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# Biochemical Properties and Regulated Gene Expression of the Superoxide Dismutase from the Facultatively Aerobic Hyperthermophile *Pyrobaculum calidifontis*

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**Superoxide dismutase (SOD) was purified from a facultatively aerobic hyperthermophilic archaeon, *Pyrobaculum calidifontis* VA1. The purified native protein from aerobically grown cells exhibited 1,960 U of SOD activity/mg and contained 0.86 ± 0.04 manganese and <0.01 iron atoms per subunit. The gene encoding SOD was cloned and expressed in *Escherichia coli*. Although the recombinant protein was soluble, little activity was observed due to the lack of metal incorporation. Reconstitution of the enzyme by heat treatment with either Mn or Fe yielded a highly active protein with specific activities of 1,970 and 434 U/mg, respectively. This indicated that the SOD from *P. calidifontis* was a cambialistic SOD with a preference toward Mn in terms of activity. Interestingly, reconstitution experiments in vitro indicated a higher tendency of the enzyme to incorporate Fe than Mn. When *P. calidifontis* was grown under anaerobic conditions, a majority of the native SOD was incorporated with Fe, indicating the cambialistic property of this enzyme in vivo. We further examined the expression levels of SOD and a previously characterized Mn catalase from this strain in the presence or absence of oxygen. Northern blot, Western blot, and activity measurement analyses revealed that both genes are expressed at much higher levels under aerobic conditions. We also detected a rapid response in the biosynthesis of these enzymes once the cells were exposed to oxygen.**

Hyperthermophiles are defined as microorganisms that grow optimally at temperatures above 80°C (39) or that can grow at temperatures above 90°C (1). A vast number of hyperthermophiles have been isolated from extreme geothermal and hydrothermal environments. Although they are diverse with respect to their phylogeny and physiological and biochemical properties, most of them are strictly anaerobic (2). Among the relatively few exceptions, we have previously isolated the hyperthermophilic archaeon, *Pyrobaculum calidifontis* VA1 (4). This strain was isolated from a water sample at the surface of a hot spring in the Philippines and grows under atmospheric air. Oxygen serves as a final electron acceptor under aerobic culture conditions, whereas oxygen can be replaced by nitrate under anaerobic conditions. Therefore, studies on *P. calidifontis* should provide valuable information on how hyperthermophiles respond to, deal with, or utilize molecular oxygen.

For the cell to adapt to oxygen and aerobic respiration, it is essential to develop mechanisms to protect its biomolecules from reactive oxygen species, such as superoxide and hydrogen peroxide. The predominant defense systems found in the aerobic eukaryotes and bacteria are the enzymes superoxide dismutases (SODs) and catalases, which catalyze the disproportionation of superoxide into hydrogen peroxide and oxygen and that of hydrogen peroxide into oxygen and water, respectively (9, 26). In the majority of *Archaea*, along with some species of anaerobic bacteria, distinct systems have evolved to protect the cell from reactive oxygen. The key enzyme is superoxide reductase (SOR), an enzyme that reduces superoxide to hydrogen peroxide with reduced rubredoxin and thereby avoids the generation of molecular oxygen (15). In *Pyrococcus furiosus*, the regeneration of reduced rubredoxin is made possible by an NAD(P)H:rubredoxin oxidoreductase (28). Although unclear at present, it has been proposed that the hydrogen peroxide is subsequently reduced to water by a rubrerythrin, which exhibits NADH peroxidase activity (10) and whose gene usually clusters with those of SOR and rubredoxin (27). In addition, members of the peroxiredoxin (Prx) family, including thioredoxins, are also presumed to be involved in the detoxification of hydrogen peroxide (16). Again, in these reactions, molecular oxygen that would otherwise be generated by a catalase is not produced.

In the aerobic or microaerobic (hyper)thermophilic archaea, SOD seems to functionally replace SOR. In general, SODs are assigned to four groups on the basis of their metal cofactors: Cu/Zn-SOD, Mn-SOD, Fe-SOD, and Ni-SOD (31, 22, 46, 47). Mn- and Fe-SODs are closely related in amino acid sequence and three-dimensional structure. Among (hyper)thermophilic archaea, Fe-SODs have been reported from *Sulfolobus solfataricus* (11, 44), *Sulfolobus acidocaldarius* (18), *Acidianus ambivalens* (17), and *Thermoplasma acidophilum* (37). Although Mn- and Fe-SODs generally exhibit a strict metal specificity for SOD activity (34), several cambialistic SODs, which are active with either Mn or Fe, have been reported. This is the case in the enzymes from *Pyrobaculum aerophilum* (42) and *Aeropyrum pernix* (45). At present, Cu/Zn-SODs have not been found in archaea. Interestingly, the archaea that harbor SOD do not necessarily utilize a catalase. In fact, the only catalases reported from hyperthermophilic archaea are the Mn catalase

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from *P. calidifontis* (3) and the heme catalase from *Archaeoglobus fulgidus*, which does not harbor an SOD (23). Genome sequence data from aerobic (hyper)thermophilic archaea also indicate the absence of catalase in the strains, with only one exception in the recently published sequence of *P. aerophilum*, which harbors an orthologue of the Mn catalase gene from *P. calidifontis* (12). Although aerobic, the genomes of *S. solfataricus* (38), *Sulfolobus tokodaii* (19), *Aeropyrum pernix* (20), *Thermoplasma acidophilum* (35), and *Thermoplasma volcanium* (21) do not harbor catalase orthologues. Furthermore, rubrerythrin orthologues are not found in these organisms, suggesting that the majority of aerobic (hyper)thermophilic archaea may utilize an SOD-Prx system.

