ISOLATION, RECONSTITUTION, AND MOLECULAR CLONING OF THE MANGANESE-CONTAINING SUPEROXIDE DISMUTASE FROM DEINOCOCUS RADIODURANS

by

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(ABSTRACT)

The superoxide dismutase from a radiation-resistant bacterium *Deinococcus radiodurans* has been purified to electrophoretic homogeneity. The superoxide dismutase has a specific activity of 3300 units/mg and an apparent molecular mass of 43,000 daltons. The enzyme contains 1.5 gram-atom of manganese per mol dimer, and is composed of two identical subunits of 23,500 daltons. The enzyme rapidly loses its catalytic activity and metal content upon dialysis in denaturing reagent, guanidine hydrochloride, and the metal ion chelator 8-hydroxyquinoline. The denatured apoprotein was renatured upon removal of the denaturant by dialysis. The renatured apoprotein assumed a gross conformation similar to the native enzyme as indicated by fluorescence spectroscopy. The renatured apoprotein was reconstituted to the native specific activity upon addition of manganese in the absence of denaturant. The manganese reconstituted enzyme contained 1.7 gram-atom of manganese per mol dimer, and had a specific
activity of 3650 units/mg. Kinetic studies revealed that the reconstitution with manganese was pH-dependent, and was inhibited by competing metal ions (iron and zinc).

The nucleotide and peptide sequences of this manganese-containing superoxide dismutase were determined by molecular cloning of the structural gene. The polymerase chain reaction (PCR) was employed for the amplification of the target superoxide dismutase gene, and two degenerate oligonucleotide primers were used. Primer SOD1 was deduced from the N-terminal peptide sequence of the D. radiodurans SOD; and primer SOD2 was deduced from the peptide sequence homologous in all of the superoxide dismutases. The amplified PCR fragment, 500 bases in length, was subcloned and sequenced, and the deduced peptide sequence confirmed the presence of the D. radiodurans superoxide dismutase coding sequence. The full length superoxide dismutase clone was obtained by probing the lambda EMBL3 genomic library with the radiolabeled PCR fragment. The sequence determination of the full length D. radiodurans superoxide dismutase clone identified an open reading frame of 630 nucleotides which encoded a peptide having a molecular mass of 23,347. The deduced peptide sequence shared 70% identity with that of the E. coli MnSOD. The sequence that could potentially form a hair-pin structure was observed at the 3’-untranslated region of the gene. This structure may be involved in the stabilization of the messenger RNA.
To Guojun and Valerie
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List of Abbreviation

A absorbance
ACES N-[2-acetamido]-2-aminoethanesulfonic acid
ATP adenosine 5′-triphosphate
bp basepair
BSA bovine serum albumin
Cu/ZnSOD copper-zinc superoxide dismutase
DAB diaminobenzidine
dATP deoxyadenosine 5′-triphosphate
dCTP deoxycytidine 5′-triphosphate
dGTP deoxyguanosine 5′-triphosphate
dITP deoxyinosine 5′-triphosphate
dTTP deoxythymidine 5′-triphosphate
DMSO dimethylsulfoxide
DNA deoxyoligonucleic acid
D. radiodurans Deinococcus radiodurans
E. coli Escherichia coli
EDTA ethylenediamine tetraacetic acid
FeSOD iron-containing superoxide dismutase
FPLC fast protein liquid chromatography
HEPES 2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HRPO horseradish peroxidase
IEF isoelectric focusing
IPTG isopropyl β-thiogalactopyranoside

x
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Kb</td>
<td>kilobasepair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese-containing superoxide dismutase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethyleneglycol</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indoly1-β-D-galactoside</td>
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Chapter 1.

Literature Review
Superoxide Radical

All aerobic cells utilize molecular oxygen as the terminal electron acceptor in respiration to gain energetic advantages over fermentation and respiratory pathways that rely on other oxidants. The bulk of oxygen reduction in respiring cells is mediated by cytochrome oxidase that transfers four electrons, one at a time, to molecular oxygen without the release of partially reduced intermediates. However, the presence of intracellular oxygen also allows formation of partially reduced oxygen metabolites that may damage critical biomolecules. Partially reduced oxygen intermediates implicated in the cellular damages include superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH·). These metabolites may be generated through sequential univalent reductions:

\[
\begin{align*}
\text{(1) } & \quad O_2 + e^- \quad \text{-----} \quad O_2^- \quad \text{<-----} \quad H^+ & \quad \text{HO}_2^- \\
\text{(2) } & \quad O_2^- + e^- + 2H^+ \quad \text{-----} \quad H_2O_2 \\
\text{(3) } & \quad H_2O_2 + e^- \quad \text{-----} \quad OH^- + OH· \\
\text{(4) } & \quad OH^- + OH· + e^- + 2H^+ \quad \text{-----} \quad 2H_2O
\end{align*}
\]

Superoxide radical, an oxygen molecule with one unpaired electron, is the first species generated on this univalent reduction pathway. Superoxide radical can be protonated to form hydroperoxyl radical (HO$_2$·) which has a pKa of 4.7 (reaction 1). Second electron reduction of the molecular oxygen generates hydrogen peroxide (reaction 2) that can be
further reduced by the third electron to form hydroxyl anion plus hydroxyl radical (reaction 3). Addition of the fourth electron will then form water (reaction 4).

Formation of Superoxide Radical in Biological Systems

Superoxide radical generated in the cells is a consequence of single electron reduction of oxygen by either enzymatic or nonenzymatic reactions. The distribution and activity of the enzymatic pathways varies with different cell types depending on their specialized function. For example, phagocytic cells, including polymorphonuclear leukocytes (PMNL), can be stimulated to generate and release free radicals to kill engulfed bacteria (respiratory burst phenomenon). The enzyme responsible for the respiratory burst in PMNL is NADPH oxidase, where NADPH is the physiological electron donor for the reaction (1, 2).

\[
\text{NADPH} + 2\text{O}_2 \longrightarrow \text{NADP}^+ + 2\text{O}_2^- + \text{H}^+
\]

NADPH oxidase is present in the membrane of phagocytic cells and has the superoxide generation site on the exterior of the membrane. However, under normal circumstance, superoxide radicals are released into the phagocytic vacuoles. This enzyme has been shown to be present only in phagocytic cells and therefore does not contribute to the intracellular oxidant load in most cells.
Xanthine oxidase has a relatively widespread cellular distribution and contains iron-sulfur centers, FAD (flavin adenine dinucleotide) and molybdenum. It can utilize molecular oxygen as an efficient oxidizing substrate for the production of superoxide radicals (3).

Other enzymes that form detectable amounts of superoxide radicals inside the cells include diamine oxidase (4), and some peroxidases (5, 6).

Superoxide is also generated nonenzymatically through autoxidation of cellular components by molecular oxygen. A major source of superoxide radical production in the intact cells appear to be related to the autoxidation of chemically reactive components produced during reductive processes. The reductive processes are associated with the mitochondrial and microsomal electron transport systems (7). The transport of electrons from substrate through the mitochondrial respiratory chain involves the sequential reduction of flavoproteins, ubiquinones, and mitochondrial cytochromes. The reduced semiquinones or flavins are largely reoxidized by the cytochrome b complex, but they may also reduce molecular oxygen to generate superoxide radicals (8). However, the reduced semiquinones represent the largest potential source for the generation of free radicals.

The electron transport system of endoplasmic reticulum (cytochrome P450-linked) also generates superoxide radicals,
and this pathway is present in a wide spectrum of bacterial and mammalian cell types (9). Normally, electron transport in this pathway involves transfer of an electron from reduced flavin to the cytochrome P450-substrate complex. A second electron is then transferred through this complex to molecular oxygen. Production of superoxide may occur through autoxidation of the partially reduced flavin cofactor or because of "leakage" of electrons from the enzyme-substrate complex to molecular oxygen.

Superoxide formation by autoxidations of thiols (10), epinephrine (11), hydropterins (12), flavins (13), and hemoglobin (14) have also been demonstrated in mammalian systems. Some of these biologically important compounds, such as the epinephrine, are found extracellularly as well as intracellularly and thus contribute to superoxide production at both sites.

The above considerations indicate that superoxide radicals can be produced at a variety of subcellular sites. These include the mitochondria (flavoprotein and ubiquinone), endoplasmic reticulum (cytochrome P450), plasma membrane and phagosomes (NADPH oxidase), and cytoplasm (xanthine oxidase, soluble components).

Properties and Reactivity of Superoxide Radical

Superoxide radical appears to be one of the major causes
of dioxygen toxicity because of its reactivity with several enzymes and biomolecules. Superoxide can directly oxidize biologically active small molecules such as epinephrine, bilirubin, α-tocopherol, and leucoflavins (15). It also inactivates catalase (16), glutathione peroxidase (17), dihydroxy isovalerate dehydratase (18) and aconitase (134). Superoxide radical is also implicated in the dioxygen-dependent toxicities mediated by viologens, quinones, dyes and nitroaromatic compounds which, by intracellular reduction followed by autoxidation, cause increased production of superoxide radicals (19).

Superoxide can be protonated under acidic condition to form hydroperoxyl radical (HO₂⁻) whose pKa is 4.7. Hydroperoxyl radical is a potent oxidant and can directly initiate peroxidation of polyunsaturated fatty acids (20). Superoxide and hydroperoxyl radical can also participate in spontaneous dismutation processes (21):

\[
\begin{align*}
\text{HO}_2^- + \text{HO}_2^- & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad k \approx 1 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \\
\text{HO}_2^- + \text{O}_2^- + \text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad k \approx 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \\
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad k < 0.3 \text{ M}^{-1}\text{s}^{-1}
\end{align*}
\]

The rate constants of the above reactions and the pKa of hydroperoxyl radical indicate that the spontaneous dismutation will be most rapid at pH 4.7 and will decrease progressively as the pH is raised to physiological pH value.
The majority of superoxide is scavenged enzymatically by superoxide dismutase (SOD) that disproportionates superoxide to hydrogen peroxide plus oxygen, and regulates superoxide concentration at $10^{-12}$ to $10^{-11}$ M (22).

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The dismutation of superoxide radicals by SOD occurs sufficiently rapidly to be of biological significance, which has a rate constant of $2 \times 10^9$ M$^{-1}$s$^{-1}$.

**Hydrogen Peroxide and Hydroxyl Radical**

Hydrogen peroxide is another important endogenous oxidant in aerobic systems, which is generated as the product of the SOD reactions in the cytosol and mitochondria. Hydrogen peroxide can also be generated nonenzymatically from the spontaneous dismutation of superoxide, or from the decomposition of peroxycytochrome P450 within the microsomal cytochrome P450 system, or from the reduction of hydroperoxyl radical by glutathione (GSH) (23). Eukaryotic peroxisomes, where several superoxide-generating enzymes reside, account for most of the hydrogen peroxide formation inside the cells. Within peroxisomes, catalase catalyzes the oxidation-reduction reaction of hydrogen peroxide to produce oxygen and water, and regulates hydrogen peroxide concentration at $10^{-9}$ to $10^{-8}$ M (22). Peroxidases also catalyze the decomposition of hydrogen
peroxide. Peroxidases prefer alkyl amines, ascorbate or glutathione as electron donors. Catalase and peroxidase activities are ubiquitous in animals, plants, and aerobic microorganisms, where they undoubtedly function physiologically to remove toxic hydrogen peroxide generated by the action of various flavoprotein oxidases indigenous to the organism.

However, hydrogen peroxide can also undergo other reactions that generate biologically active products. Within phagocytic cells, hydrogen peroxide is the substrate of myeloperoxidase (MPO) that catalyzes the reaction of hydrogen peroxide with halide (Cl\(^-\)) to form hypohalous acids (HOCl) and water (24). The hypohalous acids in addition to superoxide, hydrogen peroxide and hydroxyl radical participate in the bombardment of the engulfed microbes in phagolysosomes.

In the presence of transition metals (Fe, Cu), hydrogen peroxide can undergo a metal-catalyzed Haber-Weiss reaction, where the metal acts as a redox catalyst, and superoxide replenishes the pool of reduced metal ions (19). The result of the Haber-Weiss cycle is the ample production of a powerful oxidant: hydroxyl radical (OH\(^•\)).

\[
\begin{align*}
\text{Fe(II)} + H_2O_2 & \rightarrow \text{Fe(III)} + OH^- + OH^• \\
O_2^- + \text{Fe(III)} & \rightarrow O_2 + \text{Fe(II)}
\end{align*}
\]

Hydroxyl radical is sufficiently reactive and unstable
that it oxidizes a target within three to five molecular radii of formation site (25). However, DNA and cell membranes are polyanionic structures to which metal cations adhere. They are therefore the sites at which metal catalyzed hydroxyl radical production could occur (26, 27). Hydroxyl radicals may attack DNA at either the sugar or the base, which ultimately lead to sugar fragmentation, base loss, and strand breakage with a terminal fragmented sugar residue. Membrane damages from hydroxyl radicals are observed as accumulation of lipid peroxides, the loss of a diffusion barrier to membrane-impermeable markers, and cell lysis (28).

The relatively mild oxidative capabilities of superoxide and hydrogen peroxide belie the severity of their direct oxidative effects on biomolecules. Therefore, it has been assumed that their cytotoxic nature might be due to their ability to generate intracellular hydroxyl radical. Since hydrogen peroxide is freely diffusible across membranes and cells, and superoxide and other organic radicals are generated either intracellularly or extracellularly, hydrogen peroxide may play a role as a "mobile metabolite" in the generation of toxic species.

**Superoxide Dismutases**

Superoxide dismutases (SOD) are a group of metalloenzymes catalyzing the disproportionation of superoxide and provide
the first line of defense against reactive oxygen species. The study of SOD started half a century ago when the copper known to be present in blood was found to be associated with a protein (erythrocuprein). The function of erythrocuprein remained unknown until 1969 when McCord and Fridovich found that it inhibited the reduction of cytochrome c by the superoxide radical generated from the xanthine/xanthine oxidase reaction (29). In addition to copper, erythrocuprein was also found to contain zinc. This protein is now known as Cu/Zn superoxide dismutase as it catalyzed the dismutation of superoxide radicals. Further investigation led to the discovering of iron- and manganese-containing SODs when Fridovich and collaborators isolated the SODs from E. coli (30, 31).

The standard assay for the SOD involves the inhibition of ferricytochrome c reduction by superoxide radicals. Xanthine oxidase catalyzes the oxidation of xanthine to urate with the concomitant generation of superoxide radicals. SODs scavenge the superoxide radicals in the reactions and therefore prohibit the ferricytochrome c from reduction. The unit of SOD was defined as the amount of SOD required to inhibit the rate of cytochrome c reduction by 50% (29). Other assays evolving the inhibition of nitroblue tetrazolium reduction, hydroxylamine reduction, and epinephrine autoxidation were also widely used (32, 33, 11).
Three types of SOD divided into two evolutionary families have been described. Cu/ZnSODs have been isolated from a wide range of organisms including yeast, *Neurospora crassa*, and bovine heart (34). The Cu/ZnSODs are homodimers with a molecular mass of around 32 kDa. The two identical subunits are associated by noncovalent interactions, and each subunit contains 1 g-atom each of copper and zinc. The copper is recognized as the redox active metal at the catalytic site whereas Zn provides structural stability to the enzyme. The Cu/ZnSODs are generally found in the cytosol of eukaryotic cells and in the chloroplasts.

A tetrameric Cu/ZnSOD (135 kDa) was found to constitute 90% of the total SOD of blood plasma (35). Although the total amount of this extracellular SOD is very little compared to the intracellular SOD, the amount may be important in moderating the effects of superoxide radicals liberated from activated phagocytic cells.

The manganese-containing superoxide dismutases (MnSODs) and iron-containing superoxide dismutases (FeSODs) are believed to have evolved from the same origin. A number of MnSODs and FeSODs have been isolated, which include MnSODs from *E. coli* (30), *Streptococcus mutans* (36), *Bacillus stearothermophilus* (37), and FeSODs from *E. coli* (31), *Pseudomonas ovalis* (38). MnSODs are found in prokaryotes and in the matrix of mitochondria, and FeSODs are found in
prokaryotes and in a few families of plants. The iron and manganese enzymes have subunit molecular mass of about 23 kDa, and while the iron enzymes have been shown to exist in dimeric form, the manganese enzymes have been found to form tetramers as well as dimers. The enzymes usually contain one atom of metal per subunit.

There is another group of SODs that have similar general characteristics with those of the FeSODs and MnSODs but are able to accept either manganese or iron depending on the growth condition or the metal supply. This group of enzymes are termed "cambialistic" SODs by Martin et al. (39), and will be discussed later in this chapter.

Amino acid sequences of MnSODs and FeSODs from diverse sources are very similar, whereas little similarity is found between Cu/ZnSODs and the two other SODs. CuZnSODs therefore are believed to have evolved from an independent precursor. The marked homology between prokaryotic and mitochondria MnSODs is consistent with an endosymbiotic origin for these organelles (40). It should be noted that each of these SODs catalyze the same reaction at a comparable rate.

The best way to distinguish the various SODs is to isolate the protein and characterize the metal present. However, each isozyme has been found to have a particular characteristic sensitivity towards a number of reagents. Cyanide inhibits the Cu/ZnSOD but not the manganese or iron
SODs (41). Hydrogen peroxide inactivates both the Cu/ZnSOD and FeSOD but not the MnSOD (42). Azide inhibits the FeSODs in low concentration (< 5 mM), and inhibits MnSODs and Cu/ZnSODs at higher concentrations (>20 mM) (43).

