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A Cambialistic SOD in a Strictly Aerobic Hyperthermophilic Archaeon, *Aeropyrum pernix*[1]

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The superoxide dismutase (SOD) gene of *Aeropyrum pernix*, a strictly aerobic hyperthermophilic archaeon, was cloned and expressed in *Escherichia coli*, and its gene product was characterized. The molecular mass of the protein, based on the deduced amino acid sequence, was 24.6 kDa. The sequence showed overall similarity to the sequences of known Mn- and Fe-SODs. The metal binding residues conserved in Mn- and Fe-SODs were also found in *A. pernix* SOD. When the SOD gene was expressed in *E. coli* cells, the product formed a homodimer, and contained both Mn and Fe. Metal reconstitution experiments showed that *A. pernix* SOD is cambialistic, *i.e.* active with either Fe or Mn. The specific activities were 906 U/mg with Mn and 175 U/mg with Fe. No loss of activity of Mn-reconstituted SOD was observed at 105°C even after 5 h incubation. Sodium azide, an inhibitor of SODs, did not inhibit the Mn-reconstituted SOD from *A. pernix* even at concentrations up to 400 mM. This SOD from an aerobic hyperthermophilic archaeon, *Aeropyrum pernix*, was extremely thermostable and active with either Mn or Fe. With Mn as a metal cofactor, it was more thermostable, and less sensitive to sodium azide and sodium fluoride than with Fe.

Key words: *Aeropyrum pernix*, archaea, azide, hyperthermophile, cambialistic superoxide dismutase.

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Superoxide dismutases (SODs) catalyze the dismutation of the superoxide anion (O_2^-) to O_2 and H_2O_2, and are widely distributed among aerobic organisms. SODs play an important role in cell protection mechanisms against oxidative damage. These enzymes comprise a family of metalloproteins classified mainly into three groups: copper and zinc-containing SODs (Cu,Zn-SODs), manganese-containing SODs (Mn-SOD), and iron-containing SODs (Fe-SODs). The latter two groups are assumed to have a common evolutionary origin due to the similarity in their amino acid sequences and three-dimensional protein structures. Whereas, the Cu,Zn-SODs apparently evolved independently. Despite these similarities, metal-reconstitution studies have shown that most Mn- and Fe-SODs have strict metal binding specificities (*1, 2*). Only six groups of bacteria are known to possess cambialistic SODs that are active with either Fe or Mn as a cofactor; these include SODs from *Streptococcus mutans* (*3*), *Bacteroides fragilis* (*4*), *Methylomonas* strain J (*5*), *Propionibacterium shermanii* (*6*), *Porphyromonas gingivalis* (*7*), and *Mycobacterium smegmatis* (*8*). At present, it is not known what factors determine the metal specificity of SODs.

Hyperthermophilic bacteria and archaea occupy all the deepest and shortest branches of the universal phylogenetic tree (*9*). Therefore, they may retain primitive characteristics similar to those of the common ancestor of life on earth. The characterization of SODs from hyperthermophiles is interesting from the viewpoint of the evolution of SODs. However, only three SODs have been isolated from hyperthermophiles: *Sulfolobus acidocaldarius* (*10*), *S. solfataricus* (*11, 12*), and *Aquifex pyrophilus* (*13*). *Aeropyrum pernix* is the first strict aerobic heterotrophic neutrophile found among the hyperthermophilc archea (*14*). Therefore, we expect that *A. pernix* has a SOD with unique characteristics. Here we report the cloning and characterization of a superoxide dismutase from an aerobic hyperthermophilic archaeon, *A. pernix*, this being the first extremely thermostable cambialistic SOD.

MATERIALS AND METHODS

Microorganisms and Culture Conditions—*A. pernix* K1 (JCM9820) was grown in JXT medium at 90°C with vigorous shaking (180 rpm) (*14*). Cells in the late exponential growth phase were harvested by centrifugation and then washed twice with fresh JXT medium. The cell pellet was frozen at −85°C prior to DNA isolation.

Isolation of Genomic DNA—*A. pernix* cells (1 g wet weight) were suspended in 19 ml of lysis buffer comprising 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 100 mM EDTA, 2% SDS, and 0.1 mg/ml proteinase K, and then incubated at 65°C for 30 min. The lysate was extracted once with

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phenol, with phenol/chloroform and then with chloroform/isoamyl alcohol (24:1). After precipitation with 2 volumes of ethanol, the DNA was dissolved in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA solution was incubated with 0.1 mg/ml RNase A at 37°C for 3.5 h, and then with 0.1 mg/ml proteinase K and 0.5% SDS at 50°C for 90 min. Then, 5 M NaCl and 10% CTAB (hexadecyltrimethylammonium bromide) in 0.7 M NaCl were added to the solution to adjust the concentrations of NaCl and CTAB to 0.7 M and 1%, respectively. After incubation at 65°C for 20 min, the solution was extracted once with chloroform/isoamyl alcohol (24:1), followed by extraction with phenol/chloroform four times. After precipitation with 2 volumes of ethanol, the DNA was dissolved in 1 ml of TE buffer.

Amplification of a Part of the SOD Gene by PCR—Two PCR primers designed from conserved sequences of reported SODs were synthesized.