In the present study, we have identified and characterized the SOD from *P. calidifontis*. Since this strain also harbors a catalase, which we have recently characterized (3), *P. calidifontis* is the only hyperthermophilic archaeon known to utilize an SOD-catalase system to detoxify reactive oxygen species. Furthermore, the fact that *P. calidifontis* is a facultative aerobe allows us to study how hyperthermophiles respond to the presence or absence of oxygen at high temperatures. We therefore examined the regulation in expression of these two enzymes in *P. calidifontis*, particularly in the presence or absence of oxygen.

## MATERIALS AND METHODS

**Strains, plasmids, phages, and culture conditions.** *P. calidifontis* is a facultatively aerobic hyperthermophilic archaeon, isolated from a water sample from the surface of a hot spring in the Philippines (4). The water sample contained 0.122 mg of iron and 0.514 mg of manganese/liter. For purification of SOD, cells were cultivated aerobically with vigorous shaking in the following standard aerobic medium (per liter): 10 g of tryptone, 1 g of yeast extract, and 3 g of $Na_2S_2O_3 \cdot 5H_2O$. Cells were also cultivated anaerobically in the following standard anaerobic medium (per liter): 10 g of tryptone, 1 g of yeast extract, 1 g of $NaNO_3$, and 0.5 mg of $Na_2WO_4 \cdot 2H_2O$ as described previously (4). Resazurin (1 mg/liter) was used as a redox indicator. For the aerobic and anaerobic adaptation experiments, cells were cultivated in a combined medium containing 10 g of tryptone, 1 g of yeast extract, 3 g of $Na_2S_2O_3 \cdot 5H_2O$, 1 g of $NaNO_3$, and 0.5 mg of $Na_2WO_4 \cdot 2H_2O$ per liter.

*Escherichia coli* DH5α and the vector pUC19 were used for cloning and gene manipulation. *E. coli* XL1-Blue MRA (P2; Stratagene, La Jolla, Calif.) was used as a host strain for λEMBL3 phage (Stratagene). *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene) and a plasmid derived from pET-21a(+) (Novagen, Madison, Wis.) were used for gene expression. Luria-Bertani medium was used for cultivation of *E. coli*, and NZYM medium for amplification of phage (36).

**DNA manipulation and sequencing.** Routine DNA manipulations were performed by standard methods (36). Restriction enzymes and other modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) or Toyobo (Osaka, Japan). The Qiaex gel extraction kit (Qiagen, Hilden, Germany) was used to recover DNA fragments from agarose gel. For isolation of plasmid and phage DNA, a Plasmid Mini kit (Qiagen) and a Lambda kit (Qiagen), respectively, were used. DNA sequencing was carried out by using a BigDye terminator cycle sequencing kit and a model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, Calif.). Nucleotide and amino acid sequences were analyzed with GENETYX software (Software Development, Tokyo, Japan). The multiple alignment of amino acid sequences was performed by using the CLUSTAL W program (40) provided by DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp).

**Enzyme assays.** Catalase activity was determined spectrophotometrically with a UV-1600PC spectrophotometer with a thermal control unit (Shimadzu, Kyoto, Japan). Routine assays were performed at 70°C in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM hydrogen peroxide. Decomposition of hydrogen peroxide was monitored at 240 nm ($\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$) (8, 14). One unit of catalase activity is defined as the amount of activity required to convert 1 μmol of hydrogen peroxide to water and oxygen per min.

SOD activity was measured at 25°C by the xanthine oxidase-cytochrome *c* method (31). Xanthine, xanthine oxidase from buttermilk and cytochrome *c* from horse heart were purchased from Sigma (St. Louis, Mo.). The reduction of cytochrome *c* by superoxide anion, which was generated from molecular oxygen by reduction of xanthine, was monitored at 550 nm. One unit was defined as the amount of enzyme which inhibited the rate of cytochrome *c* reduction by 50%. The inhibition of SOD activity by sodium azide was tested by the addition of sodium azide to the reaction mixture. In the hydrogen peroxide inactivation experiment, SOD (0.25 mg/ml) was incubated with 0.25 mM hydrogen peroxide in 50 mM potassium phosphate buffer (pH 7.8) at 25°C. At various intervals, aliquots of the enzyme were taken and treated with 1 U of catalase (from bovine liver; Sigma)/μl and then assayed for residual SOD activity. In order to determine thermostability, enzyme samples (0.35 mg/ml) were incubated at 100°C in 50 mM potassium phosphate buffer (pH 7.8).

Malate dehydrogenase from *P. calidifontis* was assayed at 50°C in 50 mM potassium phosphate buffer containing 200 mM NaCl, 0.2 mM NADH, and 2 mM oxaloacetate. The oxidation of NADH was monitored at 340 nm. Blank reactions in which oxaloacetate was omitted were subtracted.

**Purification of SOD from *P. calidifontis*.** For the native enzyme, *P. calidifontis* cells were cultivated aerobically with vigorous shaking at 90°C (4). Cells (5.6 g [wet mass]) were harvested and suspended in 50 mM potassium phosphate buffer (pH 7.0). The cells were disrupted by sonication while cooled in ice water, and the supernatant was obtained by centrifugation (20,000 × *g*, 30 min, 4°C). The supernatant was subjected to ammonium sulfate fractionation at 0°C. The fraction corresponding to 65 to 95% saturation was collected and adjusted to 1.5 M (35% saturation) ammonium sulfate. Chromatographic purification procedures of SOD were performed at room temperature. The fraction was applied to a hydrophobic interaction column Resource PHE (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer containing 1.5 M ammonium sulfate (pH 7.0) and eluted with a decreasing linear gradient of 1.5 to 0 M ammonium sulfate in 50 mM potassium phosphate buffer. The fractions containing SOD activity were pooled and dialyzed to remove the ammonium sulfate and to convert the buffer to 50 mM Tris-HCl (pH 9.0). The desalted fractions were applied to an anion-exchange column Resource Q (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris-HCl (pH 9.0) and eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM Tris-HCl (pH 9.0). The fractions containing SOD activity were pooled and concentrated by using Centricon YM-30 (Millipore, Bedford, Mass.). The concentrated sample was further purified by gel filtration (Superdex 200 HR 10/30; Amersham Pharmacia Biotech) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. The fractions containing SOD activity were desalted with 50 mM potassium phosphate buffer (pH 7.8) and used as the purified enzyme in further experiments.