Although the essentiality of superoxide dismutases to the aerobic systems has been established, an aerotolerant bacterium *Lactobacillus plantarum* was found to be devoid of SOD. This organism was comparable to superoxide dismutase-containing species in its resistance toward hyperbaric oxygen and toward the oxygen-dependent lethality of plumbagin (44). However, *L. plantarum* required manganese-rich media for growth, and soluble extracts were found to contain approximately 9 μg of manganese ion per milligram of protein. Such extracts exhibited a 75-90% dialyzable manganese and EDTA inhibitable ability to scavenge oxygen radicals. It was suggested that the elevated level of manganese ion inside the cells was mimicking the superoxide dismutase scavenging activity.

**Reconstitution of the Aposuperoxide Dismutases**

Most MnSODs and FeSODs have strict specificity for the metal at their active sites although the primary structures of the enzymes are highly homologous. The way to demonstrate the metal specificity of a protein is through the reconstitution study. Several approaches have been used for the preparation
and reconstitution of aposuperoxide dismutases. Ose and Fridovich (45) prepared the apoprotein of *E. coli* MnSOD by dialyzing the protein against 0.7 M guanidine hydrochloride at pH 3.2. The full activity of the SOD was recovered upon addition of manganese in the presence of 0.1 M guanidine hydrochloride followed by the elevation of pH.

The aposuperoxide dismutase of *Pseudomonas ovalis* FeSOD was prepared by using 2 mM EDTA and 10 mM dithiothreitol in sodium carbonate buffer (38). The iron added to the apoprotein in the alkaline thiol-containing buffer restored both iron and catalytic activity.

Brock et al. (46) prepared the apoprotein by exposing the *Bacillus stearothermophilus* MnSOD to 8 M urea and 10 mM EDTA at pH 3.7. A metal-free apoprotein stable at neutral pH was obtained by further dialysis in 8 M urea and 10 mM EDTA at pH 7.5, followed by dialysis in the same buffer with 0.1 mM EDTA but lacking urea. Most of the protein was found to be precipitated if the dialysis against 8 M urea at neutral pH was omitted. The apoprotein was devoid of any residual catalytic activity, but was unable to be reconstituted with the addition of manganese at neutral pH. However, total reconstitution of fully active SOD was achieved by exposing the apoprotein to excess manganese in the presence of 8 M urea at acidic pH.

Kirby et al. (47) removed iron or manganese from several
SODs by dialysis of the cell extracts against 2.5 M guanidine hydrochloride and 20 mM 8-hydroxyquinoline. Aposuperoxide dismutases so prepared are inactive and are only reactivated by the reconstitution of the native metals. However, Gregory et al. were able to reconstitute a MnSOD from the Bacteroides fragilis FeSOD apoprotein using the same method described above (48). The FeSOD produced by anaerobically maintained B. fragilis was denatured by dialysis against 2.5 M guanidine hydrochloride and 20 mM 8-hydroxyquinoline. The apoprotein was brought to neutral pH by dialysis in the buffer lacking denaturant, and reconstitution was carried out at neutral pH by dialysis in the buffer containing either manganese or iron ion. The reconstituted FeSOD and MnSOD regained 7% and 30% of the original activities, respectively. Other aposuperoxide dismutases capable of accepting either manganese or iron ion and regaining catalytic activities were also encountered, although the method for the preparation of apoprotein was different.

Meier et al. (49) used ascorbate and o-phenanthroline at pH 5.5 to remove metal ion from Propionibacterium shermanii MnSOD. The apoprotein, which retained 34% of the original catalytic activity, was further activated upon reconstitution with either iron or manganese ion at pH 5.5. Dialysis of the apoprotein at pH 5.5 for longer than 24 hours reportedly caused precipitation of the protein. The same approach was
used by Martin et al. for the preparation of apoprotein from *Streptococcus mutans* FeSOD (39). Dialysis of the FeSOD with o-phenanthroline and sodium ascorbate generated aposuperoxide dismutase with 94% loss of activity. Subsequent dialysis of apoprotein with either manganese or iron reconstituted 37% and 30% of the original activity, respectively.

The superoxide dismutases capable of retaining their catalytic activities upon metal substitutions are termed cambialistic SODs. The cambialistic characteristic of these SODs was demonstrated not only in the reconstitution studies, but was also observed in the cultures grown under treated media. The anaerobically grown *P. shermanii* produced iron-containing SOD in a complex medium containing iron, while the same culture grown in the iron-free media supplemented with manganese produced manganese-containing SOD (49). The two SODs had the same electrophoretic mobility, apparent molecular mass, and subunit size. The amino acid compositions of the two were practically indistinguishable, and the N-terminal sequences were also identical. It was suggested that an identical protein moiety was employed for the synthesis of either iron or manganese superoxide dismutase, depending on the availability of the individual metal. Similar results were observed on the SODs of *S. mutans* (39), and *B. fragilis* (48), where the *S. mutans* was grown in untreated or manganese-supplemented media; and *B. fragilis* was grown in anaerobic or
aerobic conditions. Although the SODs in this category are able of accepting either manganese or iron, they still preferentially incorporate one type of metal versus the other under untreated culture conditions.

**Primary Structures of Iron- and Manganese-Containing Superoxide Dismutases**

The primary structures of SODs in relations to the metal binding specificity was examined by Parker et al. (50) and Isobe et al. (51). The sequences examined included FeSODs from *E. coli*, *Pseudomonan ovalis*, *Photobacterium leiognathi*, and MnSODs from *E. coli*, *Thermus thermophilus*, *Bacillus stearothermophilus*, yeast, mouse and human. The amino acid sequence identity between FeSODs and MnSODs is 34-52%, while among FeSODs and among MnSODs are 65-74% and 39-94%, respectively. However, the amino acid sequence identity among prokaryotic enzymes is between 52% and 62%. The crystal structures of *P. ovalis* (52) and *E. coli* (53) FeSODs, and *T. thermophilus* (54) and *B. stearothermophilus* (55) MnSODs have been determined. The aligned amino acid sequences and the crystal structures of the FeSODs and MnSODs revealed that the two types of SODs shared the same ligands to the metal cofactors: His 26, His 81, Asp 175, and His 179. The amino acids that formed the micro environment of the metal ions also appeared to be conserved between the FeSODs and MnSODs.
However, the active site amino acids Phe 84 and Gln 154 in the MnSODs were replaced by Tyr and Ala in FeSODs, respectively. Based on the information from the crystal structures, Isobe et al. observed that Gln 154 contributed to the formation of the hydrophobic metal-ligand environment through hydrogen bonding with Trp 133 and Tyr 34 in the MnSODs, and the substitution of Gln by Ala should cause different micro environments between the metal centers of the FeSODs and MnSODs (51). However, in addition to Phe 84 and Gln 154, Parker and Blake pinpointed three more amino acids which might be responsible for conferring the distinguishing properties of the two types of SODs (50). The aligned amino acid sequences were examined for situations where a residue was invariant or conservatively substituted in one class of enzyme but not found in the other class. Residues Gly 76, Gly 77 and Asp 155 were conserved in MnSODs, but were replaced in FeSODs by Ala, Gln, and Gly, respectively. These residues were also observed by X-ray crystal structure to be clustering around the active site of B. stearothermophilus MnSOD (55), which might also support the discrimination between iron and manganese in the SOD peptides.

**Oxygen-Dependent Induction and Biosynthesis of MnSOD and FeSOD**

Increases in cell content of SOD are often seen when conditions are imposed that increase intracellular production of oxygen radicals. Anaerobically grown *E. coli* contains only
FeSOD, while aerobically maintained culture produces three distinct isozymes of SOD. One of the SOD isozymes contains manganese, the second contains iron, and the third is a hybrid consisting of one subunit from each of the other two isozymes (56). Thus, the iron enzyme is present under both anaerobic and aerobic conditions and is, therefore, constitutive. In contrast, the manganese and the hybrid enzymes are absent under anaerobic conditions but are rapidly synthesized upon exposure to air. It has been proposed that the presence of a constitutive FeSOD in E. coli provides a back-up defense mechanism against sudden aerobic exposures to oxygen (57). The anaerobes Bacteroides fragilis (58), Bacteroides thetaiotaomicron (59) and Bacteroides gingivalis (60) contain low levels of FeSOD when grown anaerobically, and exposure to air resulted in the induction of manganese-containing SODs. However, the protein moieties of the Bacteroides iron- or manganese enzymes were identical, whereas that of the E. coli enzymes were different. Exposure to high concentrations of oxygen was also shown to induce MnSOD in Staphylococcus aureus, and Listeria monocytogenes, and induce FeSOD in Pseudomonas aeruginosa (61).

Intracellular production of oxygen radical can also be generated by several redox-cycling compounds. The compounds, including paraquat, plumbagin and streptonigrin, are able to enter the cells and generate oxygen radicals (62). Once in the
cells, the redox-cycling compounds are rapidly reduced, probably by NADH-dependent reactions, and subsequently reoxidized by oxygen to generate superoxide radical. These redox-cycling compounds, therefore, provide a cytochrome-independent pathway to oxygen, and act catalytically to increase the intracellular flux of superoxide radical to exacerbate the toxicity of oxygen. Studies of MnSOD induction by paraquat showed that the addition of paraquat to aerobically growing E. coli resulted in a rapid linear increase in MnSOD. Removal of paraquat from the growth medium causes a sharp decline in the rate of MnSOD biosynthesis (63). The induction of MnSOD by paraquat is prevented by inhibitors of transcription or translation, but not by inhibitors of replication (62). Similar inductive effects are seen with the other redox-active compounds in several bacteria (61). These results indicate that the induction of MnSOD in E. coli is under transcriptional control, and the oxygen radical could probably act as the effector for the control mechanism.

The role of iron in the regulation of MnSOD biosynthesis in E. coli was suspected by Hassan et al. during the study of MnSOD induction by paraquat (64). A twofold increase in MnSOD was observed when nalidixic acid is used as an inhibitor of DNA biosynthesis. It was shown that the induction of MnSOD by nalidixic acid was not due to its known ability to inhibit DNA gyrase nor to increase the intracellular flux of oxygen.
radical, but rather due to its ability to chelate iron. Further studies showed that the addition of excess iron to *E. coli* growth media had a repressive effect on the biosynthesis of MnSOD under aerobic growth or paraquat inducing conditions (65). Removal of iron from the growth medium by ferrous iron chelators derepressed the synthesis of MnSOD, but repletion of the medium with iron abolished this effect.

A model accommodated all of the above findings was proposed. In this model, the synthesis of MnSOD is regulated by an iron-containing repressor protein (RP). The active repressor contains ferrous iron (RP-Fe\(^{2+}\)), while the inactive form contains either ferric iron (RP-Fe\(^{3+}\)) or no iron (RP). In the absence of oxygen, the repressor is active and no MnSOD is made. On the other hand, conditions known to oxidize ferrous to ferric (i.e. oxygen radicals) or to remove ferrous from the cells (i.e. iron chelators) will generate the inactive forms of the repressor and will result in the synthesis of the enzyme, even in the absence of oxygen.

Another model has also been proposed by Pugh et al., which takes into account the effects of iron, manganese and chelators on the biosynthesis of both MnSOD and FeSOD in *E. coli* (66, 67). In this model, both FeSOD and MnSOD are viewed as autogenously regulated catalysts whose conversion from inactive apoproteins to catalytically active holoenzymes
depends on the availability of the appropriate metal cofactor and upon a prerequisite concentration of superoxide radical. The superoxide radical is capable of converting sufficient amount of Mn$^{+2}$ to Mn$^{+3}$ in order to compete effectively with Fe$^{+2}$ for the active sites in the apoprotein. Thus, under anaerobic conditions, the MnSOD apoprotein is present but the active site is occupied by iron and is therefore catalytically inactive. In the presence of oxygen radical generator (i.e. redox active compounds) or under aerobic conditions, Mn$^{+2}$ is oxidized to Mn$^{+3}$ which is a strong competitor for the active site of MnSOD apoprotein and therefore generates catalytically active MnSOD.

However, aside from the physiological studies, current research on the induction of MnSOD in *E. coli* by means of genetic tools emphasizes the coordinated global response to superoxide-mediated stress, which includes induction of defense and repair enzymes (68). It has been shown that numerous proteins in addition to MnSOD are transiently over-produced after a challenge with an intracellular superoxide generator, or permanently over-produced in strains lacking SOD (69, 70). This global response is independent of the other known heat shock, hydrogen peroxide-mediated stress or the SOS responses, and there may be specific regulatory elements for the superoxide radical-mediated stress responses.
The studies on MnSOD regulation by Touati were designed to establish the level where regulation occurred (71, 72). Several constructs containing protein and operon fusions with the lac operon were made, which dissociated transcriptional, post-transcriptional and post-translational regulatory events of the MnSOD. In the former, the lacZ gene was fused to a fragment of the MnSOD structural gene sodA and was under the MnSOD transcriptional control; in the latter, the MnSOD gene was transcribed from the oxygen-insensitive tac promoter. These, as well as plasmids carrying all or part of the sodA DNA region, were used to investigate the response to MnSOD inducers, the effect of metal ions, and to determine the type of controls of MnSOD expression. The data obtained suggested multicontrol of MnSOD, which included: (a) transcriptional regulation via ferrous ion; (b) superoxide-mediated activation of transcription; (c) autogenous regulation; and (d) post-translational modulation of the enzyme activity by manganese and iron, depending on the intracellular concentration of manganese ions. Although each model proposed by Hassan et al. or Pugh et al. seemed to accommodate part of the findings above, the overall regulation of the iron and manganese SOD was still unclear and still remained to be tested.

The study of SOD in the gram-positive bacterium D. radiodurans came to our attention due to the bacterium’s
resistance to radiation as well as its expression of high level of SOD (73). *D. radiodurans* has generated long standing interest because of its unique membrane structure and its extremely high resistance to ionizing and ultraviolet radiation (74 - 76). Recent studies of this bacterium have been focused on the genetic and molecular mechanisms involving the DNA repair enzymes (77, 78) and on the insertion and expression of drug resistance determinants (79 - 81). Although the importance of the DNA repair system in conferring the radiation resistance upon the bacterium should not be understated, the elevated levels of SOD and catalase activities (73) should also be viewed as part of the global defense system against radiation. In this study, the isolation, characterization and reconstitution of the *D. radiodurans* SOD will be described, and the molecular cloning of the gene encoding the SOD peptide will also be reported.
Chapter 2.

Isolation and Reconstitution of Superoxide Dismutase

from Deinococcus radiodurans
INTRODUCTION

Superoxide dismutases (SOD) are metalloproteins that catalyze the disproportionation of superoxide radicals to hydrogen peroxide and oxygen. These enzymes are part of the antioxidant defense system present in almost all cells (21). Three classes of superoxide dismutase have been described based on the transition metals bound at the catalytic site. The SOD found predominantly in the cytosol of eucaryotes contains copper and zinc (Cu/ZnSOD) (29). Superoxide dismutases from the cytosol of procaryotes contain either iron (FeSOD) or manganese (MnSOD) (30, 31). There is a large degree of sequence similarity among the Cu/ZnSODs and among Fe- and Mn-containing SODs but little homology between the Cu/Zn and the iron or manganese SODs (36). Moreover, the enzymes usually exhibit strict specificity for the metal bound at the active site (47).

Ose and Fridovich (45) were first to demonstrate reversible removal and replacement of manganese from the E. coli MnSOD. Denatured apoprotein was prepared in acidic guanidine hydrochloride. Catalytic activity and tightly bound Mn were restored upon addition of MnCl₂ and neutralization of the solution. These observations were extended to other Mn- and Fe-containing superoxide dismutases. In each case, the
denatured apoperoxide dismutase was reconstituted only upon addition of the intrinsic transition metal \cite{36}. Similar studies were applied to the superoxide dismutases from \textit{Bacteroides} \cite{48, 58, 59}. The iron-containing superoxide dismutases isolated from anaerobically maintained \textit{Bacteroides fragilis} and \textit{Bacteroides thetaiotaomicron} are less specific with respect to the active site transition metal. In each case cited above, reconstitution was carried out using the denatured apoprotein.

A radiation-resistant bacterium \textit{Deinococcus radiodurans} came to our attention because of the expression of high level of superoxide dismutase and catalase activities \cite{74}. This bacterium also possesses unique cell wall structure, efficient DNA repair system and high DNA GC content \cite{75-79}. Although the radiation-resistance is usually referred to the efficient DNA repair system, the elevated level of superoxide dismutase and catalase activities might also be responsible for the protection of cells from radiation. This chapter describes the isolation, characterization, and reconstitution of the superoxide dismutase from the bacterium \textit{D. radiodurans}. 
EXPERIMENTAL PROCEDURE

Materials

Acrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), N-[2-acetamide]-2-aminoethanesulfonic acid (ACES), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), ethylene diaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), guanidine hydrochloride (reconstitution study), ammonium sulfate, protamine sulfate, glucose, ammonium persulfate, N',N'-methylene-bis-acrylamide, nitroblue tetrazolium (NBT), riboflavin, xanthine, and bovine serum albumin (BSA) were obtained from Fisher Scientific Co., Pittsburgh, PA. Tris-(hydroxymethyl) aminomethane (Tris), cytochrome c, hemin, protein A- horseradish peroxidase (HRPO) conjugate, goat anti-rabbit antibody-HRPO conjugate, ovalbumin, carbonic anhydrase, o-dianisidine, lysozyme, diaminobenzidine (DAB), 4-chloro-1-naphthol, prestained molecular weight markers, ampholyte (Ph 3-10), guanidine hydrochloride (purified grade), Nonidet P-40, and hydrogen peroxide (30%) were obtained from Sigma Chemical Co., St. Louis, MO. Peptone, yeast extract, trypticase soy broth were purchased from Difco Laboratory, Detroit, MI. Guanidine hydrochloride for fluorescent and spectrophotometric studies was purchased from Pierce Chemical Co., Rockford, IL. The bacterium D. radiodurans (ATCC 13939) was obtained from The
American Type Culture Collection, Rockville, MD.

