FEBS Letters 406 (1997) 142–146 FEBS 18407

Cloning and expression of superoxide dismutase from *Aquifex pyrophilus*, a hyperthermophilic bacterium

Jae-Hwan Lim[a], Yeon Gyu Yu[a], In-Geol Choi[a], Jae-Ryeon Ryu[a], Byung-Yoon Ahn[b], Sung-Hou Kim[1,a], Ye Sun Han[a,*]

[a]*Structural Biology Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul, Korea*
[b]*Department of Genetic Engineering, Korea University, Seoul, Korea*

Received 14 February 1997

Abstract A superoxide dismutase (*SOD*) gene of *Aquifex pyrophilus*, a marine hyperthermophilic bacterium, was cloned, sequenced, expressed in *Escherichia coli*, and its gene product characterized. This is the first *SOD* from a hyperthermophilic bacterium that has been cloned. It is an iron-containing homo-oligomeric protein with a monomeric molecular mass of 24.2 kDa. The DNA-derived amino acid sequence is more similar to those of known Mn- and Fe-SODs from thermophilic archaea than of Cu, Zn-SODs. The metal binding residues found in all SOD sequences from different species are also conserved in *A. pyrophilus* SOD. The protein is biochemically active only as an oligomer and is resistant to thermal denaturation.

Key words: Hyperthermophile; Superoxide dismutase; Thermostability; *Aquifex*

1. Introduction

There are large populations of microorganisms living in an environment considered to be extreme by man. Most of them are archaebacteria, some are eubacteria, and few are algae and yeast. These organisms, extremophilis, live in habitats which are extreme in terms of temperature, pH, salinity, and/or pressure, conditions under which most normal proteins and nucleic acids will denature [1]. To understand how some microorganisms survive near 100°C, we have focused our initial attention to superoxide dismutase (SOD) from *A. pyrophilus* because biochemical and structural information of SOD from mesophilic and thermophilic organisms are available [2–4].

SOD is one of the fastest enzymes known, and operates near the diffusion limit of its substrate superoxide [5]. SODs from eucaryotes as well as mesophilic microbial sources have been studied extensively and some of their crystal structures are known [6,7]. There are three metal co-factors for SOD that have so far been discovered: copper-zinc, manganese, or iron. In general, copper-zinc SODs are found in eucaryotes, manganese SODs in eucaryotic mitochondria, and eubacteria and archaebacteria contain both manganese and iron SODs [8,9]. SOD is also extensively studied as a potential pharmaceutical against inflammation, autoimmune disease, chromosome breakage and as a general protection agent against oxidative damage of the cell [10,11].

A. pyrophilus [12] is a marine hyperthermophilic bacterium that grows between 67 and 95°C, with an optimum growing temperature of 85°C. *A. pyrophilus* was discovered to live in deep sea vents and classified as one of the most primitive eubacteria. It is rod-shaped with flagella and is a microaerophilic obligatory autotroph. This Gram-negative eubacterium uses hydrogen, sulfur, or sulfide as an electron donor and oxygen or nitrase as an electron acceptor.

Here, we present the cloning, gene structure and expression of an *SOD* gene from *A. pyrophilus*, a hyperthermophilic bacterium, and assess the thermostability of this gene product.

2. Materials and methods

2.1. Materials

A. pyrophilus [13] was obtained from Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH in Germany. Restriction enzymes and T4 DNA ligase were purchased from Promega (Madison, IL) and USB (Cleveland, OH). DNA polymerase, cytochrome *c* and xanthine oxidase were obtained from Boehringer-Mannheim (Mannheim, Germany). Phage packaging kit and λ DashII vector were obtained from Stratagene (CA, USA). Taq DyeDeoxy Terminator Cycle Sequencing Kit was from Perkin Elmer (CA, USA). *E. coli* Fe-, Mn-SOD, *Bacillus stearothermophilus* Mn-SOD and other chemicals were purchased from Sigma (Mo, USA).

2.2. Construction of plasmid and genome library and DNA sequencing

A plasmid library and a genomic λ library were constructed from *A. pyrophilus* DNA using pBluescript KS(+) plasmid, and λ DASHII from Stratagene (CA, USA), respectively.