**Analysis of purified SOD.** The homogeneity of SOD was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed according to standard procedures (36). Protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, Calif.) with bovine serum albumin as a standard. Amino-terminal amino acid sequence of the purified enzyme was determined by a protein sequencer (cLC model 491; Applied Biosystems).

Gel filtration chromatography was used to determine the native molecular mass of the purified SOD. Experiments were performed at a flow rate of 0.5 ml/min on AKTÄ explorer 10S fast-performance liquid chromatography system (Amersham Pharmacia Biotech) using a Superdex 200 HR 10/30 column. The buffer used was 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The void volume was determined with blue dextran, and a standard calibration curve was obtained by using ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) (high- and low-molecular-weight gel filtration calibration kits; Amersham Pharmacia Biotech).

Metal contents (manganese, iron, and nickel) of proteins were analyzed by plasma emission spectroscopy (ICPS-7000; Shimadzu).

**Isolation of $sod_{Pc}$ gene.** A genomic library of *P. calidifontis* was prepared by ligating genomic DNA partially digested with *Sau*3AI into *Bam*HI-digested arms of λEMBL3 (Stratagene). Two oligonucleotide primers were designed. One primer (5′-ATGCARGTNAARAARTAYGARYTRCC-3′; where R = A or G, Y = C or T, and N = A, T, G, or C) was derived from the determined N-terminal amino acid sequence of the purified enzyme, and the other primer (5′-TTRTA YTGYARRTARTANGCRTGYTC-3′) was derived from a consensus sequence obtained from an alignment among archaeal SOD genes. A DNA fragment containing a part of the SOD-encoding *P. calidifontis* gene ($sod_{Pc}$) was obtained by PCR with these primers and *P. calidifontis* genomic DNA as a template. A phage clone, which carried the complete $sod_{Pc}$ gene, was screened from the genomic library by plaque hybridization with the DNA fragment labeled by DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany) as a probe.

TABLE 1. Metal contents and specific activities of native and recombinant SODs[a]

| SOD | Metal content (Mean no. of atoms/subunit ± SD) | | | Sp act (U/mg) |
|---|---|---|---|---|
| | Mn | Fe | Ni | |
| Native SODs from cells grown: | | | | |
| Aerobically in standard medium | 0.55 ± 0.01 | <0.01 | <0.01 | 1,200 |
| Aerobically in medium supplemented with manganese | 0.86 ± 0.04 | <0.01 | ND | 1,960 |
| Aerobically in medium supplemented with iron | 0.68 ± 0.02 | 0.01 ± 0.005 | ND | 1,520 |
| Anaerobically in standard medium | 0.08 ± 0.01 | 0.43 ± 0.04 | ND | 512 |
| Recombinant SODs | | | | |
| apo-SOD | <0.01 | <0.01 | ND | 12 |
| Mn-reconstituted SOD | 0.86 ± 0.02 | <0.01 | ND | 1,970 |
| Fe-reconstituted SOD | <0.01 | 0.76 ± 0.03 | ND | 434 |

[a] The protein concentration was determined by the Bio-Rad protein assay system with bovine serum albumin as a standard. A subunit molecular mass of 24,004 Da was used to calculate the metal content. For metal reconstitution of recombinant SODs, the purified apoprotein (0.5 mM active site) was combined with 10 mM $MnCl_2$ or $Fe(NH_4)_2(SO_4)_2$ and heated at 95°C for 1 h. Unbound metals were removed by dialysis, followed by gel filtration. ND, not determined.

**Expression of *sod*$_{Pc}$ gene in *Escherichia coli*.** The full-length *sod*$_{Pc}$ flanked by the *Nde*I and *Eco*RI sites was amplified by PCR with the phage DNA and two primers (sense [5′-ATGAGACAAACGAGGGGGGTCATATGCAAG-3′] and antisense [5′-TGTAAAACGTTAAGGGAAGAATTCCACATTGAGG-3′]; the underlined sequences indicate the *Nde*I site in the sense primer and the *Eco*RI site in the antisense primer). The amplified DNA fragment was digested with *Nde*I and *Eco*RI after checking the sequence. The fragment was then ligated with the corresponding sites of plasmid pET-21a(+) to obtain pET-sod for the production of recombinant SOD from *P. calidifontis* (SOD$_{Pc}$). *E. coli* BL21-CodonPlus(DE3)-RIL harboring pET-sod was induced for overexpression with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at the mid-exponential-growth phase and further incubated for 3 h at 37°C.