The column chromatography resins DEAE cellulose was obtained from Fisher Chemical Co., and Bio-gel P-100 was obtained from Bio-Rad, Melville, NY. The fast protein liquid chromatography (FPLC) system as well as Superose 6 and Superose 12 columns were obtained from Pharmacia, Pleasant Hill, CA. The FPLC system is consisted of a GP-250 programmer, two P-500 pumps, a Frac-100 fraction collector, a UV-1 single path monitor, and a REC-481 single channel recorder.

The nitrocellulose membranes were obtained from either Hoefer Scientific Instrument, San Francisco, CA., or Schleicher and Schuell, Keene, NH. The polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad. The electrophoresis apparatus were all obtained from Hoefer Scientific Instruments. Depending on the scale of the protein separation, the mini-gel Model SE 250 or the STURDIER® vertical gel unit Model SE 400 were used. The TRANSPHOR® chamber from Hoefer Scientific Instruments was used for the transferring of proteins to nitrocellulose membrane. The spectrophotometric measurements were usually performed with a Shimadzu UV-265 spectrophotometer. The metal content of the protein was determined on a Perkin-Elmer Model 560 atomic absorption spectrophotometer by aspiration into an acetylene flame. Fluorescence emission measurements were obtained with a Perkin-Elmer Model 650-40 fluorescence spectrophotometer.
Methods

Bacterial strain and culture condition: Bacterial strain Deinococcus radiodurans (ATCC 13939) was the source of superoxide dismutase. The culture was grown in PYG medium (0.5% w/v peptone, 0.5% w/v trypticase soy broth, 1% w/v yeast extract, 1% w/v glucose, 4% v/v VPI salt) (135) supplemented with hemin (0.5% w/v). The culture was grown at 30°C overnight with shaking (200 rpm) and harvested by centrifugation. The cells were washed with 50 mM phosphate buffer containing 1 mM EDTA (pH 7.8) before lyophilization. The lyophilized cells were stored at -20°C until used.

Sonication: Suspension of D. radiodurans was disrupted by 3-s bursts (70 W) of sonic oscillation applied through the microtip at 4°C. Total sonication time was 30 min per 100 ml of suspension.

Activity assay of superoxide dismutase and unit definition: The superoxide dismutase was assayed according to the method described by Beyer and Fridovich (82). The assay was performed in 50 mM potassium phosphate buffer at pH 7.8 containing 1 mM EDTA in a cuvette maintained at 27°C. The 3 ml reaction mixture contained 1 x 10^5 M ferricytochrome c, 5 x 10^5 M xanthine, and sufficient xanthine oxidase to produce a 0.025
absorbance change per minute at 550 nm. The amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% is defined as one unit of activity (29), and standard curve which correlates percentage inhibition and units of enzymatic activity was used.

**Protein assay:** The protein concentrations were determined by the Lowry method (83), and the extinction coefficient of the purified protein at 280 nm was calculated.

**Non-denaturing polyacrylamide gel electrophoresis (PAGE):** Protein samples were routinely separated in 10% polyacrylamide gels for qualitative analysis. The protein was usually detected with Coomassie blue G-250, and superoxide dismutase was detected with histochemical activity staining (32). Superoxide dismutase inhibited the reduction of NBT by riboflavin in the presence of light and catalyst TEMED. The resulting gel showed a purple background with achromatic superoxide dismutase bands.

**Denaturing polyacrylamide gel electrophoresis:** Protein subunit molecular mass was determined in polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) (84). Samples and molecular mass markers were separated in gels containing 12% SDS. The gels were stained with Coomassie blue G-250 after
electrophoresis to visualize the proteins.

**Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE):** The isoelectric point of the superoxide dismutase was determined by IEF-PAGE (85). Protein samples were separated on a 5.5% polyacrylamide gel containing ampholyte (pH 3-10). Proteins of known pI (Pharmacia) as well as methyl red (pI 3.75) and patent blue violet (pI 2.45) dyes were included as markers. The ampholyte was extracted three times (30 min each) with 5% trichloroacetic acid, and the gel was soaked in destain solution (10% acetic acid, 25% ethanol) overnight before it was stained with Coomassie blue G-250.

**DEAE cellulose chromatography:** The DEAE-53 cellulose column (1.5 x 19 cm) was equilibrated in 5 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0). After the application of the protein sample, the column was washed with two bed volumes of the same buffer. Proteins were eluted with a 0-200 mM KCl (500 ml each) linear gradient, and fractions (4.8 ml) containing superoxide dismutase activity were pooled.

**P-100 column chromatography:** The Bio-gel P-100 column (1.5 x 90 cm) was equilibrated in 50 mM potassium phosphate buffer (pH 7.0). The sample (2 ml) applied to the column was eluted with 50 mM potassium phosphate buffer (pH 7.0) by gravity
flow, and fractions (2 ml) containing superoxide dismutase activity were pooled.

**Preparation and reconstitution of the renatured apoprotein:**
Apoprotein was prepared by dialysis in 5 M guanidine hydrochloride and 20 mM 8-hydroxyquinoline under acidic condition (pH 3.5), followed by two changes in 5 M guanidine hydrochloride (500 ml each). The denatured apoprotein was freed of denaturant by dialysis in two changes (4 liters each) of 20 mM Tris-HCl (pH 7.0, or 7.5, or 8.0). Apoprotein was also prepared in 20 mM ACES (pH 6.5, 7.0, 7.5) or 20 mM HEPES (pH 7.5, 8.0, 8.5) by subsequent dialysis. The protein thus prepared was termed renatured apoprotein, which was divided into 100 μl aliquots and stored at -20°C until used.

All the plasticware was acid-washed and rinsed in distilled deionized water for the preparation of apoprotein and the subsequent reconstitutions. Buffers were prepared from 1 M stock solutions which had been passed through Chelex-100 to diminish the trace metal content.

Reconstitution was initiated with the addition of the appropriate concentrations of metal ions to the renatured apoprotein (100 μl, 0.7-0.9 μg/ml) at room temperature. Manganese chloride, zinc chloride, or ferrous ammonium sulfate prepared as 10x concentrates in water were added. For the kinetics studies, reconstitutions were terminated at different
time points by diluting aliquots of samples into phosphate buffer (50 mM, pH 7.8) containing 2 mM EDTA. Exhaustive reconstitutions were usually terminated by dialysis after 3 h of incubation at room temperature. The reconstituted protein samples were analyzed for enzyme activities and metal contents.

**Intrinsic fluorescence spectrum:** Fluorescence excitation and emission spectra of the native- (0.21 mg/ml), manganese reconstituted- (0.31 mg/ml), and renatured apo- (0.2 mg/ml) superoxide dismutases were obtained with a Perkin-Elmer fluorometer (Model 650-40). The spectrum of the denatured apoprotein (1.5 $A_{280}$ absorbance units) in 5 M guanidine hydrochloride (sequanal grade, Pierce) was also recorded.

**Study of salt effect on superoxide dismutase activity:** An assay less sensitive to interferences by salts was used for the study of the catalytic activities of superoxide dismutases in different salts and salt concentrations. The photochemical augmentation assay (86) contained o-dianisidine (0.1 mM) and riboflavin (0.01 mM) in a 3 ml reaction buffer (25 mM potassium phosphate pH 8.0). The reactions were initiated by illumination (4 min) and the changes in absorbance at 460 nm were recorded. The presence of superoxide dismutase enhanced
the accumulation of oxidized o-dianisidine and therefore increased the absorbance at 460 nm. Native or manganese reconstituted superoxide dismutase (1.7 μg) was included in the assay, and the enzymatic activities under different salt conditions were expressed as percentage activities relative to the activity obtained in 25 mM potassium phosphate buffer (pH 8.0).

**Fast protein liquid chromatography (FPLC):** The FPLC system was used in this study to determine the apparent molecular mass of the native superoxide dismutase. Superose 6 equilibrated in 50 mM potassium phosphate (pH 7.8) was calibrated with BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and lysozyme (14 kDa). The void volume of the column was determined with blue dextran. Native (90 μg) or reconstituted (200 μg) superoxide dismutase samples were applied to the column along with lysozyme as an internal control. The apparent molecular mass of the renatured apoprotein was determined in 50 mM potassium phosphate buffer containing 2 mM EDTA. The UV monitor and recorder recorded the elution profiles, and the molecular mass of each protein was calculated accordingly.

Chromatography on Superose 12 was used to demonstrate the presence of superoxide dismutase monomers under denaturing condition. Superose 12 column was equilibrated in 50 mM Tris-
HCl (pH 8.0) and 5 M guanidine hydrochloride (purified grade, Sigma Chemical Co.). *D. radiodurans* cell extract (1 mg in 100 μl) was applied to the column and eluted with the same buffer. Fractions collected (10 x 2 ml) were dialyzed in 4 liter of 50 mM Tris-HCl containing 1 mM MnCl₂ (pH 8.0). The proteins were separated in two 10% SDS gels, where one gel was stained with Coomassie blue G-250, and the other gel was analyzed by western blotting.

**Generation of antibody and titer determination:** Polyclonal anti-MnSOD antiserum was obtained from Cocalico Biologicals Inc., where the rabbit was immunized a total of four times in the period of 50 days with the purified superoxide dismutase from *D. radiodurans*. The titer of the antibody was examined periodically by enzyme-linked immunosorbant assay (ELISA) (87). The serum of the final bleed was diluted (1:1000) and used in western analysis without further purification.

**Western analysis:** Proteins transferred to nitrocellulose membranes were detected with antibody by western analysis (88). Protein samples as well as prestained molecular mass standards (Bio-Rad or Sigma Chemical Co.) were routinely separated on 12% SDS gels, and electrophoretically transferred (1 amp, 1 h) to nitrocellulose membrane. The prestained
molecular weight markers were used to visualize the protein transfer. The membrane was blocked with 5% w/v non-fat dry milk (prepared in phosphate-buffered saline; PBS), and anti-MnSOD antibody was added followed by secondary antibody (goat anti-rabbit IgG or protein A-horseradish peroxidase (HRPO) conjugate). The blots were developed with diaminobenzidine (0.5 mg/ml Tris-buffered saline; TBS) or 4-chloro-1-naphthol (1 mg/ml TBS) in the presence of hydrogen peroxide (60 μl of 30% H₂O₂ per 100 ml). The reaction was terminated by rinsing the membranes with TBS.

Affinity purification of antibody: The procedure adapted from Olmsted (26) was used for the affinity purification of the polyclonal antibody when homogeneous antigen sample was unavailable. Proteins were separated on a 12% SDS curtain gel (Hoefer STURDIERTM vertical gel unit), and electrophoretically transferred (1 Amp) to PVDF (polyvinylidene difluoride) membrane. The membrane was blocked with 5% w/v non-fat dry milk, and western analysis was performed on a thin strip of the membrane to locate the antigen. The membrane where the antigen immobilized was excised and incubated overnight with 6 ml of rabbit antiserum buffered to pH 8.0 at 7°C with shaking. The membrane was washed once in Buffer 1 (0.25% w/v gelatin, 0.05 M Tris-HCl pH 7.5, 5 mM EDTA, 0.15 M NaCl, 0.05%
v/v Nonidet P-40), followed by three washes (0.5 hour each) with Buffer 1 lacking both Nonidet P-40 and gelatin. The membrane was incubated with 3 ml of 0.2 M glycine-HCl (pH 2.8) for 2 min in a syringe, and the solution was forced through the syringe twice. The eluted antibody was concentrated and neutralized with 1 M Tris-HCl (pH 8.0), and western analysis was performed using this purified antibody (1:50).
RESULTS

1. Isolation of *D. radiodurans* Superoxide Dismutase

Eight grams of lyophilized cells were rehydrated with 200 ml of 50 mM potassium phosphate buffer (pH 7.8) and 1 mM EDTA in a blender and disrupted by 3-s bursts (70 W) of sonic oscillation applied through the microtip. The total sonication time was 30 min per 100 ml of suspension. The suspension was clarified by centrifugation (23,000 x g, 30 min), stirred with 0.2 % w/v protamine sulfate for 45 min, and centrifuged at 23,000 x g for 15 min. The supernatant was stirred for 30 min in 50% ammonium sulfate (313 g/liter), and the mixture was clarified by centrifugation. The supernatant was brought to 80% ammonium sulfate (214 g/liter) and stirred for 30 min. The pellet, obtained by centrifugation, was dissolved in 35-50 ml of 5 mM potassium phosphate containing 1 mM EDTA (pH 7.0), and was dialyzed twice in 4 liters of the same buffer. The dialysate was applied to DEAE-53 column (1.5 x 19 cm) equilibrated in 5 mM potassium phosphate buffer (pH 7.0), and the protein was eluted with a linear KCl gradient (0-200 mM). Fractions (4.8 ml) with superoxide dismutase activity greater than 350 units/ml were pooled and dialyzed in the phosphate buffer for rechromatography on DEAE-53 under conditions identical to those described above. Fractions with superoxide dismutase activity greater than 1200 units/mg were
concentrated to approximately 2 ml over a YM-5 ultrafilter (Amicon) under N₂ pressure. The protein was applied to a Bio-Gel P-100 column equilibrated in 50 mM phosphate buffer (pH 7.0) and was eluted in this buffer. The fractions (2 ml) containing superoxide dismutase activity were pooled and concentrated over a YM-5 membrane. The superoxide dismutase from *D. radiodurans* was purified 310-fold to a specific activity of 3300 units/mg with a 46% overall yield (Table 1). This purification scheme consistently yielded enzyme with specific activity 3300 ± 480 units/mg.

2. Characterization of *D. radiodurans* Superoxide Dismutase

The isolated superoxide dismutase migrated as one major band and two faint bands on nondenaturing polyacrylamide gel stained with Coomassie blue (Figure 1 A, lane 1). A separate gel stained for superoxide dismutase activity revealed that the most intense activity migrated coincidentally with the major protein band (Figure 1 B, lane 1), and the two minor protein electromorphs displayed faint but discernible activities.

The apparent molecular mass of the native protein was 43,400 daltons, which was determined by Superose 6 gel filtration chromatography on FPLC (Figure 2). The denatured protein migrated as a single peptide in 12% SDS-PAGE
Table 1. Purification of *D. radiodurans* superoxide dismutase.

<table>
<thead>
<tr>
<th>Step</th>
<th>Units</th>
<th>Protein (mg)</th>
<th>Specific Activity (unit/mg)</th>
<th>Fold</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>23,800</td>
<td>2540</td>
<td>9.4</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Protamine</td>
<td>19,200</td>
<td>1530</td>
<td>12.6</td>
<td>1.3</td>
<td>-</td>
</tr>
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<td>sulfate 50% (NH₄)₂SO₄</td>
<td>22,200</td>
<td>1290</td>
<td>17.3</td>
<td>1.8</td>
<td>93.2</td>
</tr>
<tr>
<td>80% (NH₄)₂SO₄</td>
<td>18,300</td>
<td>90.7</td>
<td>202</td>
<td>21.5</td>
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<tr>
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<td>1520</td>
<td>162</td>
<td>69.7</td>
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<tr>
<td>Second DE 53</td>
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<td>5.1</td>
<td>2600</td>
<td>278</td>
<td>55.8</td>
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<tr>
<td>P-100</td>
<td>10,900</td>
<td>3.3</td>
<td>3300</td>
<td>351</td>
<td>45.5</td>
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Figure 1. Electrophorograms of the native-, apo-, and manganese reconstituted- superoxide dismutases from *D. radiodurans*. Protein samples separated on 10% acrylamide gels were stained for protein with Coomassie blue G-250 (panel A), or stained for superoxide dismutase activity with the method of Beauchamp and Fridovich (32) (panel B). Lanes 1: native MnSOD (panel A and B, 3.2 μg and 1 μg, respectively). Lanes 2: apoprotein (panel A and B, 4 μg and 1.1 μg, respectively). Lanes 3: manganese reconstituted protein (panel A and B, 5 μg and 1.1 μg, respectively).
Figure 2. Molecular mass determination of the native SOD. Native SOD and molecular mass markers were separated by Superose 6 column of FPLC. The elution of the proteins were detected by the absorbance at 280 nm. The markers were (solid line, from left to right): blue dextran (2,000 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa). The dotted line represented the D. radiodurans native MnSOD.
irrespective of the presence of 2-mercaptoethanol. The subunit of the superoxide dismutase from *D. radiodurans* was estimated to be between 23,000 and 24,000 daltons since it comigrated with the subunit of manganese superoxide dismutase from *E. coli* (23 kDa) and trypsinogen (24 kDa) (Figure 3).

Isoelectric focussing of the superoxide dismutase revealed two protein bands, both of which had superoxide dismutase activity. The major band focused to pI 4.0 and the minor band to pI 4.7 (Figure 4, lane 1).

The UV-visible spectrum of the native protein showed a broad band between 400 and 600 nm with a distinct peak at 415 nm and an absorbance at 280 nm with a shoulder at 288 nm (Figure 5, solid line). The extinction coefficient at 280 nm was 1.8 ml mg\(^{-1}\) cm\(^{-1}\) based on Lowry protein assay and quantitative amino acid composition. The amino acid composition is shown in Table 2, and the N-terminal sequence of the purified protein is shown in Table 3.