About 200 genomic DNA clones were sequenced by the chain termination method [14], using Taq Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer) and a model 373A ABI automated sequencer. The BLAST (Basic Local Aliqnment Search Tool) programs were used to identify homologs to each query sequence [15]. One of the clones showed high sequence homology to SOD and it was used as the probe for cloning the intact *SOD* gene.

2.3. Cloning of the SOD gene

By using the clone containing an *SOD* gene fragment from the *A. pyrophilus* plasmid library, PCR amplification was performed to make an *SOD* gene probe. A genomic library was plated on *E. coli* MRA-P2. Plaque hybridization screening was done using ECL direct system (Amersham, OH, USA). Prehybridization and hybridization were done at 42°C for 1 and 4 h, respectively, in hybridization solution. Positive plaques were pooled in SM buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin), replated at low density, and a single plaque was isolated. Phage DNA was isolated using Qiagen Lambda Kit. Phage DNA digested with *Hin*dIII, *Eco*RI, and *Sac*I were identified by Southern hybridization with the PCR-amplified fragment as a probe. The phage DNA(φ12) digested with *Sac*I was ligated with pBluescript KS(+) and transformation of *E. coli* DH5α was carried out according to the procedure by Sambrook [16]. The positive clones were tested by PCR to yield a final clone, pD2.

*Corresponding author. Fax (82-2) 958-5939

[1]As advisory director to Structural Biology Center, KIST, Seoul, Korea; Address: Department of Chemistry and Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720, USA.

PII S0014-5793(97)00262-7

The pD2 clone which had the *SOD* gene was digested with *Sac*I, *Eco*RV, *Pst*I and *Bam*HI. These fragments were subcloned into pBluescript KS(+) for DNA sequencing.

2.4. Protein expression and purification

Two PCR primers based on DNA sequence of the insert of the pD2 clone were synthesized by Bio-synthesis Inc. (Lewisville, TX):

sodF 5′-GCCTCACGTTCACCATGGGTGTGCACAAACTG-GAACCCAAAG-3′
sodR 5′-GCTAGGCATGTCGGACTTTTACTTGAT-GAAGTCCTTGAGGGC-3′

The expression construct pd27 was obtained by ligating PCR-amplified fragment digested with *Nco*I and *Bam*HI to the similarly digested pET3d vector (Novagen, Inc., WI, USA). The ligation mix was used to transform *E. coli* BL21(DE3). Expression of the *SOD* gene was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside to cells in logarithmic phase which were grown in Luria-Bertani media.

The cell pellet underwent three cycles of freeze and thaw before resuspension in lysis buffer (20 mM potassium phosphate, 20 mM NaCl, 0.1 mM phenylmethylsufonyl fluoride, pH 7.0). The cells were sonicated (Branson model 450) 5 times in 1 min bursts and placed in an ice bath. The lysate was brought to 1 mM $MgCl_2$ and DNase I at a final concentration of 20 μg/ml was added. The lysate was then incubated for 30 min in an ice bath.

The lysate was centrifuged at 25000×*g* for 1 h and the soluble extract was heated at 80°C for 1 h. The supernatant after centrifugation at 25000×*g* for 30 min was applied to a Q-Sepharose Fast Flow (Pharmacia) column. The gradient was from 0.02–0.6 M NaCl in 20 mM potassium phosphate, pH 7.0. The SOD-containing fractions were concentrated with a Centriprep 30 concentrator (Amicon, MA, USA) and applied through gel filtration chromatography (Sephacryl S-200, Pharmacia, Uppsala, Sweden) with 50 mM HEPES buffer (pH 7.0) containing 50 mM NaCl.

2.5. Metal analysis

The metal content of the purified SOD was determined with an atomic absorption spectrophotometer (Varian SpectrAA800) at 248.3 nm for iron detection and 279.5 nm for manganese.

