFLIDKNMKVRHAVINDLPLGRNADEMLRMVDALLHFEE   164
S. typhimurium  101 TGALTRNFDNMREDEGLADRATFVVDPQGIIQAIEVTAEGIGRDASDLLRKIKAAQYVAA   160
                              . *  * .*     .          **  ..  **    *.

P. horikoshii   162 K-GVAL-PHKWPNN------------ELIGDKVI-----VPPASTIEEKKQREEAKAKGE   202
M. tuberculosis 172 --ELCACN--WRKGDPTLDAG-----ELLKASA--------------------------   195
H. pylori       165 HGEVCPAG--WRKGDKGMKATHQGVAEYLKENSIKL-----------------------   198
S. typhimurium  161 HPGE-VCPAKWKEGEATLAPSLDLVGKI------------------------------   187
                          *  .           .

P. horikoshii   203 IECYDWWFCYKKLE                                             216
M. tuberculosis 195 --------------                                             195
H. pylori       198 --------------                                             198
S. typhimurium  187 --------------                                             187
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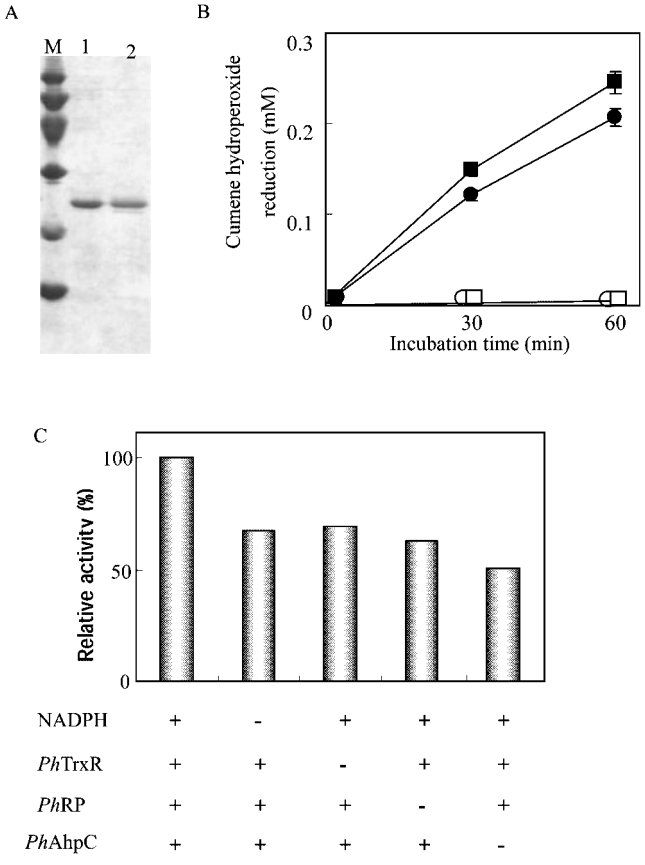
Fig. 1. **Alignment of the amino acid sequences of *P. horikoshii* AhpC (PH1217 protein) and some bacterial AhpCs.** Dashes within sequences represent gaps introduced to optimize alignment. The positions of the N-terminal Cys residue conserved in all AhpCs are boxed, and those of the C-terminal Cys residues are given in bold. Dots and asterisks under the sequence indicate the same amino acid in the three or four proteins, respectively.