The superoxide dismutase contained 1.5 g-atoms manganese per mol dimer and 0.5-0.8 g-atom iron per mol dimer. The zinc content was less than 0.1 g-atom per mol dimer (Table 4).

3. Characterization of the Renatured Aposuperoxide Dismutase

The *D. radiodurans* superoxide dismutase was denatured in 5 M guanidine hydrochloride and 20 mM 8-hydroxyquinoline. The
Figure 3. *D. radiodurans* SOD subunit molecular weight determination. Protein samples were diluted in SDS sample buffer (84) and boiled before applied to a 12% SDS gel. The proteins were stained with Coomassie blue G-250. Lanes 1 and 7: subunit molecular weight markers (Sigma Chemical Co.). Lanes 2 and 6: trypsinogen (24 kDa). Lane 3: *D. radiodurans* SOD. Lane 4: the combined proteins of *D. radiodurans* SOD and *E. coli* MnSOD. Lane 5: *E. coli* MnSOD (23 kDa).
Figure 4. Isoelectric focusing of the *D. radiodurans* superoxide dismutase. Protein samples were diluted 1:1 in application solution (4% ampholyte pH 3-10, 60% glycerol, patent blue violet marker dye) and applied to gels containing 5.5% acrylamide and ampholyte. The pH gradient was determined with pI marker proteins (Pharmacia), methyl red and patent blue violet dyes. The gel was washed consecutively in 10% trichloroacetic acid and destain solution (25% ethanol, 10% acetic acid) for several times, and stained with Coomassie blue G-250. Lane 1: pI protein markers and dye markers (arrows). Lane 2: purified native MnSOD (3.2 µg). Lane 3: apoprotein (4.0 µg). Lane 4: manganese reconstituted protein (4.0 µg).
Figure 5. UV-visible spectra of the native- and apo- superoxide dismutases. UV and visible spectra were recorded on a Shimadzu UV-265 spectrophotometer. The native MnSOD and apoprotein were prepared as described in the text. Protein concentrations of the native MnSOD were 0.98 mg/ml and 0.24 mg/ml for the visible and UV spectrum, respectively. The renatured apoprotein concentrations were 1.1 mg/ml and 0.26 mg/ml for the visible and UV spectrum, respectively. The solid line represents the native protein, and the dotted line represents the renatured apoprotein.
Table 2. Amino acid composition of *D. radiodurans* SOD.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Content&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Residues/mole&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>CM-Cys&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>14.7</td>
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<tr>
<td>Ser</td>
<td>14.9</td>
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<tr>
<td>Gl(n)</td>
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<td>37</td>
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<tr>
<td></td>
<td>10.9&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> Amino acid content based on 43,000 daltons.
<sup>b</sup> Composition given as nearest integer.
<sup>c</sup> Determined on reduced carboxymethylated protein.
<sup>d</sup> Triplicate analysis, protein hydrolyzed in 4 M methanesulfonic acid.
<sup>e</sup> Duplicate analysis, Edelhoch method (90).
Table 3. N-terminal sequence of the purified *D. radiodurans* SOD

<table>
<thead>
<tr>
<th></th>
<th>Asp</th>
<th>Tyr</th>
<th>Thr</th>
<th>Leu</th>
<th>Pro</th>
<th>Gln</th>
<th>Leu</th>
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Arg Thr Met Glu Ile His His Thr Lys His
His Gln Thr Tyr Val Asp Asn Ala Asn Lys
Table 4. Metal content of *D. radiodurans* SOD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific Activity (unit/mg)</th>
<th>g-atoms per mole dimer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mn</td>
</tr>
<tr>
<td>Native</td>
<td>3300±484</td>
<td>1.50±0.03</td>
</tr>
<tr>
<td>Apoprotein</td>
<td>50±42</td>
<td>0.25±0.16</td>
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<tr>
<td>Mn recon.</td>
<td>3560±106</td>
<td>1.70±0.08</td>
</tr>
<tr>
<td>Zn recon.</td>
<td>50±42</td>
<td>n.d.</td>
</tr>
<tr>
<td>Fe recon.</td>
<td>50±42</td>
<td>0.30±0.08</td>
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</tbody>
</table>

Notes. n.d. not determined. Data are means ± SD.
denatured apoprotein was renatured by dialysis against Tris-HCl as described. The renatured apoprotein contained no more than 2% of the catalytic activity of the native enzyme but retained 0.3 g-atom manganese, 0.4 g-atom iron, and 0.05 g-atom zinc per mol dimer (Table 4).

The renatured apoprotein retained the absorbance at 280 and 288 nm but lost the broad absorbance feature at 400-600 nm. The peak at 415 nm was markedly diminished (Figure 5, dotted line).

The apoprotein migrated to the same position and gave the same pattern of electromorphs as the holoenzyme (Figure 1A, lane 2), and yielded a small superoxide dismutase activity band (Figure 1B, lane 2). The isoelectric focusing of the apoprotein also showed the same pattern as the holoprotein (Figure 4, lane 3).

4. Reconstitution of the Renatured Aposuperoxide Dismutase

The renatured apoprotein diluted in 20 mM Tris-HCl (pH 7.5) was reconstituted with manganese ions (1 mM), zinc ions (50 μM), or ferrous ions (1 mM). Incubation of the renatured apoprotein with 1 mM manganese ions resulted in full restoration of the catalytic activity, and the reconstituted protein contained 1.7 g-atom of manganese per mol dimer. The manganese reconstituted protein migrated identically with the holoprotein in nondenaturing gels (Figure 1A and 1B, lanes
3) and IEF gel (Figure 4, lane 4). Zinc ions were able to bind the apoprotein stoichiometrically (1.7 g-atom per mole dimer) but no catalytic activity was observed. Apoprotein reconstituted with ferrous ions contained 3-7 g-atom of iron per mol dimer and no catalytic activity was observed. The summary of the reconstitution data is shown in Table 4.

It was during the above reconstitution study that the pH dependency of the reconstitution was observed. Renatured apoproteins dialyzed in ACES (pH 6.5, 7.0, 7.5), Tris-HCl (pH 7.0, 7.5, 8.0), or HEPES (pH 7.5, 8.0, 8.5) were prepared. Reconstitution was initiated with the addition of 0.1 volume of 10 mM manganese chloride to the renatured apoproteins (75 μg/ml). Aliquots were withdrawn at different time points and the reactions were terminated by diluting in 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM EDTA. The specific activities of the samples were determined, which is shown in Figure 6. Reconstitution was not affected by the different buffer components, but it was extremely sensitive to changes in pH values. Complete restoration of the enzyme activities occurred within 20 min when apoprotein was reconstituted above pH 8.0; while three hours were required when apoprotein was reconstituted at pH 7.5. The rate of reconstitution was greatly diminished when apoprotein was reconstituted below pH 7.5.

Complete restoration of enzymatic activity in
reconstitutions also depends on the availability of manganese ions. Renatured apoprotein (75 μg/ml) prepared in 20 mM Tris-HCl (pH 8.0) was incubated with 1 μM, 10 μM, 100 μM or 1 mM manganese chloride. Aliquots of the samples were withdrawn and specific activities of the samples were determined. Figure 7 indicated that the reconstitutions were completed within 20 min in all conditions, but full enzymatic activity was only recovered in the presence of 100 μM or 1 mM manganese. Although the concentration of the apoprotein was around 1.5 μM, reconstitution with 10 μM and 1 μM manganese only restored 80% and 40% of the native activity, respectively.

Although zinc and ferrous ions were unable to restore enzymatic activity of the apoprotein, manganese-dependent reconstitution of the renatured apoprotein was inhibited by either zinc or ferrous ions (Figure 8). Incubation of apoprotein (75 μg/ml, pH 7.5) with 1 mM manganese and 50 μM zinc ions restored less than 10% of the control activity after 50 minutes. Incubation of the apoprotein with 1 mM manganese and 5 μM zinc ions restored 44% of the control activity after two hours. Although presented in 20-200 fold excess, manganese ions appeared to bind apoprotein less efficiently than zinc ions did. Apoprotein incubated with 1 mM manganese and 1 mM ferrous ions also resulted in the inhibition of reconstitution, which restored 45% of the control activity within three hours of observation.
Figure 6. pH-dependent reconstitution. Renatured apoprotein (75 μg/ml) was prepared in ACES (pH 6.5, 7.0, 7.5), Tris-HCl (pH 7.0, 7.5, 8.0) or HEPES (pH 7.5, 8.0, 8.5). Reconstitutions were initiated with the addition of manganese to 1 mM final concentration. Aliquots of samples were withdrawn and diluted in 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, and the enzyme activity of each sample was determined. pH 6.5, ◦; pH 7.0, ▲; pH 7.5, ◆; pH 8.0, ◄; pH 8.5, ◆.
Figure 7. Manganese concentration-dependent reconstitution. Renatured apoprotein (75 μg/ml; 1.5 μM) was prepared in 20 mM Tris-HCl (pH 8.0). Reconstitutions were initiated with the addition of manganese to final concentration of 1 μM, 10 μM, 100 μM, or 1 mM. Aliquots of samples were withdrawn and diluted in phosphate buffer (pH 7.8) containing 2 mM EDTA, and the enzyme activities were determined. Reconstitution was carried out in the presence of 1 μM (○); 10 μM (●); 100 μM (▲); or 1 mM (▲) manganese.
Figure 8. Inhibition of manganese reconstitution by zinc or iron. Renature apoprotein (75 μg/ml) was prepared in 20 mM Tris-HCl, pH 7.5. Reconstitutions were initiated with the addition of the following metals: 1 mM MnCl₂ (○), 1 mM MnCl₂ plus 1 mM Fe(NH₄)₂(SO₄)₂ (△), 1 mM MnCl₂ plus 5 μM ZnSO₄ (●), 1 mM MnCl₂ plus 50 μM ZnSO₄ (■). Aliquots of samples were withdrawn and diluted in 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, and enzyme activity of each sample was determined.
5. Intrinsic Fluorescence Study of Superoxide Dismutase

Intrinsic fluorescence spectra of the native-, manganese reconstituted-, and renatured apo- superoxide dismutases are shown in Figure 9. The excitation maximum (292 nm) and emission maximum (329 nm) of these proteins were found to be identical. This indicated that the fluorophores within the native-, manganese reconstituted-, and renatured apo-superoxide dismutases were in a similar molecular environment. The secondary and tertiary structures of the superoxide dismutase may therefore be similar irrespective of the presence of manganese in the active sites. The fluorescence excitation and emission spectra of the denatured apoprotein in 5 M guanidine hydrochloride were recorded as control spectra. The red shift of the emission maximum (352 nm) of the denatured apoprotein is consistent with the exposure of the fluorophores to a more hydrophilic environment than that of the native superoxide dismutase.

6. Effect of Salts on Superoxide Dismutase Activity

The effect of selected salts on superoxide dismutase activity was investigated using the o-dianisidine and riboflavin augmentation assay. This assay is less sensitive to interference by salts than the xanthine-xanthine oxidase assay. Salt (Na₂SO₄, KCl, NaCl, NaNO₃, NaClO₄) solutions were
Figure 9. Intrinsic fluorescence emission spectra of *D. radiodurans* SOD. Native MnSOD (0.21 mg/ml) and manganese reconstituted SOD (0.31 mg/ml) were prepared in 50 mM potassium phosphate (pH 7.8) containing 1 mM EDTA. Renatured apoprotein (0.2 mg/ml) was prepared in 20 mM Tris- HCl, pH 7.5. The excitation spectra and emission spectra of the native MnSOD, renatured apoprotein, and manganese reconstituted SOD were identical, which were indicated by solid line and dashed line, respectively. The emission spectrum of the denatured apoprotein (1.5 A_280 absorbance unit) prepared in 5 M guanidine hydrochloride was also recorded (dotted line).
Figure 10. Effect of salts on SOD activity. The photo-augmentation assay employed riboflavin and o-dianisidine (86) was used in this study. Native SOD (1.7 μg) was present in each assay mixture, and the reaction was initiated by illumination. The absorbance changes at 460 nm were recorded, and the enzymatic activities in different salts or salt concentrations were recorded as percentage activity relative to the enzymatic activity obtained in the absence of salts (25 mM potassium phosphate, pH 8.0). The dashed line (---) represents the enzymatic activities obtained in different concentrations of phosphate buffer; and solid lines represents the enzymatic activities obtained in different salts: Na₂SO₄ (○), KCl (○), NaCl (△), NaNO₃ (▽), and NaClO₄ (◇).
prepared in 25 mM potassium phosphate (pH 8.0) to the desired concentrations, and the SOD enzymatic activity in each solution was determined as percentage activity relative to the activity obtained in 25 mM potassium phosphate (pH 8.0). As shown in Figure 10, high salt concentrations and large monoanions inhibited the enzymatic activities effectively (NaClO₄ > NaN₃ > NaCl and KCl > Na₂SO₄). This suggested that either the enzyme was sensitive to salt denaturation or the salts were interfering the catalytic processes of the enzyme, presumably by competing with the substrate for the enzyme’s active site.

6. Western Analysis of the Superoxide Dismutase

The polyclonal anti-MnSOD antiserum was obtained, and the titer of the antibody was determined by ELISA. The serum diluted 100- or 1000-fold was incubated with serial dilutions of purified D. radiodurans SOD (0.1 ng to 75 ng) immobilized on a 96-well microtiter plate. After incubation with secondary antibody (goat anti-rabbit antibody horseradish peroxidase conjugate), the peroxidase substrate ABTS (2,2’-azino-di[3-ethyl-benzthiazoline sulphonate]) was added for the chromogenic detection of the antigen-antibody complex. The serum diluted 1000-fold was able to detect 5 ng of antigen, and serum diluted 100-fold was able to detect 0.75-1 ng of
antigen. Since our studies usually involved antigens in the microgram range, the serum diluted 1000-fold was sufficient for the following studies.

Due to the high degree of similarity among the prokaryotic SODs (50), the cross-reactivities of the *D. radiodurans* antibody with SODs from *E. coli* and from other *Deinococcus spp.* were investigated. Western analysis was performed on purified superoxide dismutases from *D. radiodurans* and *E. coli*, and cell extracts from *D. radiodurans*, *D. erythromyxia* (ATCC 187), *D. radiophilus* (ATCC 27603) and *D. radiopugnans* (ATCC 19172) (Figure 11). The antibody recognized the 23 kDa peptide in the samples containing either purified MnSOD (lane 6) or cell extract (lane 2) from *D. radiodurans*. The molecular mass of the antigen recognized by the antibody is consistent with the result obtained from SDS-PAGE. The purified manganese-containing superoxide dismutase from *E. coli* (lane 7) showed cross-reactivity with the antibody, which suggested high degree of similarity between the two manganese-containing superoxide dismutases. There was no detectable cross-reactivity between the antibody and the crude extracts from either *D. erythromyxia* (lane 3) or *D. radiophilus* (lane 5). However, a protein in the *D. radiopugnans* cell extract (lane 4) was also recognized by the antibody. This protein had a molecular mass similar to the *D. radiodurans* and *E. coli*
MnSODs. Western analysis of the purified *E. coli* FeSOD showed no cross-reactivity with the antibody (data not shown). The experiment suggested that the band recognized by the antibody in the crude extract of *D. radiopugnans* might be a manganese-containing superoxide dismutase.

A minor band in the purified *D. radiodurans* SOD sample (lane 6) was also observed on western blot, which had an apparent molecular mass of 42 kDa. Since the 42-kDa peptide was present in the sample containing purified SOD, it could be (a) the dimerized SOD electromorphs, or (b) a contaminant protein which cross-reacted with the antiserum, or (c) a co-purified contaminant protein which was co-injected into the rabbit at the time of antibody production. Since there is no cysteine residue in SOD, this 42-kDa peptide is not likely to be the dimerized SOD electromorphs. However, the presence of this 42-kDa peptide on the western blot suggested that the purified *D. radiodurans* SOD sample contained both proteins, irrespective of the cross-reactivity of the antiserum with the 42-kDa peptide. Moreover, the possibility of cross-reaction could be resolved by affinity purification of the polyclonal antibody. The antibody was affinity-purified as described in the previous section, and the affinity-purified antibody was used to probe the SOD sample from the same preparation as above. The result in Figure 12 indicated that the antiserum reacted with SOD and the 42-kDa peptides (lane 1), while
Figure 11. Western analysis of SODs. Protein samples were separated on a 12% SDS gel and transferred to nitrocellulose. Prestained molecular weight markers were used to visualize the protein transfer. The blot was incubated with *D. radiodurans* MnSOD antiserum (1:1000), followed by goat anti-rabbit antibody HRPO-conjugate. The antigen-antibody complex was visualized with the addition of 4-chloro-1-naphthol and hydrogen peroxide. Lanes 1 and 8: prestained molecular weight markers. Lane 2: cell crude extract from *D. radiodurans*. Lane 3: cell crude extract from *D. erythromyxa*. Lane 4: cell crude extract from *D. radiopugnans*. Lane 5: cell crude extract from *D. radiophilus*. Lane 6: purified MnSOD from *D. radiodurans*. Lane 7: purified MnSOD from *E. coli*. 
Figure 12. Western analysis of the purified SOD using affinity-purified antibody. Purified SOD sample separated on a 12% curtain gel was transferred to nitrocellulose. The strip of nitrocellulose was incubated with antiserum or affinity-purified antibody prepared as described by Olmsted (89). The blots were incubated with protein A HRPO-conjugate, and were developed with the addition of DAB and hydrogen peroxide. Lane 1: western blot using antiserum. Lane 2: western blot using affinity purified antibody.
affinity-purified antibody only recognized the SOD peptide (lane 2). This result suggested that the antibody directed against superoxide dismutase was unable to cross-react with the 42-kDa peptide, and this 42-kDa peptide was a contaminant protein co-purified with the SOD, which also co-immunized the rabbit at the time of antibody production. However, comparing the crude extract of *D. radiodurans* and the purified SOD on western blot (Figure 11), we noticed that the 42-kDa peptide was strongly enriched during the purification of SOD. This indicated that the 42-kDa peptide had very similar ionic characteristics and apparent molecular mass with SOD, making the separation of the two proteins difficult. Also, due to the sensitivity of the detection system, the presence of the contaminant protein had not been recognized in SDS gel stained with Coomassie blue G-250 (Figure 3) until the employment of antibody in the study.