2.5.1. Preparation of apoenzyme. A ferrous ion-free apoprotein was prepared by treatment with 10 mM EDTA in 8 M urea/50 mM acetate buffer, pH 3.8, for up to 16 h at 25°C [17]. The sample was dialysed against 8 M urea/50 mM acetate buffer, pH 3.8, once and 50 mM phosphate buffer, pH 7.0, twice with and without 8 M urea. Gel filtration was performed through a desalting column (FPLC, Pharmacia) in 20 mM phosphate buffer, pH 7.0.

2.5.2. Reconstitution of active Fe-enzyme. The apoprotein was dialysed four times successively for 4 h at 4°C against; (1) 8 M urea/50 mM acetate buffer/10 mM $FeSO_4$, pH 3.8, (2) 8 M urea/50 mM phosphate buffer/10 mM $FeSO_4$, pH 3.8, (3) 50 mM phosphate buffer/1 mM $FeSO_4$, pH 7.0, (4) 50 mM phosphate buffer/0.5 mM EDTA, pH 7.0.

After dialysis, the sample was concentrated to 1 mg/ml with a Centricon miniconcentrator and the extent of reconstitution was monitored by atomic absorption spectrophotometer (Varian SpectrAA800) at 248.3 nm for iron detection.

2.5.3. Reconstitution of Mn-enzyme. The apoprotein was dialysed successively for 4 h at 4°C against: (1) 8 M urea/50 mM acetate buffer/10 mM $MnSO_4$, pH 3.8, (2) 8 M urea/50 mM phosphate buffer/10 mM $MnSO_4$, pH 7.0, (3) 6 M urea/50 mM phosphate buffer/10 mM $MnSO_4$, pH 7.0, (4) 3 M urea/50 mM phosphate buffer/10 mM $MnSO_4$, pH 7.0), (5) 50 mM phosphate buffer/1 mM $MnSO_4$, pH 7.0, (6) 50 mM phosphate buffer/0.5 mM EDTA, pH 7.0.

After dialysis, the sample was concentrated to 1 mg/ml with a Centricon miniconcentrator and the extent of reconstitution was monitored by atomic absorption spectrophotometer (Varian SpectrAA800) at 279.5 nm for manganese detection.

2.6. Enzyme assays

Protein concentrations were measured by the method of Bradford [18], using Bio-Rad protein assay kit. A qualitative test for SOD activity was done using nitro blue tetrazolium (NBT) staining method [19]. The color reaction of the NBT test is a fast and sensitive test for monitoring SOD in polyacrylamide gels. Superoxide radical reduces the colorless nitro blue tetrazolium to a blue formazan. For the inhibition experiments, nondenaturating polyacrylamide gel was presoaked for 1 h in 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM KCN or 40 mM H_2O_2 [19].

SOD activity was measured by cytochrome *c* reduction [8,20]. SOD activity unit is defined as the amount of enzyme which inhibits the rate of cytochrome *c* reduction by 50%

3. Results

3.1. Structure of A. pyrophilus SOD gene

The nucleotide sequence of the cloned genomic DNA was determined as shown in Fig. 1. We identified an open reading frame (ORF) of 639 bases which had an ATG initiation codon at position 1013 and a TAA stop codon at position 1652. The polypurine-rich sequence of GAAAAGGAGG beginning 18 bases upstream from the initiation codon is a potential Shine-Dalgarno ribosome-binding site [21]. The putative promoter sequence of TATAAA was found 70 bases upstream of the initiation codon [22].

Transcriptional regulation studies of the *sodA* gene encoding the manganese superoxide dismutase of *E. coli* suggest that the induction of the *SOD* gene may be mediated via a regulatory element near its promoter [23,24]. In the *E. coli sodA* gene, there is a 19 base palindrome at the −35 region which could be a regulatory site. In the upstream region of *A. pyrophilus* SOD, a palindromic sequence consisting of AAAAGAA (TTTTCTT) stem and a loop of TCCT which is located at the same position and is of similar size as that found in *E. coli sodA* gene, could be a binding site for a regulatory protein.