SODF3: 5′-GA(TC)GC(GATC)(TC)T(GATC)GA(AG)-CC(GATC)-3′

SODR2: 5′-(GA)TA(AG)(TA)A(GATC)(GC)(CAT)-(AG)TG(TC)TCCC-3′

The PCR reaction was performed using 1.7 ng/μl of genomic DNA as the template, with 2 μM SODF3 and 2.7 μM SODR2. The PCR reaction was started with a template denaturation step at 95°C for 4 min, followed by 30 cycles of 95°C for 0.5 min, 44°C for 0.5 min and 72°C for 1 min. The PCR product of the expected size (453-477 bp) was ligated to pT7Blue-T (Novagen, Madison, USA), and then its sequence was determined.

Construction and Screening of a Genomic Library—*A. pernix* genomic DNA was partially digested with *Sau*3AI and then ligated to SuperCos 1 (Stratagene, La Jolla, USA) digested with *Bam*HI. The ligated DNA was packaged *in vitro* and then introduced into *Escherichia coli* XL1-Blue MR according to Stratagene's instruction manual. Ampicillin-resistant transformants were obtained at a frequency of 6.4×10^4 per μg of ligated DNA. The 12,000 colonies in the genomic library were screened by colony hybridization using the PCR-amplified fragment as a probe. Labeling of the probe and hybridization were carried out with a DIG DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany).

Expression of the SOD Gene in E. coli—Two PCR primers were synthesized. AP8F contained the recognition sequence of *Nco*I upstream of the initiation codon of the ORF, and AP8R contained the recognition sequence of *Bam*HI downstream of the stop codon of the ORF.

AP8F: 5′-CAGCCATGGTGAGCTTTAAGAGGTA-3′

AP8R: 5′-TGGGGATCCCTACTGGGGGAGCAGGT-3′

After PCR-amplification of plasmid pSODAP3 containing the SOD gene, the amplified fragment was digested with *Nco*I and *Bam*HI, and then ligated into vector pET11d (Stratagene), which was digested with the same enzymes. The resulting plasmid for the expression of the *A. pernix* SOD gene in *E. coli* was designated as pSODAP11. *E. coli* BL21(DE3) was transformed with pSODAP11. The transformant was grown in LB medium containing ampicillin (50 μg/ml) at 25, 30, or 37°C to the early exponential growth phase, and then 0.2-1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture to induce expression of the SOD gene. The cells were then grown at 25, 30, or 37°C to the stationary phase. For metal-enriched cultures, 0.5 mM $MnSO_4$ or $FeSO_4$ was added to the medium.

Purification of the SOD Gene Product—Harvested cells were suspended in 50 mM Tris-HCl (pH 7.5) and then sonicated two times for 2.5 min on ice with a Sonifier 250 (Branson, Danbury, USA). After incubation at 90°C for 30 min, the lysate was centrifuged at $9730 \times g$ for 20 min to precipitate denatured *E. coli* proteins. The supernatant was concentrated with a Centriprep-10 concentrator (Amicon, Beverly, USA), and then applied to a Q-Sepharose HP HiLoad 16/10 column (Pharmacia LKB, Uppsala, Sweden) and eluted with a linear NaCl gradient (0-1.0 M) in 50 mM Tris-HCl (pH 7.5). The active fractions were collected and concentrated with a Centriprep-10 concentrator. Protein concentrations were measured by the method of Bradford (*15*) using bovine serum albumin as a standard. The proteins at each purification step were analyzed by SDS-PAGE on 16% gels.

For molecular mass estimation, the recombinant SOD (r-SOD) was applied to a Superose 12 HR 10/30 column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 7.0) + 150 mM NaCl buffer at a flow rate of 0.4 ml/min. The elution profile was monitored as the absorbance at 280 nm.

Assays for Superoxide Dismutase Activity—SOD activity was assayed at 25°C for 2 min by the xanthine oxidase-cytochrome *c* method (*16*). One unit was defined as the amount of enzyme that caused 50% inhibition of cytochrome *c* reduction when xanthine oxidase produces a rate of reduction of cytochrome *c* at 550 mμ of 0.025 absorbance units per min. To determine the effects of inhibitors, activity was measured in reaction mixtures containing the inhibitors. In H_2O_2 inactivation experiments, SOD (0.25 mg/ml) was incubated with 0.24 mM H_2O_2 in 50 mM potassium phosphate (pH 7.8) at 25°C. At appropriate intervals, aliquots were withdrawn and treated with catalase (1.0 U/μl), and then the residual SOD activity was measured. For thermal inactivation experiments, 1 mg/ml SOD in 20 mM potassium phosphate (pH 7.0) was incubated at 105 or 110°C. Aliquots were removed at intervals and chilled on ice, and then the residual activity was measured.