A chromatographic method for separating the 42-kDa protein from SOD was achieved by denaturing gel filtration on Superose 12 column of FPLC. Since the SOD peptide is able to regain its activity upon reconstitution, the SOD can be denatured in 5 M guanidine hydrochloride, separated from the 42-kDa protein as a SOD monomer, and reconstituted into a fully reactive SOD. This purification procedure involved the denaturation and reconstitution of the protein, therefore, all of the protein obtained would have
been reconstituted.

A Superose 12 gel filtration column equilibrated in 5 M guanidine hydrochloride and 50 mM Tris-HCl (pH 8.0) was prepared. *D. radiodurans* cell extract prepared in 5 M guanidine hydrochloride and 50 mM Tris-HCl (10 mg/100 μl) was applied and eluted in the same buffer. Fractions (2 ml) were collected, and protein samples were dialyzed in several changes of 50 mM Tris-HCl (pH 7.5) containing 1 mM manganese chloride. Aliquots of the fractions were separated on 10% SDS gel and the proteins were subsequently analyzed by western blotting and Coomassie blue staining. Since the majority of the proteins in the cell extract were denatured and precipitated after dialysis, the SOD was actually enriched during the denaturation and reconstitution process. Figure 13 shows the analysis of the fractions 11-14, where panel A is the Coomassie blue staining, and panel B is the western blot of the duplicate gel. The blot revealed that the majority of the superoxide dismutase was included in fractions 12-14 (lanes 3-5), and all of the 42-kDa protein was included in fraction 11 (lane 2). This experiment demonstrated that the SOD and 42-kDa peptide could be separated by denaturing gel filtration chromatography. However, the gel in panel A indicated that the fractions containing SOD were contaminated with other smaller peptides, although almost all of the peptides larger than SOD monomer were included in fraction 11.
Figure 13. Protein and western analysis of the denaturing gel filtration chromatography. Cell extract of *D. radiodurans* was applied to Superose 6 column of FPLC equilibrated in 5 M guanidine hydrochloride and 50 mM Tris-HCl (pH 8.0). The proteins were eluted with the same buffer and 2 ml fractions were collected. The proteins were dialyzed in several changes of 50 mM Tris-HCl (pH 7.5) containing 1 mM manganese chloride, and the supernatant of the dialysate of each fraction was analyzed by protein staining and western blotting. Panel A: Coomassie blue staining of the fractions 11-14 (lanes, 2-5). Panel B: western blot of the fractions 11-14 (lanes 2-5). Lane 1 in panel A and lanes 1 and 6 in panel B are prestained molecular mass markers.
Since the contamination by small peptides was not encountered using the SOD purification procedure described in the beginning of this chapter, these small peptides ought to be separated from SOD during one of the purification steps. However, detailed procedure for the novel purification of SOD needed to be worked out if this is to be pursued.
DISCUSSION

The apoprotein from *D. radiodurans* manganese superoxide dismutase, in the absence of denaturant, bound 2 gram-atom of manganese ion per mole dimer to the renatured apoprotein with full restoration of catalytic activity. Reconstitution of apoprotein with metal is one criterion of metal specificity and function in metalloproteins. The approach of reconstitution of prokaryotic superoxide dismutases used by other workers requires denaturation to remove the intrinsic metal and then addition of various metals with simultaneous removal of denaturing conditions. Reconstitution of the denatured apoprotein to 7-90% of initial specific activity has been reported for the following bacterial enzymes: *E. coli* manganese superoxide dismutase (30, 31), *B. fragilis* iron superoxide dismutase (48), and *B. thetaiotaomicron* iron superoxide dismutase (59).

The rate of reconstitution was greater at pH 8.0 than at pH 7.5, perhaps due to the deprotonation of critical residues at the metal binding sites or due to the conformational change of the protein upon binding of the metal ions.

Zinc and iron each inhibited the manganese-dependent reconstitution of the apoprotein but were bound with different stoichiometries. Zinc ions bound approximately 2 gram-atom to each mole dimer of renatured apoprotein, and might inhibit the
manganese-dependent reconstitution by competing with manganese ions to the metal binding sites on the protein. Ferrous ions bound 3 to 7 gram-atom to each mole dimer of renatured apoprotein. The non-specific binding of the ferrous ions to the renatured apoprotein might induce the conformational change of the protein and preclude the manganese-dependent reconstitution.

The variability of iron binding to apoprotein was reflected in the competition between iron and manganese for apoprotein. Reconstitution of the superoxide dismutase with equimolar iron and manganese ions resulted in the variations of specific activities from 1200 to 2200 unit/mg. The variability may result from the oxidation of iron in the reconstitution mixture. Significantly less variability was noted upon reconstitution with manganese in competition with 5 μM or 50 μM zinc.

The identical intrinsic fluorescent spectra of the renatured apoprotein, native protein and the manganese reconstituted protein suggested that the renatured apoprotein had a gross structure similar to the native and the manganese reconstituted proteins. This result is consistent with a model in which the apoprotein, freed of denaturant, assumes a gross conformation which readily accepts manganese with restoration of catalytic activity. The manganese reconstituted superoxide dismutase, like the isolated enzyme, is stable to dialysis
against EDTA-containing buffers. Binding of manganese at the active site may induce local conformational rearrangements that cause the metal to be more tightly bound.

Preparation and reconstitution of aposuperoxide dismutases have been studied using other approaches. Yamakura (38) prepared aposuperoxide dismutase from *Pseudomonas ovallys* iron superoxide dismutase using 2 mM EDTA and 10 mM dithiothreitol in sodium carbonate (pH 11.0). Iron, added to the apoprotein in the alkaline thiol-containing buffer, restored both iron and catalytic activity. In buffer at pH 7.8, the apoprotein was not catalytically reactivated upon addition of the iron salt.

Meier et al. (49) used ascorbate and o-phenanthroline at pH 5.5 to remove metal from *Propionibacterium shermanii* manganese superoxide dismutase. The apoprotein, which retained 34% of the initial catalytic activity, was further activated upon reconstitution with either iron or manganese at pH 5.5. Dialysis of the apoprotein at pH 5.5 for longer than 24 hours reportedly caused precipitation of the protein. Martin et al. (39) used the same approach to reconstitute *Streptococcus mutans* manganese superoxide dismutase. Apparently the apoprotein was not reconstituted in either case under conditions other than those used to form the apoprotein.

Procaryotic superoxide dismutases may now be divided into two classes: enzymes having an absolute metal ion
specificity and those active with either iron or manganese. Among the latter are the iron superoxide dismutases from the anaerobes *B. fragilis*, *B. thetaiotaomicron*, and *P. shermanii* (58, 59, 49) and the manganese superoxide dismutase from *S. mutans* (39). Apoprotein from each of these enzymes was reactivated upon the addition of either iron or manganese, a property termed "cambialistic" by Martin et al. (39). On the other hand, the renatured apoprotein from *D. radiodurans* was not active upon binding iron. To determine if renaturing the apoprotein precluded iron-dependent activity, I reconstituted the denatured *D. radiodurans* apoprotein with manganese or ferrous ions. Although more than 75% of the original enzymatic activity was recovered upon reconstitution with manganese ions, less than 0.3% of the original activity was recovered upon reconstitution with 1 mM ferrous ions (data not shown).

The specificity of antibody and the similar primary structures of prokaryotic SODs enable me to examine the SODs from different sources. The antiserum raised against *D. radiodurans* MnSOD cross-reacted with the *E. coli* MnSOD but did not cross-react with the *E. coli* FeSOD. Since all MnSODs share high degree of similarity within the primary structures, the cross-reactivity of the antibody with the peptide in *D. radiopugnans* cell crude extract suggested the presence of MnSOD peptide. The molecular mass of the cross-reacting peptide of *D. radiopugnans* was similar to the *E. coli* and *D.*
radiodurans MnSOD peptides, which was also consistent with the presence of MnSOD peptide. However, the lack of cross-reacting species in the cell extracts of *D. erythromyxa* and *D. radiophilus* may not necessarily mean the absence of MnSOD peptides.

The presence of the 42-kDa peptide in the purified SOD preparation was not detected until the analysis by antibody. The western blot using the affinity purified SOD antibody revealed that the SOD antibody did not cross-react with the contaminant peptide. The contaminant peptide, though present only in a minute amount, caused the production of a specific antibody in addition to the SOD antibody. Although the presence of the contaminant peptide did not interfere with the studies described above, the specific antibody against it complicated the molecular cloning of the SOD gene (see Appendix).

The application of antibody not only provides a very sensitive method to reexamine the purity of SOD, it also provides an alternative tool for the affinity purification of the enzyme. Since the affinity-purified SOD antibody was available, an affinity column could be prepared by cross-linking the affinity purified antibody to CNBr-activated Sepharose (91). The affinity column thus made is able to specifically purify the SOD in a simple one-step procedure. However, a chromatographic method for the separation of 42-kDa
protein from SOD was demonstrated by using denaturing gel filtration on Superose 12 column of FPLC. This procedure took advantage of the reconstitution character of the \textit{D. radiodurans} apoprotein which regained full enzymatic activity upon removal of the denaturant and addition of manganese, and a novel purification of SOD can be explored employing denaturing gel filtration chromatography. Although the presence of the contaminant peptide did not interfere with the studies, care should be taken, however, in examining the purity of the protein especially if the protein is to be used for antibody generation.
Chapter 3.
Molecular Cloning of the Gene Encoding
Superoxide Dismutase from Deinococcus radiodurans
INTRODUCTION

In biological systems, a variety of important processes are accompanied by the formation of superoxide radicals ($O_2\cdot^-$). These superoxide radicals and their by-products that occur spontaneously in the course of aerobic life include hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($\cdotOH$) (21). Ionizing radiation, which causes the decomposition of water, also produces a variety of free radicals. These highly reactive radicals may generate superoxide radicals in the presence of oxygen (92). Endogenous protection of the cell from these superoxide radicals can occur through the enzymatic decomposition of hydrogen peroxide by catalase, and through the enzymatic degradation of superoxide radicals by superoxide dismutases (SODs).

Superoxide dismutases are metalloenzymes that catalyze the dismutation of superoxide radicals to hydrogen peroxide. The deleterious effects which superoxide radicals have upon cells are reduced by the action of superoxide dismutases which thus protect the cells from oxygen toxicity. Three forms of superoxide dismutases, distinguished by their metal content, are found in nature: Cu/Zn-, Fe-, and Mn-containing enzymes (41). While all three forms of superoxide dismutases catalyze the same reaction, iron-containing superoxide dismutases are
confined to prokaryotes and Cu/ZnSODs to eukaryotes. Manganese-containing superoxide dismutases are present in both prokaryotes and in mitochondria of eukaryotic cells (34).

A manganese-containing superoxide dismutase has been isolated and characterized from a red-pigmented gram-positive bacterium Deinococcus radiodurans. This enzyme is a 43-kDa protein composed of two identical subunits (93). D. radiodurans has generated long standing interest because of its extremely high resistance to ionizing and ultraviolet radiation (94, 95). Its high DNA GC content (70 mol% G + C), unusual cell wall structure, and cell wall amino acid and lipid compositions have been well studied (96 – 98). Studies on radiation resistance of D. radiodurans have focused primarily on the efficient DNA repair systems (77 – 79). However, D. radiodurans also contains 2- to 5-fold and 15- to 400-fold the activity of SOD and catalase, respectively, compared to other aerobic bacteria (74). The high level of SOD and catalase are presumably important for the protection of the cells from oxidative stress produced by radiation. It is therefore of interest to investigate the molecular biology of the structural gene which encodes D. radiodurans MnSOD. Characterization of this SOD gene provides a better understanding of superoxide dismutase structure and provides tools to study the structure-function relationships as well as the regulation of SOD in D. radiodurans. In this chapter,
cloning and sequencing of the structural gene of \textit{D. radiodurans} MnSOD will be described.
EXPERIMENTAL PROCEDURE

Materials

Tris-(hydroxymethyl)aminomethane (Tris), ampicillin, kanamycin, ethylenediaminetetraacetic acid sodium salt (EDTA), hexaminecobalt (III) chloride, 2-[N-morpholino]ethanesulfonic acid (MES), sodium citrate, urea, glycerol (molecular biology grade), polyethylene glycol (MW 8000), sodium dodecyl sulfate (SDS), ammonium sulfate (molecular biology grade), casein enzymatic hydrolysate (NZ Amine A), isoamyl alcohol, isopropanol anhydrous (molecular biology grade), glucose, dimethyl sulfoxide (DMSO), sucrose (molecular biology grade), and Kodak X-OMAT™ AR film were obtained from Sigma Chemical Co., St. Louis, MO.

Chloroform, acrylamide, formamide, N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Fisher Chemical Co., St. Louis, MO.

Peptone, tryptone, yeast extract, agar, double-stranded DNA Cycle Sequencing System, DNA marker 1 Kb Ladder, agarose gel electrophoresis apparatus Horizon™ 58, agarose (electrophoresis grade), and sequencing gel electrophoresis system Model S2 were purchased from Gibco BRL, Grand Island, NY.

EMBL3 Cloning Kit, Gigapack®II Plus Packaging Extract, QuikHyb™ Rapid Hybridization Solution, Duralose-UV™ membrane,
Duralon-UV™ membrane, Prime-It™ Random Primer Kit, Stratalinker™ UV Crosslinker 1800, and the plasmid pBluescript II SK were obtained from Stratagene, La Jolla, CA.

Deoxyadenosine 5′-triphosphate (dATP), deoxyguanosine 5′-triphosphate (dGTP), deoxycytidine 5′-triphosphate (dCTP), deoxythymidine 5′-triphosphate (dTTP), N',N'-methylene-bis-acrylamide, ammonium persulfate, and Bio-Spin® 30 chromatography columns were purchased from BioRad, Melville, NY.

[α-35S]dATP (specific activity > 3000 Ci/μmol), [α-32P]dCTP (specific activity > 3000 Ci/μmol), [γ-32P]adenosine 5′-triphosphate (ATP) (specific activity > 6000 Ci/μmol) were obtained from Amersham, Arlington Height, IL.

Isopropyl-β-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal), Sequenase® Version 2.0 DNA sequencing kit, TAQuence™ Version 2.0 DNA sequencing kit, T7 gene 6 exonuclease, Tris-buffered phenol were obtained from USB, Cleveland, OH. Taq DNA polymerase, which was used in conjunction with the TAQuence™ Version 2.0 DNA sequencing kit, was obtained from Dr. Wayne Barnes in the Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis.

Magic Minipreps™ plasmid DNA isolation kit, restriction enzymes including Sau3AI, BamHI, ApaI, SalI, SmaI, XmaI, NotI,
EcoRI, XbaI and Taq DNA polymerase for polymerase chain reactions (PCR) were obtained from Promega, Madison, WI.

The GENE CLEAN® Kit for extracting DNA from agarose gel was obtained from BIO 101 Inc., La Jolla, CA. The QIAGEN Tip-20 Lambda Kit for purifying lambda DNA was purchased from QIAGEN Inc., Chatsworth, CA. The TA Cloning™ System for plasmid cloning of PCR products was purchased from Invitrogen, San Diego, CA.

The thermal cycler used in this study was TwinBlock™ System EasyCycler™ from Ericomp Inc., San Francisco, CA. PCR reaction tubes and mineral oil were purchased from Perkin-Elmer Cetus Corp., Norwalk, CT. The Bausch and Lomb Spectronic 21 spectrophotometer was used for measuring cell density. The Varian DMS200 UV visible spectrophotometer was used for the determination of DNA concentration. The ultracentrifuge used was Beckman® L8-M70, and the rotor was type SW 41. All water used in this study was purified to 18.2 MΩcm with a Milli-Q UF Plus system from Millipore, Bedford, MA.

Methods

Bacterial strains and culture conditions: Deinococcus radiodurans from ATCC (13939) was the source of DNA isolation, which was grown in PYG medium (135) supplemented with hemin (0.5% w/v) at 30°C overnight. The cells were harvested, washed with 50 mM potassium phosphate buffer (pH 7.8), and
subsequently lyophilized and stored at -20°C until use.

Propagation and selection of recombinant bacteriophage lambda EMBL3 clones were carried out in *E. coli* strains LE392 and P2392, respectively. These strains were usually maintained in lauria broth medium (LB). For infection of bacteriophage, culture was grown at 30°C in tryptone broth (TB) supplemented with 0.2% w/v maltose.