Sequence of *A. pyrophilus* Fe-SOD was compared with those of archaebacterial SODs, eubacterial Mn-SODs and Fe-SODs (not shown). Four residues involved in metal ligand binding which are known to be conserved in all SOD sequences from many species were found: His^{28}, His^{82}, Asp^{164} and His^{168}. The SOD sequence of *A. pyrophilus* showed high homology to that of *Methanobacterium thermoautotrophicum* Mn-SOD (54%), *Sulfolobus acidocaldarius* Mn-SOD (56.3%), *Thermus thermophilus* Mn-SOD (54.6%), and *E. coli* Fe-SOD (49.3%).

3.2. Expression and purification of A. pyrophilus SOD

E. coli BL21 (DE3) was transformed with pD27, a pET3d plasmid, harboring the *A. pyrophilus SOD* gene. After applying the crude lysate onto Q-Sepharose Fast Flow and gel filtration column chromatography, purified protein was subjected to electrophoresis on 16.5% Tricine SDS-polyacrylamide gel [25] and two protein bands with apparent molecular mass of 24 kDa and 96 kDa were observed. The protein band with a molecular mass of 24 kDa was consistent with the molecular mass of 24344 Da calculated from the amino acid sequence derived from the gene. The N-terminal amino acid sequence of the protein with the apparent molecular mass of 96 kDa corresponds to the deduced amino acid sequence (data not shown). This suggests that *A. pyrophilus* SOD exists as a homotetramer which is consistent with the crystal structure (manuscript submitted) and with the activity assay performed in situ on polyacrylamide gel.

The estimation of molecular mass of SOD was carried out by gel-filtration on a column of Sephacryl S-200. The