Mass Spectrometry Analysis—MALDI-TOF mass spectra were obtained in the positive ion mode with a Voyager Elite time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, USA). The matrix was prepared by dissolving sinapinic acid (10 mg/ml) in 33.3% acetonitrile and 0.0667% trifluoroacetic acid (TFA). The purified r-SOD (0.5 μl) dissolved in 0.005% TFA was mixed with 4.5 μl of the matrix to give a final concentration of 7.2 pmol/μl. The mixed sample (0.5 μl) was applied to the target plate and allowed to evaporate. Spectra were obtained by means of linear-mode measurement. The ions generated on laser (337 nm) irradiation were accelerated to 20 kV and passed through a 2.0 m flight tube to the detector. ACTH(7-38) fragment (+1): M_r 3,660.17, and myoglobin (+1): M_r 16,952.56 were used for calibration.

Amino Acid Sequence Determination—The N-terminal amino acid sequence of the purified SOD was determined with a Shimadzu PSQ-2 protein sequencer (Shimadzu, Kyoto).

Metal Analysis—The metal contents of the purified SOD

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were determined with an Inductively Coupled Plasma Emission Spectrometer (ICPS-1000 IV; Shimadzu, Kyoto).

ESR Spectra Analysis—Electron spin resonance (ESR) spectra were obtained with a JEOL JES-TE200 ESR spectrometer (JEOL, Tokyo). The purified r-SOD was diluted with 12.5 mM Tris/HCl (pH 7.5) containing sodium azide. The protein and iron concentrations were 3.48 mg/ml and 0.114 mM, respectively, in a volume of 50 μl. The samples were kept in ESR tubes (inner diameter, 4 mm), and then frozen in liquid nitrogen. The spectra were recorded at 77 K with a modulation amplitude of 1.25 mT and a microwave power setting of 1.03 mW.

Reconstitution of SOD—The purified SOD (3.0 mg) was incubated in 3 ml of denaturation buffer (50 mM acetate buffer, pH 3.8, containing 6 M guanidine hydrochloride and 10 mM EDTA) for 16 h at 30°C. The solution was chromatographed through a Sephadex G-25 gel filtration column (Pharmacia) equilibrated with the denaturation buffer to obtain the apoprotein. For preparation of the Mn-reconstituted enzyme, the apoprotein was dialyzed against 50 mM acetate buffer, pH 3.8, containing 6 M guanidine hydrochloride and 10 mM $MnSO_4$ for 4 h at room temperature, and then against 50 mM Tris-HCl buffer, pH 7.5, containing 6 M guanidine hydrochloride and 10 mM $MnSO_4$ for 4 h at room temperature. Guanidine hydrochloride was removed by dialysis against 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM $MnSO_4$ for 4 h at room temperature, and then against 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA for 12 h at room temperature. The Mn-reconstituted enzyme was obtained by gel filtration through Sephadex G-25 (Pharmacia) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Other reconstituted SODs were prepared by the same procedures using $FeSO_4$, $NiCl_2$, $CuSO_4$, or $ZnSO_4$ instead of $MnSO_4$.

Nucleotide Sequence Accession Number—The SOD gene sequence of *A. pernix* described in this paper has been deposited in DDBJ under accession number AB012621.

RESULTS

Cloning and Structure of the A. pernix SOD Gene—The PCR-amplified 468 bp fragment was thought to be a part of the *A. pernix* SOD gene because of its similarity to the amino acid sequences of known Fe- and Mn-SODs. This PCR product was used as a probe to screen a genomic library from *A. pernix*. A positive clone was selected for further research and its plasmid was designated as pSODAP3. The insert DNA fragment of the plasmid pSODAP3 was partially digested with *Sau*3AI and then ligated to pUC19. The resulting plasmids were screened by colony hybridization using the same probe. Plasmid pSODAP5 containing a 3.9 kb genomic fragment was obtained and its nucleotide sequence was determined. The open reading frame (ORF) obtained was thought to represent the SOD gene from *A. pernix* due to its similarity to those of other known Fe- and Mn-SOD genes. Its nucleotide sequence and deduced amino acid sequence are shown in Fig. 1, A and B.

A pair of conserved sequences has been found in archaeal promoters (*17*). One is highly conserved boxA, TTTA(A/T)A, and the other is weakly conserved boxB, (A/T)TG(A/C), located near the initiation codon. The distance between boxA and boxB is 22 to 27 bp. Although a boxA-like sequence, TTTAAA, was found 100 bp upstream of the initiation codon, no boxB-like sequence was found. No other promoter-like element was found upstream of the ORF in the *A. pernix* SOD gene.

The deduced amino acid sequence of the ORF comprises 214 amino acids with a molecular weight of 24,576.82. The amino acid sequence similarities to SODs of *S. acidocaldarius* (*18*), *Methanobacterium thermoautotrophicum* (*19*), and *Halobacterium cutirubrum* (*20*) are 51.4, 49.3, and 41.4%, respectively. However, it exhibits no detectable similarity to known Cu,Zn-SODs (data not shown). From structural information obtained on X-ray crystallography, the metal binding site and substrate funnel in Mn-SOD and Fe-SODs have been assigned (*21-23*). Four residues in-