**Purification of the recombinant SOD$_{Pc}$.** The overexpressed cells were harvested by centrifugation (7,000 × *g*, 10 min, 4°C), washed twice with 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, and then resuspended in the same buffer. The cells were disrupted by sonication while cooled in ice water, followed by centrifugation (20,000 × *g*, 30 min, 4°C). The resulting supernatant containing the recombinant SOD$_{Pc}$ was incubated at 85°C for 15 min and centrifuged (20,000 × *g*, 30 min, 4°C) to remove heat-labile proteins from the host *E. coli*. Chromatographic purification procedures of recombinant SOD were performed at room temperature. The supernatant was applied to an anion-exchange column Resource Q equilibrated with 50 mM Tris-HCl (pH 8.0). The flowthrough fractions were concentrated by using Centricon YM-30, and the sample was applied to a Superdex 200 HR 10/30 column equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl.

**Metal reconstitution.** Metal reconstitution by heat treatment was performed as previously reported (42, 43). For manganese reconstitution, purified apoprotein [0.5 mM active sites in 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS); pH 7.0] was combined with 10 mM $MnCl_2$ and heated at 95°C for 1 h. Excess manganese was removed by dialysis against 20 mM MOPS (pH 7.0) containing 2 mM EDTA, followed by gel filtration. Iron reconstitution was performed anaerobically using 10 mM $Fe(NH_4)_2(SO_4)_2$ and 5 mM ascorbate in 20 mM MOPS (pH 7.0). Unbound metal was removed by dialysis against 20 mM MOPS (pH 7.0) containing 2 mM ascorbate and 2 mM EDTA, followed by gel filtration. Metal reconstitution was also performed anaerobically in the presence of both metal salts.

**RNA isolation and Northern blot analysis.** Aerobically and anaerobically grown *P. calidifontis* cells were harvested at the early log phase. RNA was isolated from these cells with the RNeasy Midi kit (Qiagen). For Northern blot analysis, 30 μg of total RNA was denatured by heat treatment at 65°C for 15 min, separated by 1% agarose gel electrophoresis, and transferred to nylon membranes with a vacuum blotting apparatus (AE-6680 Genopirator; ATTO, Tokyo, Japan). Labeling of DNA fragments, hybridization, and detection were performed by using the DIG DNA labeling and detection kit. DNA fragments corresponding to the internal 0.4-kbp region of the *sod*$_{Pc}$ gene and the 0.7-kbp region of the *kat*$_{Pc}$ gene, which encoded the catalase of *P. calidifontis*, were used as probes.

**Western blot analysis.** *P. calidifontis* cells grown under various conditions were disrupted by sonication. The crude cell extracts were obtained by centrifugation (20,000 × *g*, 30 min, 4°C). Ultracentrifugation (110,000 × *g*, 2 h, 4°C) was performed to separate the cytosol and membrane fractions. Each fraction (5 μg), along with the purified enzymes (0.05 μg), was subjected to SDS-PAGE, followed by Western blot analysis with specific antisera (rabbit) against the recombinant enzymes. Protein A-peroxidase conjugate was used to visualize the specific proteins, together with 4-chloro-1-naphthol and hydrogen peroxide.

**Nucleotide sequence accession number.** The *sod*$_{Pc}$ gene sequence is available under the accession no. AB109302 in the GenBank/EMBL/DDBJ databases.

## RESULTS

**Purification of SOD from *P. calidifontis*.** As in the case of the catalase from *P. calidifontis* (3), SOD activity was detected in the cell extracts of aerobically grown *P. calidifontis*. We purified the SOD from the crude extracts of cells grown under atmospheric air with vigorous shaking. The SOD was purified by ammonium sulfate fractionation, followed by hydrophobic interaction, anion-exchange and gel filtration column chromatography. The N-terminal amino acid sequence of the purified enzyme was MQVKKYELPPLPYAYNALEP. It showed high similarity to the N-terminal regions of Fe- and Mn-SODs from various archaeal species. The purified SOD (SOD$_{Pc}$) exhibited a specific activity of 1,200 U/mg and contained only 0.55 ± 0.01 manganese atoms per subunit (a subunit molecular mass of 24,004 Da was used to calculate the metal content) (Table 1). Iron and nickel were not present at detectable levels (<0.01 atoms per subunit).

Because of the low metal content, we purified SOD$_{Pc}$ from cells which were grown in a medium supplemented with manganese (Table 2). When 0.1 mM $MnCl_2$ was added to the medium, the activity of the purified SOD$_{Pc}$ increased up to 1,960 U/mg and the manganese content also increased up to 0.86 ± 0.04 atoms per subunit (Table 1). This phenomenon

TABLE 2. Purification of SOD from *P. calidifontis* cells grown aerobically in medium supplemented with manganese[a]

| Purification step | Total activity (U) | Total protein (mg) | Sp act (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 25,600 | 390 | 65.0 | 100 | 1 |
| Ammonium sulfate (65 to 95%) | 19,700 | 60 | 329 | 77 | 5 |
| Resource PHE | 11,800 | 7.3 | 1,620 | 46 | 25 |
| Resource Q | 6,840 | 3.8 | 1,780 | 27 | 27 |
| Superdex 200 | 4,280 | 2.2 | 1,960 | 17 | 30 |

[a] The medium was supplemented with 0.1 mM $MnCl_2$.

was also observed in the case of the Mn catalase from *P. calidifontis* ($Kat_{Pc}$). Crystal structure of Mn catalases indicate that each subunit possesses two manganese atoms in their active site (5, 7). Addition of manganese to the medium led to an increase in catalase activity in the cell extract, and the manganese content of $Kat_{Pc}$ increased from 1.32 ± 0.04 (3) to 1.78 ± 0.03 atoms per subunit.

The subunit molecular mass of $SOD_{Pc}$ estimated by SDS-PAGE was ca. 24 kDa, which was in good agreement with the value predicted by the deduced amino acid sequence of its gene (24,004 Da, see below). The molecular mass of the native protein was determined to be 61 kDa by gel filtration chromatography.