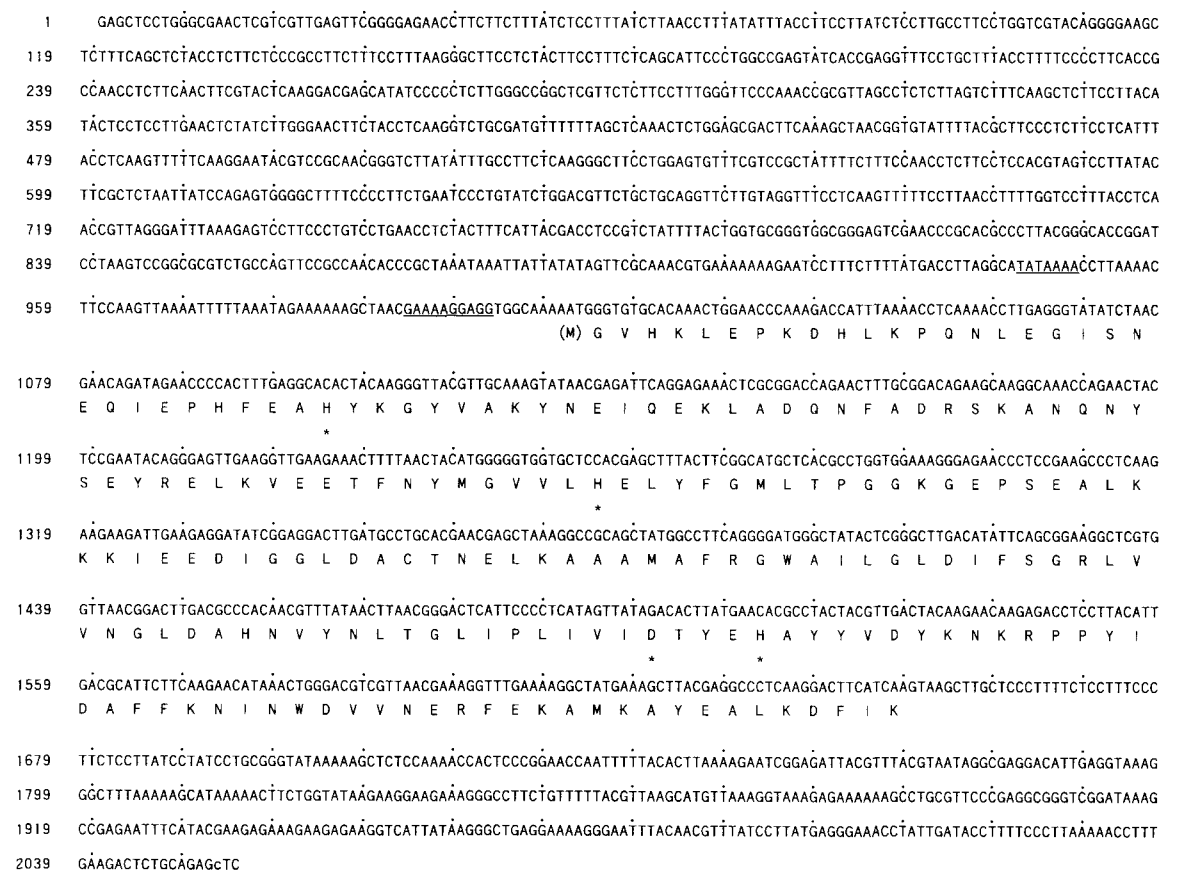

Fig. 1. Gene sequence and deduced amino acid sequence of *A. pyrophylus* SOD. The structural gene for SOD begins with an ATG start condon at position 1013 and ends with a TAA stop condon at position 1652. In the upstream region of SOD, a putative ribosomal binding site and promoter sequence are underlined. Residues identified by an * are likely to be metal ligands based on the crystal structures of highly homologous SODs from *Thermus thermophilus*.

apparent molecular mass was determined to be about 89 000 Da.

3.3. Metal content

The purified enzyme was subjected to metal analysis by atomic absorption spectroscopy to identify the metal type. The purified SOD contains 0.75 atom of iron per subunit and negligible amounts of manganese and copper.

Reconstitution of Fe- and Mn-SOD was achieved by addition of Fe^{2+} and Mn^{2+} to apoprotein in 8 M urea at acid pH to identify metal type of *A. pyrophilus* SOD. Reconstituted Fe-SOD contained 0.85 atom of iron per subunit and gave a specific activity of 1500 U/mg. The specific activity of Mn-SOD which contained 0.82 atom of manganese per subunit was 80 U/mg at 25°C. Another reconstituted Fe-SOD which has 0.165 of iron atom per subunit had a specific activity of 530 U/mg. The specific activity of Fe-SOD depended on the reconstituted iron amount per subunit. Therefore, *A. pyrophilus* SOD appears to be a Fe-SOD.

X-ray crystallography of *A. pyrophilus* SOD shows that each subunit has a metal binding site (manuscript submitted). The purified SOD which contained 0.75 atom of iron per subunit indicated that some of iron was lost during the purifications of SOD.

3.4. SOD activity assays: NBT test

Two inhibitors, KCN and H_2O_2, were added to the staining solution to differentiate the types of SOD (Fig. 2). SOD inhibits color formation by scavenging O_2^- and appears as a colorless spot. Cyanide is a specific inhibitor of Cu, Zn-SOD and H_2O_2 inactivates Fe-SOD. *A. pyrophilus* SOD was not inhibited by KCN, but was inhibited by H_2O_2, confirming that it is an Fe-SOD. The *E. coli* Fe-SOD and Mn-SOD on nondenaturing gel electrophoresis was easily differentiated from each other by the different migration behavior [26].

Fe-free apoprotein was subjected to electrophoresis on a 12% Tricine SDS-polyacrylamide gel (Fig. 3). Fe-free apoprotein exists as an inactive monomer (Fig. 3, lane 1) and reconstituted Fe-SOD has two forms: active tetramer and inactive monomer (Fig. 3, lanes 2 and 3). The protein with no heat treatment (Fig. 3, lane 3) shows the same bands as the protein heated at 95°C for 10 min (Fig. 3, lane 2).

Denaturation of the purified enzyme with different concentrations of urea (2, 4, 6 and 8 M) indicates that SOD forms an oligomeric protein conformation. NBT staining showed that oligomeric proteins are active and monomers are inactive.