arated by isoelectric focusing using ReadyStrip IPG strips (pH 3–11, 11 cm length; Biorad, Hercules, CA, USA). The second electrophoresis was performed on a 15% SDS–polyacrylamide gel. As a control, a cell homogenate prepared by the same procedure without air-stimulation was also electrophoresed. After staining with Coomassie-Brilliant Blue R250, an increasing protein band was only detected when the cells were stimulated by air (data not shown). The N-terminal amino acid sequence of the protein was determined to be VVIGEK, and PH1217 in the genome database of *P. horikoshii* (http://www.bio.nite.go.jp:8080/dogan/MicroTop) was found to be a corresponding protein. PH1217 was predicted to be a DNA encoding alkyl hydroperoxide reductase (AhpC) from the sequence homology. The deduced amino acid sequence of the PH1217 protein contains three cysteine residues (Fig. 1). The consensus sequence around Cys46 of the PH1217 protein (DFTPVCTTE) exhibits high homology to the sequence of other AhpCs (DFTFVCPTE). The C-terminal region of the protein is larger than those of the other AhpCs, and the region around the other two cysteine residues (Cys205 and Cys211) exhibits no homology to those of the others. The PH1217 protein induced by oxygen may play an important role in the resistance against oxygen.

To characterize the PH1217 protein, we cloned the gene and prepared a recombinant protein using *E. coli*. The complete coding sequence of PH1217 was amplified by 30 cycles of PCR [denaturing at 96°C, annealing at 60° and extension at 74°C, with KOD DNA polymerase (Toyobo, Osaka)] using the genomic DNA of *P. horikoshii* as a template. Primers, 5′-GAGGAGATCCATATGGTAG-TGATTGGAGAA-3′ and 5′-GGAAGGAATTCATTCAAG-CTTCTTATAGCAGA-3′, containing recognition sites for restriction enzymes (underlined) were used for amplification of the gene. The resulting DNA fragment was digested with *Nde*I and *Eco*RI (Takara, Kyoto), and then inserted into the corresponding site of expression vector pET21a (Novagen, Madison, WI, USA). The plasmid obtained was introduced into *E. coli* Rosetta(DE3) (Novagen, Madison, WI, USA). The nucleotide sequence of the inserted gene was confirmed by DNA sequencing. Preparation of the genomic DNA of *P. horikoshii* was reported previously (*25*).

E. coli Rosetta(DE3) harboring the constructed plasmid was grown in LB broth supplemented with 0.4% glucose at 37°C under the following two different conditions. Under aerobic conditions, cells were grown until O.D.600 = 0.6 with shaking, and then the recombinant protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h. Under semi-anaerobic conditions, cells were grown aerobically until OD600 = 0.3, and then the bottle was capped and sealed. After further incubation for 1 h at 37°C without shaking, the recombinant protein was induced with 1 mM IPTG for 16 h. The two kinds of cell paste obtained on centrifugation at 7,000



rpm for 5 min were re-suspended in 20 mM Tris-HCl (pH8.0), and then homogenized by ultrasonication. The homogenates were treated at 85°C for 30 min, and then centrifuged at 15,000 rpm for 20 min. The supernatants were dialyzed against 20 mM Tris-HCl (pH8.0). All extraction steps were performed under nitrogen gas, and de-gassed and N_2-saturated buffer was used. From the *E. coli* cells under both conditions, the recombinant PH1217 protein, of which the molecular weight was 25 kDa, was prepared (Fig. 2A). We recently found two redox proteins that form a thioredoxin-like system composed of homologues of glutaredoxin (*Ph*RP) and thioredoxin reductase (*Ph*TrxR) in *P. horikoshii* (manuscript submitted). The recombinant *Ph*RP and *Ph*TrxR were expressed with the pET vector system in *E. coli*. The alkyl hydroperoxide reductase activity in the obtained crude enzyme solutions was examined (Fig. 2B). When the PH1217 protein was expressed in *E. coli* cells under aerobic conditions, no degradation of cumene hydroperoxide was observed (Fig. 2B). This means that the expressed protein exhibited no alkyl hydroperoxide reductase activity. On the other hand, the protein prepared under semi-anaerobic conditions exhibited DTT-dependent alkyl hydroperoxide reductase activity. The protein also exhibited NADPH-dependent alkyl hydroperoxide reductase activity in the presence of *Ph*RP and *Ph*TrxR (Fig. 2, B and C). In addition, there is no AhpF homologous protein in the *P. horikoshii* genome. These results indicated that the PH1217 protein (hereafter referred to as *Ph*AhpC) prepared under semi-anaerobic conditions exhibited thioredoxin-like protein-dependent peroxidase activity (Fig. 3), as reported for *Helicobacter pyloli* (*18*). Basically, *P. horikoshii* does not seem to have strong resistance systems for high concentrations of oxygen such as that in air, because of its obligate anaerobic characteristics. Accordingly, PhAhpC seems to be inactivated by air, probably oxygen.