***sod*$_{Pc}$ gene.** In order to obtain a homologous probe to isolate the gene encoding $SOD_{Pc}$, two PCR primers were designed: one from the N-terminal amino acid sequence of the purified protein and the other based on a conserved C-terminal region of archaeal SODs. PCR with the two primers and the *P. calidifontis* genomic DNA as a template led to specific amplification of a DNA fragment with the expected length of ca. 0.5 kbp. The entire gene was then isolated from the genomic library of *P. calidifontis* with the amplified DNA fragment as a probe. DNA sequence analysis identified an open reading frame consisting of 627 bp encoding a protein of 209 amino acids with a calculated molecular mass of 24,004 Da. The N-terminal amino acid sequence deduced from the open reading frame was identical to the determined N-terminal sequence of the purified enzyme. The primary structure of $SOD_{Pc}$ displayed ca. 53 to 85% identity to previously characterized SODs from hyperthermophiles. A putative TATA-like element (5′-TTTAAA, box A [48]) and a putative ribosomal binding site (5′-GAGG) were located 28 and 12 nucleotides upstream of the initiation codon, respectively.

**Expression and purification of recombinant $SOD_{Pc}$.** We expressed the $sod_{Pc}$ gene in *E. coli* BL21-CodonPlus(DE3)-RIL cells harboring the expression plasmid, pET-sod. Cells were grown at 37°C, and 0.4 mM IPTG was added for gene expression, along with 1 mM $MnCl_2$. Since the gene product was produced in a soluble form, the cell extract was then heat treated at 85°C for 15 min to precipitate the heat-labile proteins from the host cell, including the SODs from *E. coli*. The supernatant was further subjected to anion-exchange chromatography, followed by gel filtration chromatography. However, the purified recombinant $SOD_{Pc}$ displayed only 134 U of SOD activity/mg and 0.05 ± 0.01 manganese atom and <0.01 iron atom per subunit. The cultivation temperature (37 or 30°C), the concentration of IPTG (0.1 or 0.4 mM), and/or the point in time of manganese addition were examined but did not lead to a highly active enzyme with sufficient incorporation of manganese. We therefore set out to reconstitute the holoenzyme in vitro. The addition of manganese to the medium was omitted, and 1 mM EDTA was added to all buffers after cultivation. Consequently, a purified, recombinant SOD with only negligible amounts of metal, and only trace activity, was obtained (apo-SOD; Table 1). Metal reconstitution was performed by adding the desired metal cations to the purified enzyme sample and subjecting the mixture to heat treatment (42, 43). Mn-reconstituted SOD exhibited a specific activity of 1,970 U/mg and contained 0.86 ± 0.02 manganese atoms per subunit (Table 1). These results were identical to those of the native

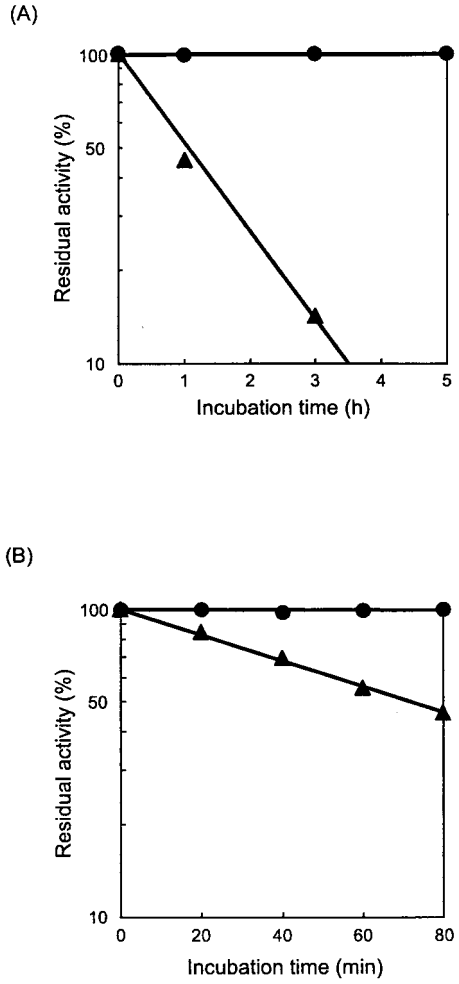


FIG. 1. Enzymatic properties of the recombinant SODs. Symbols: ●, the Mn-reconstituted SOD; ▲, the Fe-reconstituted SOD. (A) Thermostability of the SODs at 100°C. Enzyme samples (0.35 mg/ml) were incubated at 100°C in 50 mM potassium phosphate buffer (pH 7.8). Aliquots were removed at the desired time and chilled on ice, and residual activities were measured. (B) Hydrogen peroxide inactivation of the SODs. SODs (0.25 mg/ml) were incubated with 0.25 mM hydrogen peroxide in 50 mM potassium phosphate buffer (pH 7.8) at 25°C. At various intervals, aliquots of the enzyme were drawn, treated with 1 U of catalase/μl, and then assayed for residual SOD activity.

Mn-SOD purified from *P. calidifontis*. It may be that these values correspond to those of the enzyme fully charged with manganese. Fe-reconstituted SOD was also active. It displayed 434 U of SOD activity/mg and contained 0.76 ± 0.03 iron atoms per subunit. Iron was not present at detectable levels (<0.01 atoms per subunit) in the Mn-reconstituted SOD, and neither was manganese in the Fe-reconstituted SOD.