A

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1     AGGTATCCATGGCTGCGGGTGAGGGGTAAGGCCTGCCGTAGCGCTAGCGGCCGCCCAAGC
61    GTTTTAAATAGCGCCTGCTCTTAAACTGGCGCCCGAAATATTTATCCTAACTATTAAACT
121   ATTCTTTAACTCCGTTAACATACTATTACCCACGGTTCGAGAAGGTGAGTTGGAATGGTG
181   AGCTTTAAGAGGTACGAGCTCCCCCCGCTACCCTACAACTACAACGCCCTGGAGCCCTAC
241   ATTATAGAGGAGATAATGAAGCTGCACCACCAGAAGCATCACAACACGTATGTCAAAGGG
301   GCTAACGCCGCACTCGAGAAGATAGAGAAGCATCTCAAGGGCGAGATACAGATAGACGTT
361   AGGGCTGTCATGAGGGACTTCAGCTTCAACTACGCAGGCCACATAATGCACACCATATTC
421   TGGCCCAACATGGCCCCGCCCGGCAAGGGCGGTGGAACACCTGGCGGCAGGGTGGCTGAC
481   CTCATAGAGAAGCAGTTCGGCGGCTTCGAGAAGTTCAAGGCCCTCTTCAGCGCCGCTGCG
541   AAGACGGTGGAGGGCGTCGGGTGGGGCGTGCTCGCGTTCGACCCTCTGACAGAGGAGCTC
601   AGGATACTGCAGGTGGAGAAGCACAACGTCCTCATGACGGCGGGCCTTGTGCCCATACTA
661   GTTATTGACGTGTGGGAGCACGCCTACTACCTCCAGTACAAGAACGACAGGGGCAGCTAC
721   GTCGAGAACTGGTGGAACGTGGTCAACTGGGACGACGTTGAGAAGAGGCTGGAGCAGGCT
781   CTAAACAACGCGAAGCCCCTCTACCTGCTCCCCCAGTAGCTCCCCACTAGCCGCGGCTTC
841   CCAGCACTTTTTTAACGGTCCTACCCGCTATGTAGTCCATGAGCACCTCCTTCGCCCCCT
901   CGCCTATGTCAATAACCT
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Fig. 1. **(A) Nucleotide sequence of the *A. pernix* SOD gene.** The initiation codon is underlined. The stop codon is double underlined. A boxA-like sequence is indicated by a wavy line. **(B) Alignment of the amino acid sequence of *A. pernix* SOD with those of other SODs.** The sequences were obtained from the SWISS-PLOT and DDBJ databases and aligned using the program, CLUSTAL W (*40*). Positions are numbered in accordance with the sequence of *A. pernix* SOD. Residues that may be important for the metal specificity are boxed. Abbreviations: /Mn, Mn-SOD; /Fe, Fe-SOD; H, SODs from hyperthermophiles; T, SODs from thermophiles; M, SODs from mesophiles; C, cambialistic SODs with about the same activity with Mn and Fe; Cm, cambialistic SODs with higher activity with Mn.; A.per, *A. pernix*; S.sol, *S. solfataricus*; A.pyr, *Aquifex pyrophilus*; T.aqu, *Thermus aquaticus*; B.ste, *Bacillus stearothermophilus*; E. col, *E. coli*; P.aer, *Pseudomonas aeruginosa*; B. fra, *Bacteroides fragilis*; P.gin, *Porphyromonas gingivalis*; P.she, *Propionibacterium shermanii*; S.mut, *Streptococcus mutans*; M.J, *Methylomonas* strain J; M.sme, *Mycobacterium smegmatis*.

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volved in the metal binding site which are conserved in all Fe- and Mn-SODs were found in *A. pernix* SOD (His-31, His-79, Asp-165, and His-169). The four residues postulated to comprise the substrate funnel were also found in *A. pernix* SOD (His-36, Tyr-39, His-79, and Trp-83). These data indicate that *A. pernix* SOD is a Mn-SOD or Fe-SOD, and that it has a substrate funnel (Fig. 1B).

Expression and Purification of A. pernix SOD—Transformant *E. coli* cells harboring the expression plasmid, pSODAP11, were sonicated and heated to remove *E. coli* proteins including endogenous SOD. The concentrated supernatant was applied to a Q-Sepharose column, *A. pernix* SOD being eluted at 0.16 M NaCl. As shown in Fig. 2, the *A. pernix* SOD was purified to homogeneity. The