3.5. Cytochrome c reduction assay

When measured by the cytochrome *c* reduction assay, *A. pyrophilus* SOD had a specific activity of 1400 U/mg at 25°C. This is comparable to 1623 U/mg for *E. coli* Fe-SOD and 1387 U/mg for *B. stearothermophilus* Mn-SOD. Thus, at room temperature, the activity of *A. pyrophilus* SOD is similar to the other SODs of mesophiles and thermophiles. *A. pyro-*

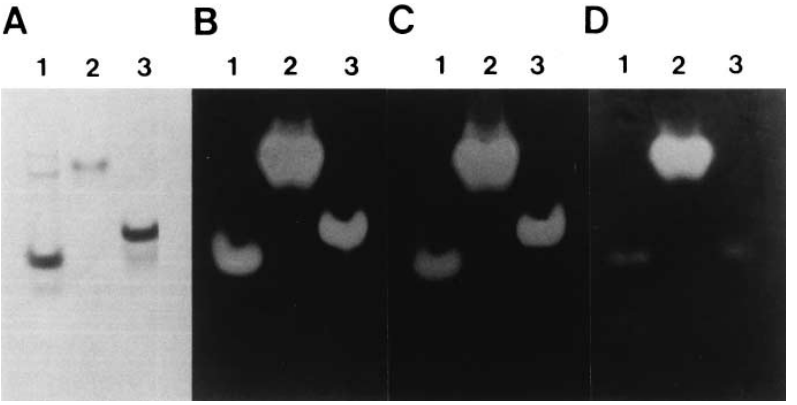

Fig. 2. Nondenaturating gel electrophoresis of *A. pyrophilus* SOD activity. Gel (A) was stained by Coomassie Brilliant Blue and gels (B–D) were stained with NBT (see Section 2) after presoaking in 20 mM potassium phosphate, pH 7.0 (B), or 20 mM potassium phosphate, pH 7.0, containing 10 mM KCN (C) or 20 mM potassium phosphate, pH 7.0, containing 40 mM H_2O_2 (D) for 1 h. SOD activity is represented by a decolorized zone ageinst a dark background. Lane 1, *E. coli* Fe-SOD; lane 2, *E. coli* Mn-SOD; lane 3, *A. pyrophilus* SOD.

philus SOD retained 80% of initial activity after heating to 95°C for 2 h (Fig. 4) when assayed by the cytochrome *c* reduction method *E. coli* Fe-SOD and *B. stearothermophilus* Mn-SOD lost their activity almost immediately upon 95°C heating. This confirms the remarkable thermostability of *A. pyrophilus* SOD. When measured by the cytochrome *c* reduction method at room temperature, no loss of activity was observed in the pH 4.0–10.0 range.

4. Discussion

A. pyrophilus SOD is the first SOD of hyperthermophilic bacteria cloned and expressed in *E. coli*. This enzyme is an iron-containing homo-oligomeric protein with a monomeric molecular mass of 24.2 kDa.

Many studies show that metal plays a very important role both for catalytic activity and for stability of SOD [27,28]. Fe-reconstituted *A. pyrophilus* SOD remains as an active tetramer after heating at 95°C for 10 min (Fig. 3). But Fe-free apoenzyme exists as an inactive monomer under the same condition on a 12% Tricine SDS-polyacrylamide gel. This conforms that metal binding could effect not only activity but also stability.

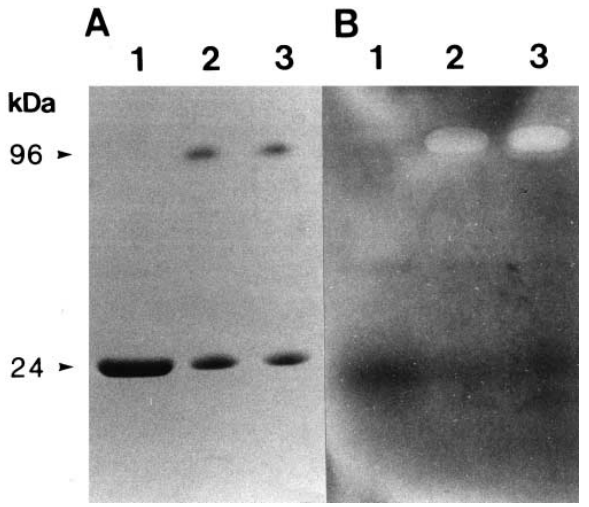

Fig. 3. Fe-free *A. pyrophilus* SOD and Fe reconstituted active protein on Tricine-SDS polyacrylamide gel. A: Stained with Coomassie Brilliant Blue. B: Stained with NBT. M, molecular size marker; lane 1, Fe-free *A. pyrophilus* SOD (50 μg); lane 2, Fe-reconstituted active protein (20 μg) was heated at 95°C for 10 min before running the gel; lane 3, Fe-reconstituted active protein (20 μg), not heated.