**Enzymatic properties of $SOD_{Pc}$.** Thermal inactivation experiments were performed at 100°C, which is the upper limit of growth for *P. calidifontis* (4). There is a clear difference between the Mn-reconstituted SOD and the Fe-reconstituted SOD, as shown in Fig. 1A. The Mn-reconstituted SOD was a very thermostable enzyme, which was not inactivated at all after 5 h of incubation at 100°C. On the contrary, a 1-h incu-

TABLE 3. Metal contents of reconstituted SODs[a]

| Metal reconstitution condition | | Metal content (mean no. of atoms/subunit ± SD) | |
|---|---|---|---|
| $MnCl_2$ concn (mM) | $Fe(NH_4)_2(SO_4)_2$ concn (mM) | Mn | Fe |
| 1 | 0 | 0.77 ± 0.05 | 0.01 ± 0.004 |
| 0 | 1 | 0.01 ± 0.006 | 0.65 ± 0.05 |
| 5 | 5 | 0.08 ± 0.01 | 0.57 ± 0.03 |
| 1 | 1 | 0.11 ± 0.02 | 0.64 ± 0.06 |

[a] For metal reconstitution, the purified apoprotein (0.5 mM active site) was combined with metal salts and heated at 95°C for 1 h. Unbound metals were removed by dialysis, followed by gel filtration.

bation led to a 50% decrease in the activity of the Fe-reconstituted SOD.

Azide is known as an inhibitor of Mn- and Fe-SODs. In general, iron-containing SOD is more sensitive to inhibition by azide than manganese-containing enzymes (33). We investigated azide sensitivities of both reconstituted SODs. Both enzymes displayed relatively strong resistance against azide (Mn- and Fe-SOD activity decreased 50% with 380 and 340 mM azide, respectively). This resistance against azide has also been observed in other archaeal SODs (44, 45).

It has been reported that hydrogen peroxide inactivates Fe-SODs but not Mn-SODs (6). Therefore, addition of hydrogen peroxide is often used to distinguish between Mn- and Fe-SODs. As shown in Fig. 1B, the Fe-reconstituted SOD was inactivated by hydrogen peroxide, whereas the Mn-reconstituted SOD was not affected at all.

**Metal reconstitution.** As mentioned above, in vitro metal incorporation into the recombinant apoenzyme (0.5 mM active site) with 10 mM $MnCl_2$ or $Fe(NH_4)_2(SO_4)_2$ led to highly active enzymes. Metal reconstitution was also successful at concentrations of 1 mM $MnCl_2$ or $Fe(NH_4)_2(SO_4)_2$ (Table 3). Since the native enzyme purified from aerobically grown *P. calidifontis* cells exclusively harbored Mn, we performed the metal reconstitution experiments in the presence of both metal salts. We found that both metals were incorporated with the recombinant apo-SOD, and surprisingly, with a preference for Fe (Table 3).

**Metal contents of native SODs from *P. calidifontis* grown under various conditions.** Since *P. calidifontis* SOD was found to be a cambilalistic SOD in vitro, we examined whether this feature also applied in vivo. We first purified SOD from cells grown aerobically in iron-supplemented medium. In this case, the enzyme exhibited a specific activity of 1,520 U/mg and contained 0.68 ± 0.02 Mn and 0.01 ± 0.005 Fe atoms/subunit (Table 1). We further purified the SOD found in anaerobically grown cells. The level of SOD activity in anaerobically grown cells is low, corresponding to ca. 6% of the activity was found in aerobically grown cells (see below). In this case, the purified enzyme displayed a specific activity of 512 U/mg and contained 0.08 ± 0.01 Mn and 0.43 ± 0.04 Fe atoms/subunit (Table 1). Therefore, the *P. calidifontis* SOD was a cambialistic SOD in vivo, containing only manganese under aerobic growth conditions and mainly iron under anaerobic conditions.

**SOD and catalase activities in *P. calidifontis* cells grown under aerobic and anaerobic conditions.** *P. calidifontis* was cultivated under various culture conditions, and SOD and catalase activities were measured in the respective crude extracts. Anaerobically grown cells showed low levels of SOD activity (2.8 U/mg) and catalase activity (≤10 U/mg). Cells grown under atmospheric air but without shaking also displayed similar levels of both activities. However, when cells were grown under atmospheric air with vigorous shaking, the crude extracts exhibited activities of 49.9 and 62.5 U/mg for SOD and catalase, respectively. Therefore, not only the catalase but also the SOD is induced when *P. calidifontis* is grown under aerobic or oxidative conditions.

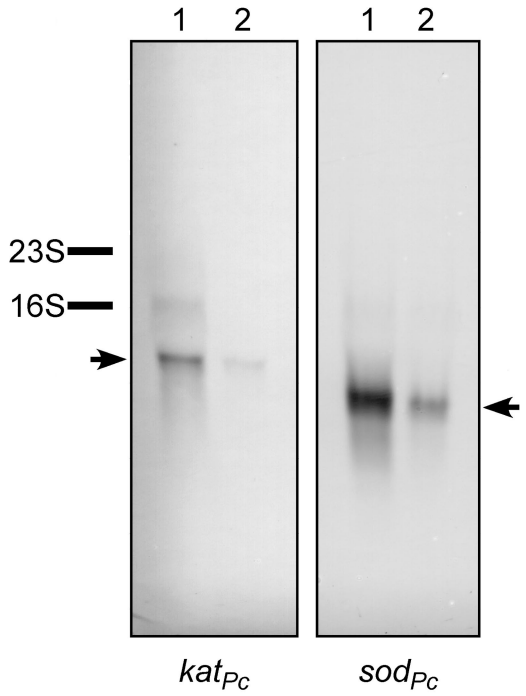


FIG. 2. Northern blot analysis of RNA from *P. calidifontis* cells grown aerobically (lanes 1) and anaerobically (lanes 2). The $kat_{Pc}$ (left panel) and the $sod_{Pc}$ (right panel) genes were used as probes. Each lane contains 30 μg of total RNA.