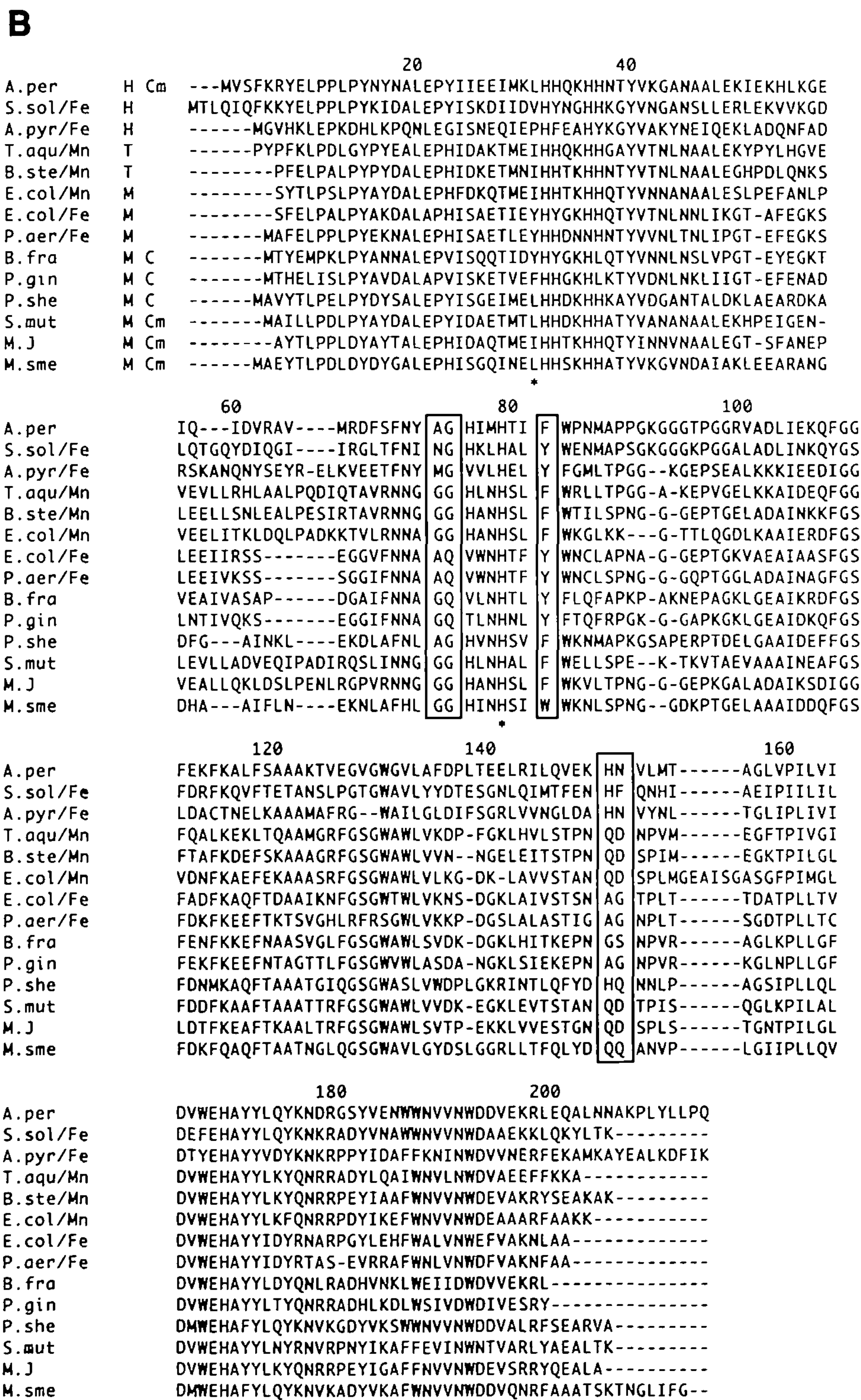

Fig. 1B

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apparent molecular weight of the monomer was estimated to be 25,600 on SDS-PAGE, which agrees approximately with the value, 24,576.82, calculated from the deduced amino acid sequence. The N-terminal amino acid sequence of the purified SOD showed it to be a mixture of a peptide starting with an initial Met and a truncated peptide without an initial Met (1:1). The molecular weights estimated from the nucleotide sequences agreed well with the values, 24,577.3 and 24,446.3, obtained on MALDI-TOF mass spectrometry, thus suggesting that the *A. pernix* SOD is not modified in *E. coli*. The apparent molecular weight of the whole enzyme estimated by gel filtration was approximately 60 kDa (data not shown). This indicates that *A. pernix* SOD forms a homodimer in *E. coli* cells.

Metal Cofactor—The recombinant *A. pernix* SOD produced in *E. coli* always contains both Mn and Fe, although its metal contents vary according to the conditions for gene expression and culture. Its activity appeared to depend on the Fe and Mn contents. To determine the metal-specificity of *A. pernix* SOD, metal-reconstitution experiments were carried out. The apoprotein of *A. pernix* SOD showed no SOD activity. As shown in Table I, the Mn-reconstituted SOD contains 0.76 atom of manganese per subunit and exhibits a specific activity of 906 U/mg. The Fe-reconstituted SOD contains 0.8 atoms of iron per subunit and exhibits a specific activity of 175 U/mg. This indicates that *A. pernix* SOD is a cambialistic SOD which is active with either Fe or Mn as a cofactor. Although nickel was bound to *A. pernix* SOD during refolding, the resultant protein had no SOD activity. Under the same refolding conditions, neither Cu nor Zn was bound to *A. pernix* SOD. The specific activities of the Mn- and Fe-reconstituted *A. pernix* SODs are lower than the reported values for mesophilic eubacterial Fe- and Mn-SODs (*24*). Because xanthine oxidase in the assay mixture for SOD is not thermostable, SOD activity is always measured at 25°C (*10, 11, 25-27*). This temperature is probably much lower than the optimum temperature for *A. pernix* SOD activity because the optimum growth temperature for *A. pernix* is 95°C (*14*).