Denaturation of the enzyme by different concentrations of urea shows that oligomeric conformations have activity but the monomer lose all activity. The activity between different oligomeric forms were almost the same. The secondary structure of *A. pyrophilus* SOD denatured with the different concentrations of Urea were analyzed by Circular Dichroism (CD) spectroscopy (data not shown). There were no changes of CD spectra between oligomeric forms.

The SOD of *A. pyrophilus* is extremely stable to heat compared with all other known SOD enzyme. The activity of *A. pyrophilus* SOD was reduced only by 20% after incubation at 95°C for up to 2 h (Fig. 4).

The amino acid composition of *A. pyrophilus* was compared with those of *M. thermoautotrophicum*, *S. acidocaldarius*, *Th. aquaticus*, and *E. coli*. The number of charged residues — aspartic acid, glutamic acid and lysine — of *A. pyrophilus* SOD were almost twice that of *E. coli* SOD. Many charged residues were also found in thermophilic SODs, *M. thermoautotrophicum* and *Th. aquaticus*, compared to *E. coli* SOD. This is consistent with the earlier observations [29,30] that large networks of ion-pairs found in hyperthermophilic glutamate dehydrogenase and indol-3-glycerolphosphate synthase.

There might be several other factors to contribute in hyperthermophilic protein besides the increase number of ion-pairs, for example, hydrogen bonds or hydrophobicities [31,32]. The X-ray crystal structure of *A. pyrophilus* SOD shows that tetrameric SOD had dramatically increased buried surface area per monomer compared to other SODs, *Thermus thermophilus*, *Human mitochondria*, *Mycobacteria tuberculosis* and *Escherichia coli* (manuscript submitted). The increased buried surface area results the formation of hydrogen bonds and hydrophobic interactions in tetramer of *A. pyrophilus* SOD, and this could be another factor of hyperthermostability.

Acknowledgements: We thank Dr. Rosalind Kim for her advise and participation in the preparation of the manuscript. We gratefully acknowledge the support of Korea Institute of Science and Technology

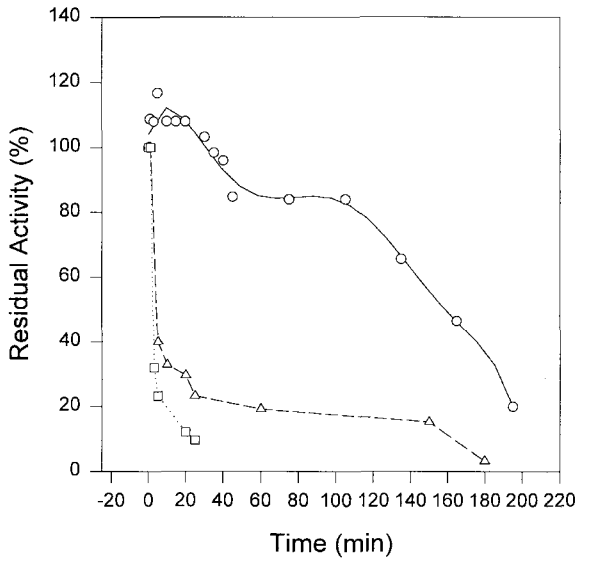

Fig. 4. Thermal inactivation of *A. pyrophilus* SOD (○), *E. coli* Fe-SOD (□) and *B. stearothermophilus* SOD (△). Reaction mixtures contained 1 μg/μl SOD in 20 mM potassium phosphate, pH 7.0, and incubated at 95°C. Aliquots were removed at intervals, chilled in an ice bath, and assayed for residual SOD activity.

and a grant from Ministry of Science and Technology, Korea. The work of S.-H. Kim was supported by US Department of Energy (DOE-AC03-76S00098).