*E. coli* strain DH5α was routinely used for transformation studies. The culture was usually maintained in M9 minimal salt medium, and grown in SOB medium for transformation. The *E. coli* strain DH5αF' was used as the host of transformation in subcloning DNA fragments generated from polymerase chain reactions (PCR). The culture conditions for DH5αF' were the same as for DH5α. The genetic makeup of the bacterial strains are listed below:

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LE392 F' *hsdR514*(r<sup>K</sup>; m<sup>K</sup>*) supE44 supF58 lacY1 or Δ(lacIZY)6 galK2 galT22 metB1 trpR55

P2392 F' *hsdR514*(r<sup>K</sup>; m<sup>K</sup>*) supE44 supF58 lacY1 or Δ(lacIZY)6 galK2 galT22 metB1 trpR55 P2<sup>+</sup>

DH5α F' φ80dlacZΔM15 endA1 recA1 *hsdR17*(r<sup>K</sup>; m<sup>K</sup>*) supE44 thi-1 λ' gyrA96 relA1 Δ(lacZYA-argF)U196

DH5αF' F' φ80dlacZΔM15 endA1 recA1 *hsdR17*(r<sup>K</sup>; m<sup>K</sup>*) supE44 thi-1 λ' gyrA96 relA1 Δ(lacZYA-argF)U196

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The components of the media used in this study are listed below:
PYG medium: 0.5% w/v peptone, 0.5% w/v trypticase soy broth, 1% w/v yeast extract, 1% w/v glucose, 4% v/v VPI salt

VPI salt: 0.02% w/v CaCl₂, 0.02% w/v MgSO₄, 0.1% w/v K₂HPO₄, 0.1% w/v KH₂PO₄, 1% w/v NaHCO₃, 0.2% w/v NaCl

LB medium: 1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl

TB medium: 1% w/v tryptone, 0.5% w/v NaCl, 10 mM MgSO₄

M9 medium: 50 mM Na₂HPO₄, 20 mM KH₂PO₄, 0.05% w/v NaCl, 20 mM NH₄Cl, 0.4% w/v glucose

SOB medium: 2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄

NZYM medium: 1% w/v NZ Amine, 0.5% w/v yeast extract, 0.5% w/v NaCl, 15 mM MgSO₄

Phage and plasmids: Bacteriophage lambda EMBL3 was used as cloning vector for genomic library construction. The lambda EMBL3 system takes advantage of SpI (sensitive to P2 inhibition) selection which allows the recombinant phages to grow on host strains which are P2 lysogens (99). Non-recombinant EMBL3 contains active red and gam genes in the stuffer fragment and is therefore unable to grow in host strains lysogenic for P2. The lambda EMBL3 vectors were prepared so that they were capable of accepting BamHI compatible DNA fragments.
Plasmid pBluescript II SK was a high copy number plasmid which was used for subcloning and sequencing of the cloned gene. pBluescript II SK carried ampicillin resistant gene which allowed only the transformed host to grow on medium containing ampicillin. It also contained the lacZ α-complementation fragment for blue/white color selection of recombinant plasmids.

Linearized plasmid PCR™1000 from Invitrogen was used for the subcloning of PCR fragments. The thermostable polymerase used in PCR reactions usually adds a single deoxyadenosine (dA) to the 3’-end of all duplex molecules in a template-independent fashion. The PCR™1000 digested with endonuclease HphI provided a single 3’ dT-overhang at both ends of the insertion site. Thus, the PCR fragments were able to be cloned in this vector with high efficiency.

Agarose gel electrophoresis: Gels ranging from 0.3% to 1.5% (w/v) agarose were routinely used depending on the sizes of the DNA fragments to be separated. Agarose gels were prepared by dissolving agarose in TAE buffer (40 mM Tris-acetate pH 8.5, 1 mM EDTA), and the DNA was electrophoresed in the same buffer at 60 volts, room temperature. Gels were stained with 0.5 μg/ml ethidium bromide, destained with TAE, and DNA bands were visualized with a UV transilluminator.
Transformation: Transformation of *E. coli* hosts with plasmid DNA was carried out using a modified Hanahan procedure (100). A fresh overnight culture was diluted to an A_{600} of 0.1 with fresh SOB medium. The culture was grown at 37°C with shaking until A_{600} reached 0.3. The culture was chilled on ice and pelleted by centrifugation. The cells were washed twice with TFB (10 mM MES pH 6.3, 45 mM MnCl₂, 10 mM CaCl₂, 100 mM KCl, 3 mM hexaminecobalt (III) chloride), and resuspended in TFB (0.08 of original volume) followed by DMSO (0.0028 of original volume). The cells were incubated on ice for 15 min before another aliquot of DMSO was added. The competent cells were prepared fresh for each transformation.

Transformation was initiated with the addition of DNA (less than 100 ng) to 200 µl of competent cell suspension. The cells were heat-shocked (42°C for 90 sec) after 30 min of incubation on ice. SOB medium containing 20 mM glucose (800 µl) was added and the cells were incubated at 37°C with shaking for 45 min before plating.

Plasmid purification: Small scale purification of plasmid DNA was routinely carried out by using the Magic Miniprep™ (Promega) DNA purification system. The instruction protocol from the manufacturer was followed. The quality and concentration of the plasmid DNA were verified by agarose gel
electrophoresis.

If the plasmid DNA was to be used for subcloning, a scale-up procedure was followed. Overnight culture (50 ml) was harvested and resuspended in 5 ml of 50 mM Tris-HCl pH 7.5, 10 mM EDTA. The cells were lysed with 5 ml of 0.2 N NaOH and 1% SDS, and the RNase A (2 μg/ml) was added to the supernatant after the cell debris was pelleted. The mixture was extracted twice with equal volume of phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with ethanol, and the pellet was dissolved in a solution containing 1.67 ml of water, 0.33 ml of 5 M NaCl, and 2 ml of 13% w/v PEG (MW 8,000). The mixture was incubated on ice for 1 h and the plasmid DNA was pelleted by centrifugation (10,000 x g, 15 min). The plasmid DNA was washed with 80% ethanol and dissolved in 500 μl of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Spectrophotometric quantitation of plasmid DNA was performed by using the extinction coefficient of 0.02 ml/μg at 260 nm.

Genomic DNA isolation: The procedure described by Maniatis et al. (101) was followed for the isolation of genomic DNA with some modifications.

Lyophilized D. radiodurans cells (0.05 g) were resuspended in 50 ml of butanol-saturated phosphate buffer
(4.7 g Na₂HPO₄, 4.6 g KH₂PO₄, 3.7 g EDTA, 60 ml n-butanol, distilled water to 1 liter). Butanol pretreatment rendered the cells sensitive to lysozyme degradation (102). The cells were pelleted and resuspended in 5 ml 50 mM Tris-HCl (pH 8.0) containing 50 mM EDTA. Lysozyme (5 mg) was added to the cell suspension and the mixture was incubated on ice for 45 min. One ml of STEP solution (0.5% w/v SDS, 50 mM Tris-HCl pH 7.5, 0.4 M EDTA, 1 mg/ml Proteinase K) was added to the cell suspension and the mixture was incubation at 50°C for the lysis to occur. The DNA was extracted with phenol (3-4 times), then with chloroform/isoamyl alcohol (24:1). The DNA was precipitated and spooled out of the solution in the presence of 0.1 M sodium acetate and 50% ethanol. The DNA was washed in 80% ethanol and subsequently dissolved in 10 ml of TE buffer. The DNA concentration was determined from A₂₆₀.

Genomic library construction: Genomic DNA was partially digested with various amounts of endonuclease Sau3AI. The enzyme/DNA ratios for the digestions were calculated to be 0.250, 0.125, 0.063, 0.031, and 0.016 unit/µg DNA. The reactions were incubated at 37°C for 30 min, and terminated by the addition of EDTA (5 mM) as well as heating (70°C, 15 min). Aliquots of the samples were analyzed on a 0.4% agarose gel.

The digested DNA fragments were fractionated on a 10%--
30% sucrose gradient by centrifuging at 35,000 rpm in a SW 41 rotor (Beckman) for 18 hours at 4°C. The DNA fractions (6-7 drops) were collected from the bottom of the centrifuge tubes, and DNA sample (35 μl) from each fraction was analyzed in a 0.3% agarose gel. The fractions containing DNA 9 to 23 Kb in size were pooled and concentrated on Centricon-30 (Amicon).

Ligation was performed by mixing the size fractionated genomic DNA (0.45 μg) with Bam HI predigested lambda EMBL3 arms (1 μg) as well as 2 units of ligase. The ligation mixture was incubated at 4°C for 3 days before it was examined by agarose gel electrophoresis.

Packaging was achieved by mixing the ligated DNA with packaging extracts from Stratagene (Gigapack® II Plus Packaging Extract). The instructions provided by Stratagene were followed for this process. The packaged phage particles were stored in 500 μl of SM buffer (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl pH 7.5, 0.01% w/v gelatin) and 20 μl of chloroform before the titer was determined.

**Titer determination and amplification of lambda EMBL3 library:**

E. coli strain P2392 was used for initial amplification and titer determination of the packaged lambda EMBL3 genomic library. P2393 was grown in TB medium containing 0.2% w/v maltose at 30°C overnight. The cells were harvested and
resuspended in 10 mM MgSO₄. Diluted cells (λ₆₀₀ ~0.5, 200μl) were mixed with 20 μl of the appropriate dilutions (10³ to 10⁻⁴) of the packaged lambda, and the mixture was incubated for 30 min at room temperature. The infected cells were plated on a 90 mm TB plate which was incubated at 37°C for 6-8 hours. The titer of the bacteriophage was determined as pfu/μl (plaque forming unit/μl).

The EMBL3 library was amplified by plating 500,000 plaques in ten 150 mm plates. After incubation, each plate was covered with 8 ml SM buffer and incubated overnight at 4°C with gentle shaking. The SM buffer containing the bacteriophages was recovered, and 0.01 volume of chloroform was added. The amplified library was titered and stored at 4°C until use.

After initial amplification, E. coli strain LE392 was used as the host for lambda EMBL3 propagation in the subsequent studies.

Oligonucleotide synthesis: The oligonucleotides used in this study for both polymerase chain reaction and sequencing experiments were synthesized by Protein Chemistry Laboratory, Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis. The sequences of the oligonucleotides are presented in page 103.
Polymerase chain reaction (PCR): PCR is a technique utilizing flanking oligonucleotide primers and repeated cycles of DNA synthesis to amplify picogram quantities of target DNA sequences in vitro (103, 104). The method is based on the repetition of three consecutive steps: denaturation of templates, annealing of primers, and the polymerase-mediated 5'→3' extensions of the primer-template complexes. Since the melting of DNA templates takes place at elevated temperatures, a thermal stable Taq DNA polymerase isolated from Thermus aquaticus is used in the reaction.

The concentrations of the components for polymerase chain reactions were derived from the standard reaction described by Saiki et al. (105). The following condition was optimized for the amplification of the D. radiodurans superoxide dismutase gene:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA 50 ng/µl</td>
<td>5</td>
</tr>
<tr>
<td>10 X Taq DNA polymerase reaction buffer</td>
<td>10</td>
</tr>
<tr>
<td>Primer (SOD 1) 100 ng/µl</td>
<td>2</td>
</tr>
<tr>
<td>Primer (SOD 2) 100 ng/µl</td>
<td>2</td>
</tr>
<tr>
<td>Mixture of 2.5 mM each of dA, dT, dC, dG</td>
<td>8</td>
</tr>
<tr>
<td>Taq DNA polymerase 3 unit/µl</td>
<td>1</td>
</tr>
<tr>
<td>Milli-Q water</td>
<td>72</td>
</tr>
</tbody>
</table>

Each reaction mixture was overlaid with 50 µl of mineral oil and then placed into the thermal cycler. The cycles for this reaction were the following:
1 cycle: 92°C 3 min.
35 cycles: 55°C 30 sec.
72°C 1 min.
92°C 30 sec.
1 cycle: 55°C 30 sec.
72°C 10 min.

The reaction mixture (5 µl) was analyzed on a 1.5% agarose gel after the cycles were completed.

**TA Cloning™ of PCR products:** PCR products were subcloned into plasmid by TA Cloning™ system which contained linearized pCR™1000 plasmid. The resulting recombinant superoxide dismutase clones were designated as TA clones. TA Cloning™ instruction manual was followed for the process.

**Isolation of DNA separated on agarose gel:** DNA fragments separated on agarose gels were recovered by excising the gel containing the desire DNA bands and purified by GENECLEAN® Kit following instructions provided by the manufacturer.

**DNA random primers labeling:** DNA fragments isolated from agarose gel was labeled by [α-32P]dCTP (specific activity > 3000 Ci/mmol) with PRIME-IT™ random primer kit. The instruction manual from the manufacturer was followed. The unincorporated nucleosides were separated from the labeled DNA using Bio-Spin® 30 Column. The labeled DNA was collected by
centrifugation (4 min, 1,500 x g), while the unincorporated nucleosides remained in the column. The radioactivity of the labeled DNA was measured on Probe Count™ from Oncor.

**Southern hybridization:** Southern hybridization, which was first described by Southern (106), involved the probing of the immobilized DNAs with radiolabeled probes. Three different methods for DNA immobilization on nitrocellulose or nylon membranes were used in this study, which were adapted from Molecular Cloning (107) with modifications.

DNA fragments separated on agarose gels were denatured in denaturation solution (0.5 M NaOH, 1.5 M NaCl), followed by several changes in neutralization solution (0.5 M Tris-HCl pH 7.4, 1.5 M NaCl). The gel was placed against a piece of Duralon-UV™ nylon membrane, and the DNA was transferred in 10 X SSPE (1.5 M NaCl, 0.1 M NaH₂PO₄, 10 mM EDTA) with capillary action. After overnight transfer, the DNA was UV-crosslinked to the membrane by Stratalinker™ 1800 (auto crosslink mode: 120,000 microjoules/cm²).

Plaque lift hybridization involved the transferring of DNA from plaques to nylon or nitrocellulose membranes. A membrane was overlaid on a plate, and the DNA on the membrane was denatured and neutralized, followed by crosslinking as described above.
Dot blot hybridization was also performed in this study. Cell cultures grown in 96-well microtiter plate were aliquoted into the Minifold® dot blot transfer chamber (Schleicher and Schuell) where a piece of Duralon™-UV membrane was previously assembled. Suction was applied, the cells adsorbed onto the membrane, and the membrane was processed as described above.

Membrane prehybridization was carried out by incubation with prehybridization solution (QuikHyb™ from Stratagene, 0.04 ml/cm²) at 68°C for 15-30 min. Radiolabeled probe was boiled and mixed with the prehybridization solution to 2 × 10⁶ dpm per ml prehybridization solution. The blot was hybridized at 68°C for 60 min, washed 3 times (15 min each) with 0.1 X SSPE/0.1% sarkosyl at 60°C, and exposed to a film.

To strip the probe, the membrane was incubated with 0.5 N NaOH solution for 30 min with shaking, and rinsed in several changes of 6 X SSPE.

Propagation and DNA isolation of EMBL3 clones: Fresh overnight culture of LE392 grown in TB medium containing 0.2% w/v maltose was used for the propagation of lambda EMBL3. LE392 (0.5 ml) and phage (0.5 ml of 10⁶⁷ pfu/ml) were combined with 9 ml of NZYM medium, which was incubated at 37°C with shaking until lysis occurred. Fresh LE392 grown in NZYM/maltose (0.2% w/v) medium was added until the desired
volume of lysate was obtained, and chloroform (0.01 volume) was added at the end of the incubation.

Phage DNA was extracted and purified using QIAGEN Lambda Kit following the instructions provided by the manufacturer.

**DNA sequencing:** DNA sequence determination was performed by the combination of several DNA sequencing kits, all of which employed the chain-termination method (108). Radioactive nucleoside [α-³⁵S]dATP (specific activity > 3000 Ci/mmol) was used with TAQuence™ Version 2.0 DNA sequencing kits, and [γ-³²P]ATP (specific activity > 6000 Ci/mmol) was used with the dsDNA Cycle Sequencing System. Whenever applicable, TAQuence™ Version 2.0 was used in conjunction with KT5 Taq DNA polymerase obtained from Dr. Wayne Barne in the Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis.

Double-stranded plasmid DNA (2-4 µg) was denatured with 0.4 M NaOH for 5 min, and neutralized with 0.3 M sodium acetate (pH 4.5). The DNA was ethanol precipitated and dried. The DNA pellet was rehydrated in the presence of primers (1 µl of 2.5 ng/µl) and reaction buffer, and the DNA was heated to 70°C for 2 min. The DNA prepared was used with TAQuence™ Version 2.0. Protocols from the vendors were followed for the sequencing reactions.
Special areas of sequencing compression were resolved by using bacteriophage T7 gene 6 exonuclease to generate single-stranded DNA template (109). Gene 6 exonuclease has 5'→3' exonuclease activity that degrades only double-stranded DNA (110, 111). The degradation of double-stranded DNA stops when single-stranded DNA is encountered, where two half molecules of single-stranded DNA are generated. Pairs of restriction endonucleases flanking both ends of the target DNA to be sequenced were used to linearize the plasmid DNA. After exhaustive digestion with gene 6 exonuclease, both of the single-stranded templates were obtained. Endonucleases ApaI and NotI were used with pJDS clones (full length superoxide dismutase gene subcloned into pBluescript II SK), and ApaI and EcoRI were used with TA clones.

Plasmid DNA (2-4 μg) was incubated with 10 units of restriction endonuclease and 40 units of T7 gene 6 exonuclease at 37°C for 30 min. The mixture was heated at 80°C for 10 min to inactivate the enzymes, and sequencing reaction was carried out.

The dsDNA Cycle Sequencing System was also used in this study, which permits direct sequencing of lambda DNA. The primers (2.5 μl of 2.5 ng/μl) were 5' end-labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP (10 μCi). Once the primers were labeled, the sequencing reactions were
carried out as suggested by the manufacturer in a thermal cycler.