Thermal Inactivation—*A. pernix* grows between 70 and 100°C, with an optimum growth temperature of 90 to 95°C, but no growth is observed at 102°C (*14*). As shown in Fig. 3, no loss of activity of the *A. pernix* Mn-reconstituted SOD was observed at 105°C even after 5 h incubation. After heating at 110°C for 5 h, 56.4% of the initial activity remained. On the other hand, the Fe-reconstituted SOD is less thermostable than the Mn-reconstituted enzyme. The Fe-reconstituted SOD was slightly inactivated at 95°C, which is the optimum growth temperature for *A. pernix*. However, the half-life of its activity at 95°C was found on extrapolation to be 9.9 h, which was significantly longer than the doubling time (3.5 h). These results show that *A. pernix* Mn-SOD is the most thermostable among the known SODs.

Effects of Inhibitors and Hydrogen Peroxide—Sodium azide is known to competitively inhibit all types of SODs. As shown in Fig. 4A, sodium azide caused 50% inhibition of the Fe-reconstituted SOD at 41 mM. This is higher than the corresponding concentrations of *E. coli* Fe-SOD and Mn-SOD, which are 4 and 20 mM, respectively (*28*). As shown in Fig. 4B, the ESR spectrum of the Fe-reconstituted SOD represents high-spin ferric iron with $g=4.26$ at the active site. The shapes and g values of the ESR signals slightly changed on the addition of azide ($g=4.23$). This revealed that azide bound to Fe and slightly changed the coordination of Fe. This suggests that the reaction mechanism of the Fe-reconstituted SOD from *A. pernix* is similar to that of *E.*

TABLE I. **Specific activities and metal contents of reconstituted *A. pernix* SODs.**

Sample	Metal content (atom/subunit)			Specific activity (U/mg)
	Mn	Fe	Ni	
Mn-reconstituted SOD	0.76±0.03	<0.01	–	906±30
Fe-reconstituted SOD	<0.01	0.80±0.02	–	175±4
Ni-containing SOD	<0.01	–	0.61±0.03	0
Apoprotein	0	0	0	0

Data are averages of two to three independent experiments.

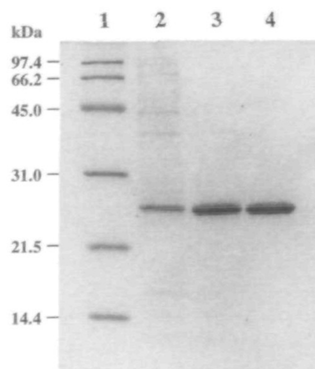

Fig. 2. **SDS-PAGE analysis at various purification steps.** Aliquots of samples at obtained at the various purification steps were analyzed by SDS-PAGE on a 16% gel. Each sample lane contained 5 μg of protein. The gel was stained with Coomassie Brilliant Blue. Lane 1, protein size markers; lane 2, supernatant of a cell lysate after sonication; lane 3, supernatant of a cell lysate after heat treatment (for 30 min at 90°C); lane 4, active fraction from the Q-Sepharose column.

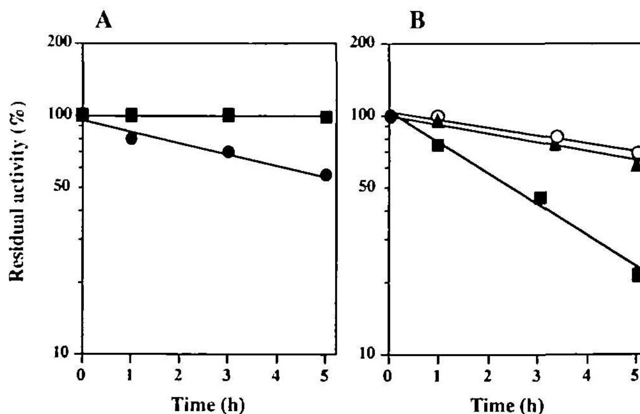

Fig. 3. **Thermal inactivation of the metal-reconstituted SODs from *A. pernix*.** The SODs were incubated in 20 mM potassium phosphate (pH 7.0). Aliquots were removed at intervals and chilled on ice, and then the residual activity was measured. Residual activity is expressed on a logarithmic scale. (A) Mn-reconstituted SOD. Closed squares, 105°C; closed circles, 110°C. (B) Fe-reconstituted SOD. Open circles, 95°C; closed triangles, 100°C; closed squares, 105°C.

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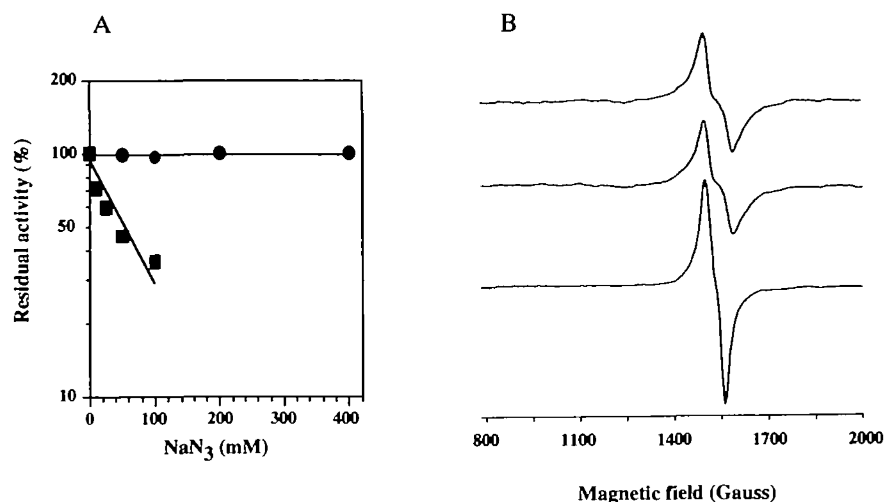