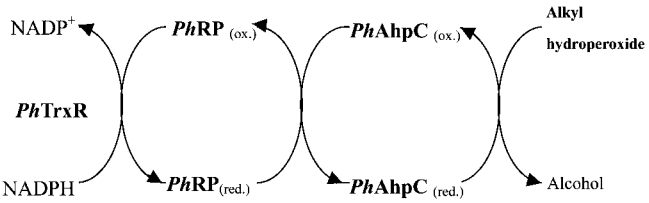


Fig. 3. **Proposed pathway for the transfer of reducing equivalents from NADPH to hydroperoxide in *P. horikoshii*.**

Fig. 2. **SDS-PAGE profile of the enzyme solutions used for measurement of the enzyme activity (A), alkyl hydroperoxide reductase activity of the protein (B), and the activity in the presence of various components (C).** (A) Crude extracts of *E. coli* harboring aerobically and semi-anaerobically expressed PH1217 protein in *E. coli* were extracted and treated at 85°C or 30 min. After centrifugation (15,000 rpm, 20 min), the supernatants (2.5 μg of protein) were loaded onto a 17% polyacrylamide gel. M, molecular weight markers: phosphorylase b (97 kDa), albumin from bovine serum (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa); 1, Extract of aerobic cultured cells; 2, Extract of semi-anaerobic cultured cells. (B) For measurement of DTT-dependent activity, the reaction mixture contained 50 mM MOPS (pH 7.0), 20 μM aerobically (open squares) or semi-anaerobically (solid squares) produced PH1217 protein, 0.3 mM DTT, and 1 mM cumene hydroperoxide. The reaction mixture for *Ph*RP-dependent activity contained 50 mM MOPS (pH 7.0), 20 μM aerobically (open circles) or semi-anaerobically (solid circles) produced PH1217 protein, 10 μM *Ph*RP, 50 nM *Ph*TrxR, 0.3 mM NADPH, and 1 mM cumene hydroperoxide. As a negative control, a mixture contained 50 mM MOPS (pH 7.0), and 1 mM cumene hydroperoxide was also prepared. The reaction mixtures were incubated at 85°C, and aliquots were removed at the indicated times. The results are expressed as the decrease-rate in the reaction subtracted from that in the negative control. To monitor the disappearance of cumene hydroperoxide from the solution, experiments were conducted essentially as described elsewhere (*18*)**.** Reactions were terminated by the addition of 0.95 ml of 10% trichloroacetic acid to 50 μl of the reaction mixture, and then to determine the peroxide content as ferrithiocyanate complex formation, 0.2 ml of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 0.1 ml of 2.5 M KSCN were added. After centrifugation to remove the precipitated protein, red ferrithiocyanate complex was measured at 480 nm, and the peroxide concentration was determined from a standard curve obtained with cumene hydroperoxide. The activity was expressed as the decrease in cumene hydroperoxide in the reaction mixtures. The results are the averages of four trials. The values are the means of four experiments ± SD (C) Each component was present (+) or absent (–). Conditions: 1 mM cumene hydroperoxide, 0.3 mM NADPH, 20 μM *Ph*AhpC, 10 μM *Ph*RP, and 50 nM *Ph*TrxR were incubated in 50 mM MOPS (pH 7.0) at 85°C, and then the concentration of cumene hydroperoxide was determined as ferrithiocyanate complex formation. The concentration of the anaerobically expressed *Ph*AhpC was determined by determination of the density of the band on SDS-PAGE with reference to that of the aerobically expressed *Ph*AhpC protein purified to homogeneity by salting out, Hi-Trap Q (Amersham Biosciences, Picataway, NJ, USA), and Hi-load phenyl (Amersham biosciences, Picataway, NJ, USA). Comparison of the densities of two bands was performed with an Gel-Pro analyzer (MediaCybernetics, Silver Spring MD, USA). The results are presented as relative activity as to the reaction with all the components, and are the averages of four trials. 100% activity corresponds to a decrease of 0.12 mM cumene hydroperoxide per 30 min.