The sequencing reaction products were analyzed on denaturing (7-8 M urea) polyacrylamide gels (5%-6%) or HydroLink™ gels (6%-8%). The sequencing gel electrophoresis system from BRL (Model S2) was used, and the gel was electrophoresed at 60 watts for 2-4 hours. The polyacrylamide gels were fixed in 10% acetic acid and 25% ethanol for 15 min before they were dried. The films were exposed to the sequencing gel overnight before they were developed. The DNA sequences obtained were analyzed by GCG software (112) and Compugene programs (113) on a Digital VAX 8530 computer.
RESULTS

1. Genomic Library Construction

Genomic DNA from *D. radiodurans* was isolated as described in the previous section. The isolated DNA was examined by electrophoresis on a 0.4% agarose gel, where the size of the genomic DNA isolated from *D. radiodurans* was larger than 23 Kb (Figure 1, lane 6).

1.1. Partial digestion of genomic DNA

Bacteriophage lambda EMBL3 was used as the cloning vector for genomic library construction, and DNA sized between 9-23 Kb with *BamHI* compatible ends can be inserted into this vector. Restriction endonuclease *Sau3A*I cuts DNA sequences containing \( \uparrow\text{GATC}\downarrow \), which generates cohesive ends compatible with *BamHI* digest. Since *Sau3A*I cuts DNA very frequently, it was necessary to partially digest the DNA for the random generation of larger DNA fragments.

Genomic DNA samples digested with various amount of *Sau3A*I were analyzed on a 0.4% agarose gel (Figure 1). Samples in lane 4 and 5 were found to contain more DNA fragments ranging in size 9-23 Kb than others. The two DNA samples were combined, and the DNA was fractionated on a 10-30% sucrose gradient by ultracentrifugation. Fractions were collected and aliquots of the samples were analyzed on a 0.4% agarose gel
Figure 1. Partial digestion of *D. radiodurans* genomic DNA with *Sau3AI*. The DNA samples were digested with *Sau3AI* (37°C, 30 min), and the resulting DNA fragments were analyzed on a 0.4% TAE agarose gel. Lane 1 to lane 5: genomic DNA digested with 0.25, 0.125, 0.063, 0.031, and 0.016 units of *Sau3AI* per microgram of DNA respectively. Lane 6: undigested genomic DNA. M: lambda DNA *HindIII* marker, the size of each marker was as indicated.
Figure 2. Sucrose gradient size fractionated genomic DNA digest. Fractionation of the genomic DNA digest was described in the text. Aliquots of the fractions were analyzed on a 0.4% TAE agarose gel. The numbers on the top of the figure represented the fractions analyzed. M: lambda DNA HindIII markers. U: undigested genomic DNA.
1.2. Ligation, packaging and amplification of EMBL3 library

Size fractionated genomic DNA prepared above was dissolved in 0.1 X TE buffer to a concentration of 0.4–0.5 μg/μl. Ligation was carried out by mixing 1 μg of EMBL3 predigested arms and 0.45 μg of fractionated genomic DNA as well as 2 units (Weiss) of ligase. A parallel test ligation was also performed, where fractionated genomic DNA was substituted by PME test insert (~12 Kb). After three days of incubation at 4°C, aliquot of ligation mixtures were removed and analyzed on a 0.3% agarose gel. Results shown in Figure 3 indicated the formation of high molecular weight DNA (lane 2 and 4).

The ligated DNA was packaged into lambda phage as described in the previous section by using GIGAPACK®II PLUS packaging extract. Once the lambda DNA was packaged, the titer of the bacteriophage was determined. By propagating the packaged lambda in either P2393 or LE392, the ratio of the recombinant versus non-recombinant EMBL3 was determined. The titers of the packaged lambda propagated in P2392 and LE392 were $2.4 \times 10^5$ pfu/μl and $2.6 \times 10^5$ pfu/μl respectively, which indicated that 92% of the packaged lambda were recombinant.

The EMBL3 genomic library was amplified on strain P2392.
Figure 3. Ligation of lambda EMBL3 arms with inserts. The ligation reactions were carried out as described in the text. The resulting samples were analyzed on a 0.3% TAE agarose gel. Lane 1 and 2: before and after three days of ligation of EMBL3 arms with *D. radiodurans* DNA insert, respectively. Lane 3 and 4: before and after three days of ligation of EMBL3 arms with PME test insert, respectively. M: lambda DNA *HindIII* markers.
by propagating 500,000 recombinant plaques. The amplified phage was stored at 4°C in the presence of chloroform, which was used in the subsequent studies.

2. Oligonucleotides

Seven oligonucleotides were synthesized during the study, which were designated SOD1-SOD7. The sequences of the oligonucleotides are listed in Table 1.

Since the surface HPI-layer protein of *D. radiodurans* (114) was the only gene from *D. radiodurans* ever reported in the GenBank, the codons of the 1,036 amino acids were analyzed and preliminary codon usage information was obtained to assist the design of the oligonucleotides. Mixed oligonucleotides were synthesized, where the third position of most of the codons in SOD1 and SOD2 were either degenerate or replaced by deoxyinosine 5′-triphosphate (dTTP).

Oligonucleotide SOD1 was derived from the N-terminal sequence of *D. radiodurans* superoxide dismutase, which corresponded to the coding strand of the superoxide dismutase gene. Oligonucleotide SOD2 was derived from a conserved region of peptide common to all of the superoxide dismutases near the C-terminal ends. The conserved region contained 7 amino acids, which had a sequence of WEHAYYL. SOD2 was synthesized complementary to the coding strand. Oligonucleotides SOD3 to SOD7 were primers for sequence determination. They were
Table 1. Sequences of the synthetic oligonucleotides.

<table>
<thead>
<tr>
<th>SOD1</th>
<th>5′-d(CC(C/T)&lt;sup&gt;a&lt;/sup&gt; CAC AT(C/T)&lt;sup&gt;a&lt;/sup&gt; GA(C/T)&lt;sup&gt;a&lt;/sup&gt; GC(C/T)&lt;sup&gt;a&lt;/sup&gt; CG)-3′</th>
</tr>
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<tbody>
<tr>
<td>SOD2</td>
<td>5′-d(AGG TAG TAI&lt;sup&gt;b&lt;/sup&gt; GCG TG(C/T)&lt;sup&gt;a&lt;/sup&gt; TCC CAI&lt;sup&gt;b&lt;/sup&gt; ACI&lt;sup&gt;b&lt;/sup&gt; TC)-3′</td>
</tr>
<tr>
<td>SOD3</td>
<td>5′-d(GTC GTG TCC ACC GCC AAC)-3′</td>
</tr>
<tr>
<td>SOD4</td>
<td>5′-d(CTT GTT GGC GTT GTC CAC)-3′</td>
</tr>
<tr>
<td>SOD5</td>
<td>5′-d(ACT TCG TCC CAG TTC AC)-3′</td>
</tr>
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<td>SOD6</td>
<td>5′-d(CAC CAC ACC AAG CAT CA)-3′</td>
</tr>
<tr>
<td>SOD7</td>
<td>5′-d(GCG TCG AAG CTG CCG AA)-3′</td>
</tr>
</tbody>
</table>

<sup>a</sup> The third position of the codons are degenerate.

<sup>b</sup> The third position of the codons are replaced with dITPs.
generated based on the sequence derived from TA clones using SOD2, M13 forward, and T7 primers.

3. Polymerase Chain Reaction (PCR)

In order to probe the superoxide dismutase gene by PCR, several parameters were examined to optimize the condition for the reaction. These parameters include melting temperature (Tm) of the primer/DNA complex, probe concentration, magnesium concentration, and the cycle program for the reaction. The standard condition for PCR described by SaiKi et al. (105) was followed in examining the above parameters. In this study, oligonucleotides SOD1 and SOD2 were the primers and genomic DNA of *D. radiodurans* was the template for the PCR reactions.

3.1. Optimization of polymerase chain reactions

Since both oligonucleotides SOD1 and SOD2 were degenerate, the Tms of the two oligonucleotides were calculated as the highest and lowest possible melting temperatures. The calculated Tms of SOD1 and SOD2 for priming *D. radiodurans* superoxide dismutase gene were 52-60°C and 68-74°C respectively; and for priming *E. coli* manganese superoxide dismutase gene were 30-36°C and 64-70°C respectively. Because of the design of SOD2 enables this oligonucleotide to hybridize to most of the superoxide dismutases at approximately the same temperature, the only
specific primer for probing *D. radiodurans* SOD has to be SOD1. It is important to control the annealing temperature of the primers for the specific probing of *D. radiodurans* SOD because the N-terminals of the *D. radiodurans* and *E. coli* MnSODs have 85% identity in the first 40 residues. This is especially important when the template DNA are propagated and prepared form *E. coli* mini-prep lysate. To decrease the possibility of mispriming and increase the signal-to-noise ratio, annealing temperature of 55°C was found to be optimum in accommodating both priming specificity and primer/template complex stability of *D. radiodurans* SOD.

The effective primer concentrations were examined since both primers are degenerate. Reactions containing 0.35 µM or 1.75 µM (5 times more) primers were examined. Results in Figure 4 indicated that 1.75 µM of primers caused more non-specific priming (lane 2) compared to 0.35 µM of primers (lane 3).

Magnesium concentration is critical for the stringency of the polymerase chain reactions, and magnesium ion concentration of 1.5 mM was found to be given less non-specific DNA bands. Figure 4 lane 1 and lane 4 were the polymerase chain reactions containing 2.5 mM and 1.5 mM magnesium respectively.

The superoxide dismutase gene to be amplified by SOD1 and SOD2 was expected to be around 0.5 Kb judging from other
superoxide dismutase sequences. Figure 4 lane 4 showed that the superoxide dismutase gene amplified under optimal condition generated DNA of correct size, and was devoid of non-specific background.

3.2. TA Cloning™ of PCR product

Optimized polymerase chain reaction (100 μl) was carried out as described above, and 0.5 μl of the PCR DNA sample was removed. The DNA was combined with linearized TA Cloning™ vector pCR™1000 (50 ng), and the DNA mixture along with T4 DNA ligase were incubated at 12°C overnight before transformation was carried out. The transformed E. coli host DH5αF’ was grown on LB medium containing kanamycin, IPTG and X-Gal at 37°C overnight. The recombinant (white) colonies were selected and inoculated into LB medium (150 μl) containing kanamycin on a 96-well microtiter plate. The cultures were grown for 6 hours, and 50 μl of each culture was transferred to the Duralon™-UV membrane through dot blot transfer chamber. The cells were lysed and the DNA was denatured followed by southern hybridization.

The probe was prepared by isolating the 0.5 Kb PCR fragment separated on agarose gel. The DNA was extracted and purified, and was subsequently labeled by [γ-32P]dCTP. The labeled DNA was separated from unincorporated [γ-32P]dCTP by
Figure 4. Optimization of the polymerase chain reactions. The polymerase chain reactions were performed using *D. radiodurans* genomic DNA template and oligonucleotides SOD1 and SOD2 primers. The resulting DNAs were analyzed on a 1.5% TAE agarose gel. Lane 3 and 4: PCR under optimized conditions. Lane 1: PCR under optimized conditions except the magnesium concentration was 2.5 mM. Lane 2: PCR under optimized conditions except the primer concentration was 1.75 mM. Lane M: 1 Kb ladder DNA markers (Gibco, BRL).
Figure 5. Autoradiogram of the screening of TA clones. Aliquots (50 μl) of TA clones grown in 96-well microtiter plate were transferred to Duralon™-UV membrane by dot blot transfer chamber. The probe was PCR 0.5 Kb DNA random primer labeled with [γ-32P]dCTP. The blot was hybridized, and washed under stringent conditions. The dark spots represented the positive clones. The clone in H-12 was a non-recombinant negative control.
spin column (Bio-Spin® 30), and the radioactivity of the DNA was measured. The probe was added to the hybridization solution, and the blot was hybridized (68°C, 60 min), washed three times (15 min each) under stringent condition (0.1 X SSPE, 0.1% sarkosyl), and autoradiographed. Figure 5 is the southern hybridization of the TA clones after overnight exposure at -80°C, where dark spots represent the positive clones. A non-recombinant clone (blue colony) was also included at well A-12 as negative control.

Since E. coli host did not seem to introduce background interference to the blot, it was clear that the probe did not cross-hybridize with the host DNAs.

3.3. Preliminary sequence analysis of TA clones

Although Southern hybridization suggested that the PCR product had been subcloned, there was no indication that these clones contained SOD gene. Preliminary sequence analysis was carried out to confirm that the positive TA clones were indeed superoxide dismutase clones. Six of the positive clones were chosen for preliminary sequence analysis, which included A-6, C-4, A-10, B-10, C-10, and D-11. Plasmid DNA from the six clones were prepared and double-stranded DNA sequencing was performed using T Aquence™ Version 2.0 DNA sequencing kit in conjunction with K75 Taq DNA polymerase. Primer flanking either end of the insert, T7 or M13 forward primer, was used
on each clone for sequence analysis.

The DNA sequences obtained were translated in all six reading frames, and the deduced peptide sequences were compared with the N-terminal sequence of *D. radiodurans* superoxide dismutase. Peptide sequences not compatible with the N-terminal sequence of *D. radiodurans* superoxide dismutase were compared with other peptides of superoxide dismutases for C-terminal homology. Table 2 showed the deduced preliminary peptide sequences of the TA clones (TA peptides), and the N-terminal peptide sequence of *D. radiodurans* MnSOD obtained from chemical method. The corresponding regions of peptide sequences from *E. coli* MnSOD are also shown for comparison.

Due to the sequencing compressions and PCR errors, the DNA sequences of the TA clones could not be translated without frame shifts. Patches of conserved residues among MnSODs observed by Parker and Blake (50) assisted the allocation of the residues of TA peptides. The general homology between the TA peptides and other MnSOD peptides suggested that the TA clones were SOD clones. The complete identity between TA peptides and the N-terminal sequence of *D. radiodurans* indicated that the TA clones were indeed *D. radiodurans* SOD clones. The 63% GC content of TA clone DNA was in agreement with that of *D. radiodurans* DNA (60%-70% GC), which was clearly different from that of *E. coli* DNA (50% GC). Since the TA clones did not include the full length SOD
### Table 2. Sequence comparison of the SOD peptides.

#### N-terminals

<table>
<thead>
<tr>
<th>Residue numbers&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>26</th>
</tr>
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<td>PHIDARTMEIHHT</td>
<td>PHFDKQTMEIHHT</td>
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<td><em>D. radiodurans</em> SOD&lt;sup&gt;c&lt;/sup&gt;</td>
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<td><em>E. coli</em> MnSOD&lt;sup&gt;d&lt;/sup&gt;</td>
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#### C-terminals

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<td>AFGSFDACKKFX</td>
<td>AFGSVDNFKAEEFE</td>
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<tr>
<td><em>E. coli</em> MnSOD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>AFGSFDACKKFX</td>
<td>AFGSFDACKKFX</td>
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<tr>
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<td>K AAASRFGSGWAWLWKLGKD</td>
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<td>LDVVPSTANQDNPLMX'X'X'TAG</td>
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<td>VSGLTPIILGVVDVWHAYYLYL</td>
<td>ASGFPPPILGLDLVDWHAYYLYL</td>
<td>ASGFPPPILGLDLVDWHAYYLYL</td>
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</table>

<sup>a</sup> Residue numbers corresponded to the full length MnSOD of *D. radiodurans*.

<sup>b</sup> The deduced peptide sequence of TA clones.

<sup>c</sup> The N-terminal peptide sequence determined by chemical method.

<sup>d</sup> The derived peptide sequence in Reference 116.

<sup>e</sup> The residues could not be deduced from the DNA sequence.
sequence, subsequent cloning of SOD gene from the genomic library was necessary. Also since the DNA sequence obtained from PCR product was prone to error (115), the representative DNA sequence should be obtained from a genomic clone. However, the DNA sequence information obtained from the TA clones of \textit{D. radiodurans} MnSOD was used for the synthesis of oligonucleotides primers, and the primers were used for the sequence determination of genomic SOD clone.

4. Genomic Library Screening and Subcloning

Since a library does not always represent the whole composition of a genome, the titer of the SOD gene in the genomic library was determined.

4.1. Titration of SOD gene in genomic library

Polymerase chain reaction optimized for the amplification of superoxide dismutase gene was employed in this study owing to its great sensitivity. Amplified EMBL3 (10^4-10^5 pfu) was propagated by infecting \textit{E. coli} host LE392. The phage DNA was extracted and purified using a QIAGEN column, and the concentration of the DNA was determined. The DNA was diluted so that an aliquot (5 μl) of each dilution contained 5 x 10^3 to 5 x 10^2 μg of DNA in ten fold increments. Polymerase chain reactions were performed using SOD1 and SOD2
as primers and dilutions of recombinant phage DNA as templates.

Figure 6 showed the result of the polymerase chain reactions, where the amplified superoxide dismutase gene (0.5 Kb band) was present in all the dilutions. Although the amplified SOD band became less intense as the template DNA became more diluted, the result suggested that at least one copy of the SOD gene was still present in $5 \times 10^{-3}$ µg of the recombinant EMBL3 DNA. Since each bacteriophage contained $\sim 5 \times 10^{11}$ µg of DNA (117), at least one positive SOD clone would be obtained if one thousand EMBL3 plaques were screened.

4.2. Genomic library screening

Two 150 mm plates each containing 5000 plaques were prepared, and duplicate membrane blots were probed with the 0.5 Kb PCR fragment labeled with [$\gamma^{32}$P]dCTP. The membranes were washed under stringent condition and autoradiographed.

Figure 7 is the autoradiogram of a set of duplicate filters, where the dark spots represents the plaques that were hybridized to the probe. Only the plaques that hybridized in duplicate blots were identified as positive clones, and the spots present in one of the blots were considered non-specific hybridizations.