Fig. 4. **Effects of sodium azide on the metal-reconstituted SODs from *A. pernix*.** (A) Inhibition of the metal-reconstituted SODs by sodium azide. SOD activity was assayed in the standard reaction mixture with or without sodium azide. Residual activity is expressed on a logarithmic scale. Closed circles, Mn-reconstituted SOD; closed squares, Fe-reconstituted SOD. (B) ESR spectra of the Fe-reconstituted SOD. The spectra were recorded at 77 K in 12.5 mM Tris/HCl (pH 7.5) with or without sodium azide. The protein concentration was 3.48 mg/ml. Top, 100 mM NaN_3; middle, 50 mM NaN_3; bottom, 0 mM NaN_3.

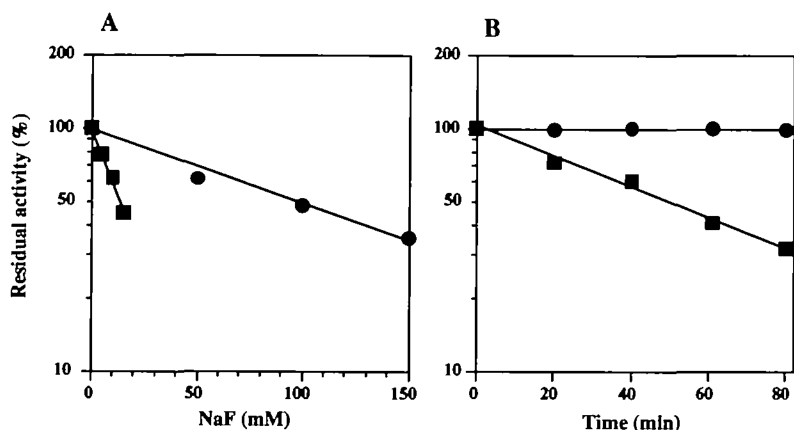

Fig. 5. **Inhibition and inactivation of the metal-reconstituted SODs from *A. pernix*.** (A) Inhibition by sodium fluoride. SOD activity was assayed in the standard reaction mixture with or without sodium azide. Residual activity is expressed on a logarithmic scale. Closed circles, Mn-reconstituted SOD; closed squares, Fe-reconstituted SOD. (B) Inactivation by hydrogen peroxide. SOD (0.25 mg/ml) was incubated with 0.24 mM H_2O_2 in 50 mM potassium phosphate (pH 7.8) at 25°C. At appropriate intervals, aliquots were withdrawn and treated with catalase (1.0 U/μl), and then the residual SOD activity was assayed. Closed circles, Mn-reconstituted SOD; closed squares, Fe-reconstituted SOD.

coli Fe-SOD, in which Fe at the active site alternates between five- and six-coordination during turnover (*29*).

On the other hand, sodium azide did not inhibit the Mn-reconstituted SOD even at concentrations up to 400 mM (Fig. 4A). This is the most resistant SOD to sodium azide among known SODs. In order to clarify the mechanism underlying the insensitivity of the Mn-reconstituted SOD to sodium azide, the effect of sodium fluoride on the SOD activity was studied. The fluoride ion (F^-) of which the ionic radius is smaller than that of N_3^-, binds to the metal at the active site of SOD and is reported to inhibit SODs (*24*). As shown in Fig. 5A, sodium fluoride inhibited both the Mn- and Fe-reconstituted SODs. The concentrations of sodium fluoride causing 50% inhibition of the Mn- and Fe-reconstituted SODs were 89 and 13 mM, respectively (Fig. 5A).

It has been reported that Mn-SOD is not inactivated by hydrogen peroxide but that Fe-SOD is. We examined the effect of hydrogen peroxide on the metal-reconstituted SOD with either Mn or Fe. As shown in Fig. 5B, H_2O_2 inactivated the Fe-reconstituted SOD in a time-dependent manner, but not the Mn-reconstituted enzyme. The incubation time for 50% inactivation of the Fe-reconstituted SOD in the presence of 0.24 mM H_2O_2 was 50 min, which was longer than that for *E. coli* Fe-SOD (*30*). The inactivation curve fitted first order kinetics as in the case of *E. coli* Fe-SOD (*30*). While the Fe-reconstituted SOD was less sensitive to H_2O_2, this result shows that the process of its inactivation is similar to that of *E. coli* Fe-SOD.