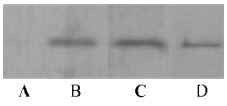


Fig. 4. **Western blot analysis of *Ph*AhpC expression.** For the preparation of anti-*Ph*AhpC antisera, the *Ph*AhpC protein was purified to homogeneity as mentioned in the legend to Fig. 2. Antisera against *Ph*AhpC were obtained using the purified protein in Freund's adjuvant. 50 μg of *Ph*AhpC protein was injected into BALB/c mice. Booster immunizations were given weekly under the same conditions but with the use of incomplete Freund's adjuvant until anti-*Ph*AhpC antibodies were detected in the sera. It was confirmed that the obtained antisera did not bind to protein in the extract of *E. coli* Rosetta(DE3) on ELISA. The cells of *P. horikoshii* were stimulated with 20 ml of air at the early logarithmic phase (9 h) for 0 (lane A), 30 (with air; lane B), and 60 min (with air, lane C), and with 1 mM hydrogen peroxide for 30 min (lane D). The cells were harvested and homogenized in 20 mM Tris-HCl (pH 8.0). In the case of stimulation with hydrogen peroxide, the cells were grown in the medium without sulfur. Extracts of the cells were analyzed by Western blotting. Following SDS-PAGE of the homogenates, proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h with a blocking solution (Roche Diagnostics, Basel, Switzerland). The membranes were then incubated with the mouse anti-*Ph*AhpC antisera in PBS, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO, USA). The antibody to *Ph*AhpC was detected using an AP conjugate substrate kit (Biorad, Hercules, CA, USA). Cell extracts (9 μg each) were loaded on a 15% gel.

We investigated how the expression of *Ph*AhpC was affected by oxygen (Fig. 4, lanes B and C). *Ph*AhpC was apparently increased in *P. horikoshii* cells stimulated by air. In addition, *Ph*AhpC was induced by hydrogen peroxide (Fig. 4, lane D) like Prx from yeast (*26*). However, the induction of the *Ph*AhpC was slight because most of the hydrogen peroxide might have reacted with the sodium sulfate that had been added to the medium to eliminate oxygen. In the genome database of *P. horikoshii*, catalase-related proteins were not found. These results suggest that the expression of *Ph*AhpC is also induced by hydrogen peroxide produced through reduction of the superoxide anion *via* SOR.

In *P. furiosus*, an SOR-rubredoxin system for elimination of the oxygen anion has been reported (*23–24*). *P. horikoshii* also has a homologue of SOR (PH1083; 74% identity to SOR of *P. furiosus*). Antioxidant systems may be critical for the defense against ROS generated by oxygen also for *P. horikoshii*. Therefore, the Trx system-dependent AhpC is also important for the antioxidant system in *Pyrococcus* species. Interestingly, no homologues of catalase or glutathione reductase systems such as gamma-L-glutamyl-L-cysteine synthethase and glutathione reductase were found in the genome of *Pyrococcus* species. Therefore, *P. horikoshii* does not have other main reductase systems such as glutathione-dependent redox system (*11–13*). Accordingly, *Ph*AhpC presumably plays an important role in the resistance against oxygen, even if it is inactivated by oxygen. Furthermore, anaerobic conditions for the production of recombinant proteins from *Pyrococcus* species should be important for clarifying the mechanism of resistance against oxygen, as described here.

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