**Transcriptional regulation of $sod_{Pc}$ and $kat_{Pc}$ genes.** Since SOD and catalase activities were induced under aerobic conditions, we further examined the regulation of gene expression of $sod_{Pc}$ and $kat_{Pc}$. Total RNA was extracted from cells cultivated independently under aerobic and anaerobic conditions, and Northern blot analysis was performed with $kat_{Pc}$ and $sod_{Pc}$ probes (Fig. 2). Both genes were actively transcribed under aerobic growth conditions and only slightly under anaerobic growth conditions. This result was in good agreement with the levels of enzyme activity. By comparing the mobilities of the transcripts with those of 16S (1.5 kb) and 23S rRNA (3.0 kb), the lengths of the transcripts were estimated to be ca. 1.1 and 0.7 kb. Considering the length of the $kat_{Pc}$ (894 bp) and $sod_{Pc}$ (627 bp) genes and their flanking genes (not shown), it is most likely that both $kat_{Pc}$ and $sod_{Pc}$ are transcribed monocistronically. By Western blot analysis with anti-$SOD_{Pc}$ and anti-$Kat_{Pc}$ antibodies, we also examined the protein levels of the two enzymes under aerobic and anaerobic conditions. Whereas intense bands were observed under aerobic conditions, proteins were barely detectable in cells grown in the absence of

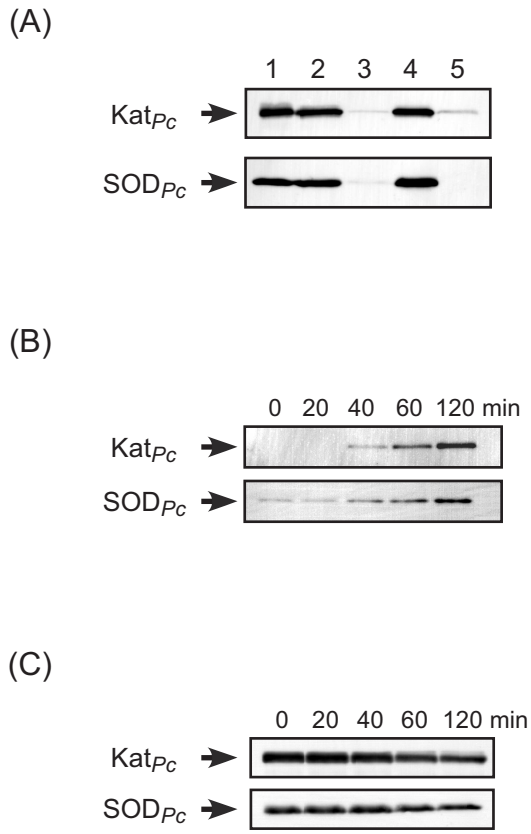


FIG. 3. Western blot analysis with anti-Kat$_{Pc}$ and anti-SOD$_{Pc}$ antibodies. Each lane contains 5 μg of protein unless mentioned otherwise. (A) Lane 1, purified Kat$_{Pc}$ (0.05 μg) or SOD$_{Pc}$ (0.05 μg); lane 2, crude extract of aerobically grown cells; lane 3, crude extract of anaerobically grown cells; lane 4, cytosolic fraction from the crude extract of aerobically grown cells; lane 5, membrane fraction from the crude extract of aerobically grown cells. Adaptive responses of *P. calidifontis* cells to anaerobic-to-aerobic (B) and aerobic-to-anaerobic (C) changes. Anaerobically or aerobically cultivated cells in their log phase were transferred to aerobic or anaerobic conditions, respectively. After 0, 20, 40, 60, and 120 min, cells were harvested and then analyzed by Western blotting with anti-Kat$_{Pc}$ and anti-SOD$_{Pc}$ antibodies.

oxygen (Fig. 3A, lane 2 [crude extract of aerobically grown cells] and lane 3 [crude extract of anaerobically grown cells]).

**Aerobic and anaerobic adaptive responses of *P. calidifontis* cells.** As mentioned above, the levels of transcription, proteins, and activities of SOD$_{Pc}$ and Kat$_{Pc}$ were higher in cells grown in the presence of oxygen. We further examined how the levels of these two proteins responded in *P. calidifontis* when cells were exposed to oxygen. *P. calidifontis* cells were cultivated anaerobically until the log phase and then transferred to an aerobic culture condition. After 0, 20, 40, 60, and 120 min, cells were harvested and analyzed by Western blotting (Fig. 3B). SOD$_{Pc}$ and Kat$_{Pc}$ were induced remarkably after cell exposure to oxygen. This was not due to the presence of resazurin in the medium, which can form hydrogen peroxide under aerobic conditions, since we observed the same induction of SOD$_{Pc}$ and Kat$_{Pc}$ in its absence. We also investigated the response to cells transferred from aerobic to anaerobic conditions (Fig. 3C). Within 20 min, the red color of resazurin, an oxygen indicator, in the media turned clear, indicating the consumption of residual oxygen. Levels of Kat$_{Pc}$ decreased gradually, whereas a change in the levels of SOD$_{Pc}$ was not apparent.