References

[1] M.W.W. Adams, Annu Rev Microbiol 47 (1993) 627–658.
[2] W.S. Campbell, D.E. Laudenbach, J Bacteriol 177 (1995) 964–972.
[3] A. Carlioz, M.L. Ludwig, W.C. Stallings, J.A. Fee, H.M. Steinman, D. Touati, J Biol Chem 263 (1988) 1555–1562.
[4] M. Takao, A. Yasui, A. Oikawa, J Biol Chem 266 (1991) 14151–14154.
[5] D. Klug, J. Rabani, I. Fridovich, J Biol Chem 247 (1972) 4839–4842.
[6] J.B. Cooper, K. Mcintyre, M.O. Badasso, S.P. Wood, Y. Zang, T.R. Garbe, D. Young, J Mol Biol 246 (1995) 531–544.
[7] M.L. Ludwig, A.L. Metzger, K.A. Pattridge, W.C. Stallings, J Mol Biol 219 (1991) 335–358.
[8] I. Fridovich, Adv Enzymol 58 (1986) 61–87.
[9] I. Fridovich, Annu Rev Biochem 64 (1995) 97–112.
[10] I. Fridovich, Annu Rev Pharmacol Toxicol 23 (1983) 239–257.
[11] J.M. McCord, B.B. Keele Jr., I. Fridovich, Proc Natl Acad Sci USA 68 (1971) 1024–1027.
[12] R. Huber, T. Wilharm, D. Huber, A. Tricone, S. Burggraf, H. Koning, R. Rachel, I. Rockinger, H. Fricke, K.O. Stetter, Syst Appl Microbiol 15 (1992) 349–351.
[13] W.E. Balch, G.E. Fox, L.J. Magrum, C.R. Woese, R.S. Wolfe, Microbiol Rev 43 (1979) 260–296.
[14] F. Sanger, S. Nicklen, A.R. Coulson, Proc Natl Acad Sci USA 74 (1977) 5463–5467.
[15] K. Samuel, S.F. Altschul, Proc Natl Acad Sci USA 90 (1993) 5873–5877.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, vol. 1, pp. 1-110. 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[17] S. Sato, J.I. Harris, Eur J Biochem 73 (1977) 373–381.
[18] M. Bradford, Anal Biochem 72 (1976) 248–254.
[19] C. Beauchamp, I. Fridovich, Anal Biochem 44 (1971) 276–287.
[20] J.M. McCord, I. Fridovich, J Biol Chem 244 (1969) 6049–6055.
[21] L. Gold, D. Pribnow, T. Schneider, S. Shinedling, B. Singer, G. Stromo, Annu Rev Microbiol 35 (1981) 365–407.
[22] W. Zillig, P. Palm, W.D. Reiter, F. Gropp, G. Puhler, H.P. Klenk, Eur J Biochem 173 (1988) 473–482.
[23] H.M. Hassan, H.C.H. Sun, Proc Natl Acad Sci USA 89 (1992) 3217–3221.
[24] Y. Takeda, H. Avila, Nucl Acids Res 14 (1986) 4577–4591.
[25] H. Schagger, G. Von Jagow, Anal Biochem 166 (1987) 368–379.
[26] C. Spiegelhalder, B. Gerstenecker, A. Kersten, E. Schiltz, M. Kist, Infect Immun 61 (1993) 5315–5325.
[27] M.L. Ludwig, A.L. Metztger, K.A. Pattridge, W.C. Stallings, J Mol Biol 219 (1991) 335–358.
[28] Fee, J.A. (1980) Metal ion activation of dioxygen in: (Spiro, T.G., ed.), Superoxide, Superoxide Dimutases and Oxygen Toxicity, pp. 209-237, John Wiley, New York.
[29] M. Hennig, B. Darimont, R. Sterner, K. Kirschner, J.N. Jansonius, Structure 3 (1995) 1295–1306.
[30] K.S.P. Yip, et al. Structure 3 (1995) 1147–1158.
[31] L.F. Delboni, et al. Prot Sci 4 (1995) 2594–2604.
[32] R.J.M. Russel, D.W. Hough, M.J. Danson, G.L. Taylor, Structure 2 (1994) 1157–1167.