DISCUSSION

Several SODs have been isolated from thermophiles or hyperthermophiles: *Sulfolobus* spp.(*10-12*), *Thermoplasma acidophilum* (*31*), *Methanobacterium thermoautotrophicum* (*26*), and *Aquifex pyrophilus* (*13*). Although the optimum temperatures for growth of these strains are 70-87, 59, 65-70, and 85°C, respectively, that of *A. pernix* is 90-95°C, which is the highest among these strains. With the exception of *Sulfolobus* spp, these strains are microaerobic or anaerobic. *A. pernix*, a strict aerobe, seemed to be exposed to stronger oxidative stress than these microaerobes and anaerobes. The high growth temperature and aerophily of *A. pernix* may be factors responsible for the higher thermostability of its SOD. However, no specifically conserved amino acids are observed among thermostable SODs (Fig. 1B).

The Mn-reconstituted SOD from *A. pernix* is extremely resistant to azide, but sensitive to fluoride. The Fe-SOD from *S. solfataricus* was reported to be insensitive to azide (*12*). It was thought that in the case of *S. solfataricus* Fe-SOD there may be some steric hindrance in the sub-

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strate funnel which allows O_2^- and F^- to gain access to the active site but prevents N_3^- doing so, because the molecular size of N_3^- is greater than those of O_2^- and F^- (*12*). Because *A. pernix* SOD forms a homodimer and has four residues postulated to comprise the substrate funnel, it may have a "size-selective" substrate funnel similar to that of *S. solfataricus* Fe-SOD. On the other hand, the Fe-reconstituted SOD from *A. pernix* is more sensitive to azide. In *A. pernix* SOD, replacement of Mn with Fe at the active site may cause slight conformational changes of the SOD which decrease the steric hindrance in the substrate funnel. These conformational changes may also cause a decrease in thermostability.

Amino acid sequence analysis of native and hydrogen peroxide-treated Fe-SODs from *Pseudomonas ovalis* (*32*) and *E. coli* (*30*) has shown that hydrogen peroxide inactivation accompanies the specific loss of tryptophan residues, leaving other amino acid residues substantially unchanged. Beyer and Fridovich (*30*) have proposed that the iron in the active center may induce the disruption of tryptophan residues close to the iron, possibly through a Fenton-type reaction, which generates a strong oxidizing species through the reduction of peroxide by Fe^{2+} (*32*). Multiple alignment of the known amino acid sequences of Fe- and Mn-SODs showed that most of the SODs that are resistant to hydrogen peroxide lack a tryptophan residue at position 73 (numbering based on the *P. shermanii* sequence), which is close to metal-binding His-75 (*33, 34*). This tryptophan residue is not present at the corresponding position, 77, of *A. pernix* SOD, as shown in Fig. 1B. The absence of this tryptophan residue may be the reason why the Fe-reconstituted SOD has a lower H_2O_2 sensitivity.

According to the differences in activity between Mn-forms and Fe-forms, cambialistic SODs can be divided into two groups. The SODs from *Bacteroides fragilis* (*4, 35*), *Propionibacterium shermanii* (*36*), and *Porphyromonas gingivalis* (*7*) show almost the same activity in both the Mn- and Fe-forms. On the other hand, the SODs from *Streptococcus mutans* (*3*), *Methylomonas* strain J (*5*), and *Mycobacterium smegmatis* (*8*) exhibit low activity in the Fe-form and high activity in the Mn-form. The former group are anaerobes and the latter are aerobes or facultative anaerobes. Because the SOD from *A. pernix* exhibits low activity in the Fe-form and high activity in the Mn-form, it apparently belongs to the latter group. Thus, the aerophily of microorganisms is related to the differences in activity between the Mn-forms and Fe-forms of cambialistic SODs. Generally, aerobes and facultative anaerobes contain Mn-SOD and/or Fe-SOD, while anaerobes and microaerobes possess Fe-SOD (*37*). It is likely that microorganisms prefer Mn-SOD to Fe-SOD in aerobic environments (*4, 35, 38, 39*). Aerated cells of *Bacteroides fragilis* and *Porphyromonas gingivalis* preferably produce Mn-forms rather than Fe-forms. The major metal in SOD from aerobically grown *Mycobacterium smegmatis* cells is Mn. The metal contents of the SODs from *Propionibacterium shermanii*, *Streptococcus mutans*, and *Methylomonas* J are directly related to the metal concentration in the medium. While aqueous Fe is usually limited in aerobic environments at neutral pH, manganese may be easily incorporated into cells because of its solubility. Cambialistic SODs are likely to bind Mn in aerobic environments, while they bind Fe in anaerobic environments.

Parker and Blake compared the amino acid sequences of Mn- and Fe-SODs, and found that five amino acid residues are important for the metal specificity (*25*). These residues are boxed in Fig. 1B. In Mn-SODs, they are G, G, F, Q, and D at positions 74, 75, 82, 150, and 151, respectively. In Fe-SODs, they are A, Q, Y, A, and G, respectively. In *A. pernix* SOD, these residues are A, G, F, H, and N, respectively, and thus are different from these consensus residues in these two types of SODs. The corresponding residues in the Fe-SODs from hyperthermophiles, *Sulfolobus* and *Aquifex*, are also different. It is not clear which residues are important for the metal specificity of *A. pernix* SOD. The distribution of cambialistic SODs is not restricted to specific bacterial groups. No specifically conserved amino acids and apparent phylogenetic relationships are observed among cambialistic SODs. The mechanism underlying the metal-substitution in cambialistic SODs remains to be studied.

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