**Localization of SOD$_{Pc}$ and Kat$_{Pc}$.** With the anti-SOD$_{Pc}$ and anti-Kat$_{Pc}$ antibodies, we determined the subcellular localization of SOD$_{Pc}$ and Kat$_{Pc}$. The cytosol and membrane fractions were separated from the crude extract of aerobically grown *P. calidifontis* cells by ultracentrifugation and were then subjected to SDS-PAGE and Western blot analysis. Malate dehydrogenase activity of *P. calidifontis* was found exclusively in the cytosolic fraction after ultracentrifugation. The results clearly indicated that SOD$_{Pc}$ and Kat$_{Pc}$ were localized exclusively in the cytosol fraction (Fig. 3A, lane 4 [cytosol fraction] and lane 5 [membrane fraction]).

## DISCUSSION

We have characterized here the native and recombinant SODs from the facultatively aerobic hyperthermophilic archaeon *P. calidifontis*. We have also examined the regulation of SOD$_{Pc}$ and Kat$_{Pc}$ syntheses in *P. calidifontis* cells, particularly in terms of their response to oxygen.

The subunit molecular mass of SOD$_{Pc}$ estimated by SDS-PAGE was ca. 24 kDa, which was in good agreement with the value predicted by the deduced amino acid sequence of its gene (24,004 Da). The molecular mass of the native protein was estimated to be 61 kDa by gel filtration chromatography, a little larger than the value expected for a dimeric structure. However, it has previously been reported that SODs from *Sulfolobus*, although displaying similarly small molecular masses in gel filtration experiments (11, 24), are actually composed of tightly packed tetramers (25, 41). Since other SODs from hyperthermophilic archaea also exhibit the same tendency (42, 45), this may also be the case for SOD$_{Pc}$.

Cambialistic SODs function efficiently with either manganese or iron at their active centers but can be divided into two groups. One group is comprised of cambialistic SODs that have comparable activities with both metals, for example, the SODs from *Propionibacterium shermanii* (32) and *Bacteroides fragilis* (13). Members of the other group exhibit higher activity with manganese than with iron (e.g., the SODs from *Streptococcus mutans* [29] and *Methylomonas* sp. strain J [30]). Our results indicate that SOD$_{Pc}$ falls into the latter group, along with the enzymes from the obligate aerobe *Aeropyrum pernix* (45) and the facultative microaerobe *P. aerophilum* (42). We have further revealed the cambialistic nature of SOD$_{Pc}$ in vivo; the enzyme is an Fe-SOD under anaerobic conditions, whereas it is a Mn-SOD in aerobically grown cells. This is similar to several cambialistic SODs from bacteria. Since the metal content of native SODs from *Aeropyrum pernix* and *P. aerophilum* has not been examined, the in vivo cambialistic nature of SODs in these archaeal strains has not yet been determined.

An interesting observation was that in the in vitro reconstitution experiments, recombinant SOD$_{Pc}$ displayed a preference to uptake Fe rather than Mn. This was unexpected considering the fact that, although Fe was much more abundant (20-fold) in the cell extracts of *P. calidifontis*, the native enzyme from aerobically grown cells specifically contained Mn. The metal contents of *P. calidifontis* cell extracts were as follows: aerobically grown cells contained 744 ± 6 μmol of Mn and 14,400 ± 200 μmol of Fe/mg of extract, and anaerobically

grown cells contained 75 ± 2 μmol of Mn and 14,600 ± 300 μmol of Fe/mg of extract. Analysis by plasma emission spectroscopy cannot distinguish free and sequestered metal cations, so the majority of intracellular Fe in aerobically grown *P. calidifontis* may not be in a free state. There is a large difference in solubility constants between ferrous [$Fe(OH)_2$, $1.6 \times 10^{-14}$] and ferric [$Fe(OH)_3$, $1.1 \times 10^{-36}$] iron. Under anaerobic conditions, where ferrous iron is stable, there may be a sufficient amount of free iron to be incorporated by the SOD, whereas under aerobic conditions, where ferrous iron is oxidized to ferric iron, the availability of free iron is most likely to be too low, and therefore the incorporation of manganese becomes dominant.

Although several biochemical examinations of SODs from (hyper)thermophilic archaea have been reported, studies on their expression and regulation have not been performed. Many of them have focused primarily on the properties of recombinant enzymes. Facultatively aerobic hyperthermophiles, including *P. calidifontis*, enable us to study how these proteins and/or genes respond when hyperthermophile cells are exposed to oxygen or oxidative conditions. We have found that the SOD and the catalase of *P. calidifontis* were regulated at the transcriptional level (Fig. 2), and their proteins were abundant under aerobic conditions and negligible under anaerobic conditions (Fig. 3A). Once cells were exposed to oxygen, rapid synthesis was observed (Fig. 3B). In contrast, rapid protein degradation was not observed when cells were transferred from aerobic to anaerobic conditions (Fig. 3C). Levels of $Kat_{Pc}$ decreased gradually, whereas a change in the levels of $SOD_{Pc}$ was not apparent. The response to the removal of oxygen, in terms of catalase and SOD, seems not to be an active or sensitive response. This is not surprising, since their presence will provide an advantage in natural environments, where it is often the case that the amount of oxygen consistently changes. The difference in the degradation rates of the two enzymes may simply reflect their different thermostabilities. The in vitro half-lives of the $Kat_{Pc}$ were 50, 114, and 432 min at 100, 95, and 90°C, respectively (3); these values are much shorter than that of $SOD_{Pc}$ (Fig. 1A) and therefore $Kat_{Pc}$ is likely to be more susceptible to protein degradation pathways in the cell.

In *P. calidifontis*, SOD and catalase were found not to be constitutive enzymes but regulated at transcriptional levels by the presence or absence of oxygen; this is the first such report in (hyper)thermophilic archaea. This is another interesting parameter for studying the regulation of gene expression and signal transduction in hyperthermophilic archaea. Investigations of the factors involved and the actual compound(s) that trigger this response to oxygen or the oxidative state are now under way.

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