Five positive clones were individually isolated, and
Figure 6. Titration of the superoxide dismutase gene in the genomic library. The recombinant EMBL3 genomic library DNA was isolated and diluted. Polymerase chain reactions were performed using SOD1, SOD2 primers and diluted EMBL3 DNA templates. The resulting DNA was analyzed on a 1.5% TAE agarose gel. Lane 1-7: polymerase chain reactions containing 5 x 10^{-2} to 5 x 10^{8} microgram of template DNA in ten fold increments, respectively. Lane M: 1 Kb ladder DNA markers.
Figure 7. Autoradiogram of genomic library screening. Duplicate filters were blotted against a plate containing 5000 plaques. The plaques were screened with radiolabeled 0.5 Kb SOD DNA fragment obtained from PCR. The blots were hybridized and washed under stringent condition as described in the text. The resulting autoradiogram was obtained from overnight exposure at -80°C with an intensifying screen.
secondary screening were performed until homogeneous clones were obtained. These clones were propagated, and DNA from each clone was purified.

4.3. Southern hybridization of the lambda clones

To confirm the presence of SOD gene in all of the clones isolated, Southern hybridization was performed. Complete digestion of the recombinant lambda DNA with restriction endonuclease SalI liberated the lambda arms, while the insert DNA (~15-20 Kbp) remained intact. Additional restriction digestions of the insert were necessary for the generation of smaller DNA fragments for subcloning. Since the GC content of the insert DNA was ~63% and the lambda DNA was ~50%, restriction endonucleases which recognized GC-rich sequences would digest the insert more frequently. ApaI and XmaI, which recognized -GGGCC- and -C-CGGG- respectively, were found to generate satisfactory digestion patterns on the isolated clones. Digestion of the isolated clones with the above enzymes in conjunction with SalI generated several DNA fragments (Figure 8). The variations in the digested patterns suggested that the clones were in different constructs. The insert DNA were digested to less than 6.6 Kbp, while the lambda arms were digested to 3.5 Kbp, 5.5 Kbp, 9.5 Kbp, and 10.5 Kbp. The 18 Kbp fragments represented the undigested DNA.

The digested clones separated on the agarose gel were
used for Southern hybridization, where the DNA fragments were denatured and transferred to a nylon membrane. The radiolabeled probe and hybridization conditions were identical to that described in the screening process. The autoradiogram in Figure 9 indicated that the SOD gene was present in all five lambda clones. The estimated sizes of the DNA fragments that contained the SOD gene were 3.0 Kb, 6.0 Kb, 3.5 Kb, 1.5 Kb and 5.5 Kb in clone 1-5, respectively. The additional band (8.5 Kb) present in clone 2 might be the result of incomplete digestion.

4.4. Subcloning of superoxide dismutase gene

The superoxide dismutase gene was subcloned into pBluescript II SK vector by purifying the 1.5 Kb DNA fragment from the agarose gel. Since the restriction termini of the DNA fragment were unknown, Southern hybridization on lambda clone 4 DNA digested with pairs of restriction endonucleases were carried out. The DNA was digested with either ApaI/SalI or ApaI/XmaI or SalI/XmaI. The digested DNA samples were separated on agarose gel along with the triple-digested (ApaI/SalI/XmaI) clone 4 DNA. The DNA fragments were transferred to a membrane, and Southern hybridization was performed. The autoradiogram of the Southern hybridization (Figure 10) revealed that the SalI/XmaI combined digestion of lambda clone 4 DNA generated a 1.5 Kb southern positive band.
Figure 8. Restriction digestion of isolated lambda clones. Five independently isolated lambda clones were digested with ApaI, SalI, and XmaI. The DNA fragments separated on a 0.7% TAE agarose gel were stained with ethidium bromide and visualized on a UV transilluminator. Lane 1-5: five independently isolated lambda clones digested with ApaI, SalI, and XmaI.
Figure 9. Autoradiogram of Southern hybridization of the lambda clones. The DNA fragments separated on the agarose gel of Figure 8 were denatured and transferred to a piece of Duralon™-UV membrane. Southern hybridization were performed using the radiolabeled PCR 0.5 Kb SOD fragment. The blot was washed under stringent condition after hybridization. The resulting autoradiogram was obtained after 10 min of exposure to Hyperfilm™-MP (Amersham). The DNA fragments contained the SOD gene were 3.0 Kb, 6.0 Kb, 3.5 Kb, 1.5 Kb, and 5.5 Kb from lane 1 to lane 5 respectively.
Figure 10. Southern hybridization of the digested lambda clone 4 DNA. Purified lambda clone 4 DNA was digested with pairs of restriction endonucleases and separated on a 0.7% TAE agarose gel. Southern hybridization of the DNA was performed using the radiolabeled 0.5 Kb SOD DNA fragment obtained from PCR. The blot was hybridized and washed under stringent condition. The autoradiogram was obtained after 10 min of exposure. Lane 1: ApaI and SalI digested DNA. Lane 2: ApaI and XmaI digested DNA. Lane 3: SalI and XmaI digested DNA. Lane 4: ApaI, SalI, and XmaI digested DNA.
(lane 3) which was identical to the Southern positive band on the triple-digested sample (lane 4). The DNA digested with ApaI/SalI or ApaI/XmaI generated Southern positive bands around 4.0 and 4.4 Kb respectively. It was concluded that the cohesive ends of the 1.5 Kb DNA fragment were generated by the combination of SalI and XmaI.

The lambda clone 4 DNA digested with SalI and XmaI was separated on agarose gel, and the 1.5 Kb DNA fragment was extracted and purified from the gel. Vector pBluescript II SK digested with the same two enzymes was gel purified, and ligation of the vector and the 1.5 Kb DNA was carried out followed by transformation of E. coli strain DH5α. The culture was grown on LB medium containing ampicillin, IPTG and X-Gal, and sixteen recombinant clones (white colonies) were selected and the plasmids were isolated. The polymerase chain reaction optimized for the amplification of SOD was employed for the screening of the plasmid clones.

All sixteen clones were positive by the screening, Figure 11 shows three of the positive clones (lanes 3-5) along with the positive and negative controls for the polymerase chain reactions. Positive controls, including D. radiodurans genomic DNA and the gel-purified 1.5 Kb southern-positive DNA fragment, were amplified by PCR, respectively (lane 1 and lane 2). There was no amplification of the samples containing either vector pBluescript II SK along (lane 6) or E. coli
Figure 11. Screening of the superoxide dismutase subclones. Plasmid clones were screened using polymerase chain reactions optimized for the amplification of *D. radiodurans* SOD gene. The resulting DNA samples were analyzed on a 1.5% TAE agarose gel and stained with ethidium bromide. Lane 1: *D. radiodurans* genomic DNA. Lane 2: the Southern positive 1.5 Kb DNA fragment. Lane 3-5: positive plasmid subclones (pJDS). Lane 6: vector pBluescript II SK. Lane 7: *E. coli* genomic DNA.
genomic DNA (lane 7). The plasmid subclone of *D. radiodurans* SOD gene (pJDS) was used for DNA sequence determination.

5. DNA Sequence Determination

The DNA sequence was determined by the dideoxy chain-termination procedure, which involves the synthesis of a DNA strand by DNA polymerase using a single-stranded DNA template. DNA synthesis is initiated at where the primer anneals to the template, and is terminated by the incorporation of a 2',3'-dideoxynucleoside 5'-triphosphate (ddNTP) which will not support continued DNA elongation. Since the GC content of the DNA to be sequenced was around 63%, DNA sequencing performed at 37°C (SEQUENASE®) introduced a series of compressions and generated unreadable sequences. The DNA sequencing using Taq DNA polymerase was performed at 70°C, which generated satisfactory DNA sequences with high GC templates. In this study, two different sequencing systems both incorporated Taq DNA polymerase were used for the sequence determination of the SOD DNA.

The plasmid clone of SOD, pJDS, was denatured by treating the double-stranded DNA with NaOH, followed by neutralization and ethanol precipitation. The resulting single-stranded DNA template was annealed with the sequencing primers (SOD3-SOD7), and DNA sequencing was performed using TAQuence™ Version 2.0 and KT5 Taq DNA polymerase. The
sequencing primers SOD3–SOD7 were derived from the TA clones. Degenerate primers SOD1 and SOD2 were also used in the sequencing reactions, and only SOD2 was able to generate readable DNA sequences. The sequencing strategy is shown in Figure 12.

Special areas of sequencing compressions were resolved by pretreatment of the pJDS DNA with bacteriophage T7 gene 6 exonuclease. Since bacteriophage T7 gene 6 exonuclease only degrades double-stranded DNA from 5' to 3', exhaustive digestion of linearized double-stranded DNA with this exonuclease will generate two half-molecules of single-stranded DNA templates. Both strands of the pJDS templates were generated by using either ApaI or NotI to linearize the plasmid, followed by digestion with bacteriophage T7 gene 6 exonuclease. The sequences of the single-stranded templates so generated were determined by TAQ™uence Version 2.0 DNA sequencing kit and KT5 Taq DNA polymerase. The partial restriction map of pJDS is shown in Figure 13. The nucleotide sequence encompassing the D. radiodurans superoxide dismutase gene and the derived amino acid sequence are shown in Figure 14. The single open reading frame contains 630 nucleotides, which encodes a peptide of 23,347 calculated molecular mass.

Comparison of the deduced D. radiodurans SOD peptide sequence with nine SOD peptide sequences aligned by Parker and Blake (50), one conserved residue (Leu 88) was not observed in
the *D. radiodurans* SOD. Instead, a methionine residue was present (Met 87). The possibility of frame shift was ruled out since patches of conserved residues flanking the methionine residue were observed. Also because the obtained DNA sequence in the region was devoid of compression, the possibility of missing three bases in a short stretch of sequence (~30 basepairs) was very small. We therefore concentrated our investigation on the possibility of mutation of this residue and the residues surrounding it. The double-stranded DNA Cycle Sequencing System enabled the direct sequencing of lambda DNA, which bypassed the subcloning of the gene and proofread the sequence obtained form pJDS. Direct sequencing on two other lambda clones revealed the same sequence obtained from pJDS in the region of suspicion. Additional proofreading sequencings were obtained from TA clones. The TA clones were the subclones of genomic DNA amplification, which bypassed the lambda cloning procedure. The TA clone was linearized with ApaI or EcoRI, followed by bacteriophage T7 gene 6 exonuclease digestion. The single-stranded DNA was sequenced, and the sequencing result also supported the DNA sequence obtained from pJDS clone. We therefore concluded that the presence of Met 87 instead of Leu 87 was not the result of mutation or sequencing error. Moreover, the MnSOD genes from *Halobacterium halobium* (118) was also found to contain methionine instead of leucine at the aligned positions, which suggested that this
residue was not strictly conserved.

The amino acid composition deduced from the gene product is compared with the amino acid composition analysis obtained from the purified protein (Table 3). The amino acid compositions derived from the two methods show general agreement with each other. The deduced N-terminal sequence of the gene agrees completely with that of the purified protein listed in page 105. The codon frequency of _D. radiodurans_ SOD gene and _D. radiodurans_ phosphoglycerate kinase (PGK) gene are listed in Table 4. The codon frequency of the strongly expressed (S) and moderately to weakly expressed (W) _E. coli_ genes (119) are also listed for comparison.

Preliminary experiment for the expression of the cloned _D. radiodurans_ SOD gene in _E. coli_ was achieved by subcloning the 1.5 Kb DNA fragment containing the SOD gene to pBluescript II KS in contrast to pBluescript II SK. The transcription of SOD gene became under the control of _E. coli_ lac promotor. By inducing the transformed cultured with IPTG, the _E. coli_ strain DH5α was able to express the cloned _D. radiodurans_ SOD gene. The expressed _D. radiodurans_ SOD migrated differently with the _E. coli_ MnSOD and FeSOD on non-denaturing gel, and the activity of each protein could be identified by SOD activity staining (32) of the gel (Figure 15).
Figure 12. Sequencing strategy of superoxide dismutase gene. The arrows represent the directions and lengths of each sequencing reaction. The short bars represent the oligonucleotide primers. The open box is the coding region of the SOD gene and the direction of transcription is from the left to the right.
Figure 13. Partial restriction map of pJDS. A 1.5 Kb SalI to XmaI fragment containing superoxide dismutase from D. radiodurans was subcloned into pBluescript II SK. The restriction sites used for single-stranded sequencing (ApaI and NotI), as well as the direction of transcription of superoxide dismutase gene are shown.
Figure 14. The complete DNA coding sequence and the deduced peptide sequence of *D. radiodurans* MnSOD. The putative ribosome binding site (Shine-Delgarno sequence) is indicated, and the region homologous to the *E. coli* -10 sequence is underlined. The N-terminal sequence obtained by chemical method is from Ala 1 to Lys 40.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Deduced residue/mol$^a$</th>
<th>Residue/mol$^b$</th>
</tr>
</thead>
<tbody>
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<td>0</td>
</tr>
<tr>
<td>As(n)</td>
<td>58</td>
<td>53</td>
</tr>
<tr>
<td>Thr</td>
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<td>16</td>
</tr>
<tr>
<td>Ser</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Gl(n)</td>
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</tr>
<tr>
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<tr>
<td>Val</td>
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</tr>
<tr>
<td>Met</td>
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<td>5</td>
</tr>
<tr>
<td>Leu</td>
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<td>35</td>
</tr>
<tr>
<td>Tyr</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>Trp</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ The number of amino acid residue deduced from the nucleotide sequence per dimer.

$^b$ The number of amino acid residue obtained from amino acid analysis of the purified protein normalized to 46.6 kDa molecular weight per dimer.
Table 4. Codon frequency\(^a\) of *D. radiodurans* SOD gene.

<table>
<thead>
<tr>
<th>Amino acid and codons</th>
<th>SOD</th>
<th>PGK</th>
<th>(E. coli)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Ala GCG</td>
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<td>25</td>
</tr>
<tr>
<td>GCU</td>
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<td>8</td>
<td>33</td>
</tr>
<tr>
<td>GCC</td>
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<td>44</td>
<td>9</td>
</tr>
<tr>
<td>GCA</td>
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<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Arg AGG</td>
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<td>3</td>
<td>0</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>0</td>
<td>4</td>
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<td>5</td>
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</tr>
<tr>
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<td>21</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>1</td>
</tr>
<tr>
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<td>10</td>
<td>13</td>
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</tbody>
</table>

Note: the numbers underlined are less than 0.4.

\(a\): The codon frequency is given in the number of codon used per 1000 codons.

\(b\): The codon usage of strongly expressed (S) and moderately to weakly expressed (W) *E. coli* genes.
Figure 15. The expression of the cloned *D. radiodurans* SOD gene. Cell extracts of the transformed *E. coli* cultures were separated on a 10% non-denaturing polyacrylamide gel and stained with the SOD activity staining (32). Lane 1-4: cell extracts of the transformed *E. coli* cultures. Lane 5-7: purified *E. coli* FeSOD, MnSOD and *D. radiodurans* MnSOD, respectively.
DISCUSSION

The gene encoding a manganese-containing superoxide dismutase from *D. radiodurans* was cloned, and the nucleotide sequence and the deduced peptide sequence were determined. The 1.5 Kb *SalI*-*XmaI* DNA fragment contains a single open reading frame of 630 nucleotides which encodes a peptide of 210 amino acids. The peptide has a calculated molecular mass of 23,347. The translated N-terminal peptide sequence is identical with the N-terminal sequence of the purified protein, and the amino acid composition of the deduced (dimeric) peptide is in agreement with that of the purified protein. The calculated molecular mass of the deduced peptide also agrees with the molecular mass of the SOD monomer obtained from SDS-PAGE (23,500 daltons).

Comparison of the peptide sequence of *D. radiodurans* MnSOD with the MnSODs from *E. coli* (120), *Bacillus stearothermophilus* (121) and *Thermus aquaticus* (122) reveals general homology among these peptides (Figure 16). Among them, *E. coli* MnSOD shares 70.6% identity with the *D. radiodurans* SOD. FeSOD peptide sequences from *E. coli* (123) and *Bacteroides gingivalis* (124) are also shown. The ligands to the metal cofactor His 28, His 83, Asp 176, His 180 (double underlined) are invariant amino acids (50), which are also observed in the *D. radiodurans* SOD. The invariant amino acids
Figure 16. The aligned SOD peptide sequences. The SOD peptide sequences are (from top to bottom): E. coli MnSOD (120), D. radiodurans MnSOD, Bacillus stearothermophilus (121) MnSOD, Thermus aquaticus MnSOD (122), E. coli FeSOD (123), and Bacteroides gingivalis FeSOD (124). The identical amino acids are shown in asterisks. The double underlined amino acids are ligands to the metal cofactor and the underlined are primary discriminators between the iron and manganese SODs. The amino
acids at the 78 and 174 positions are bold.
among the five peptides are indicated by asterisks.

The amino acids proposed by Parker and Blake (50) for
the discrimination of manganese versus iron in SOD were
examined. These amino acids were selected based on the crystal
structure of *Bacillus stearothermophilus* MnSOD of being
clustering around the metal binding site (55). They were also
selected based on the conservation within the FeSODs or MnSODs
but difference between the two groups. There were 22 amino
acids proposed to play a role on the discrimination of iron
versus manganese on the SOD active site. However, five
residues surrounding the metal on the active site were chosen
as primary candidates, they included amino acids 77, 78, 86,
155, and 156 (underlined).

The FeSOD peptide sequence from *E. coli* (123) represents
the typical FeSOD, which contains all of the 115 conserved
FeSOD amino acids. The FeSOD peptide of *Bacteroides gingivalis*
(124) represents one of the cambialistic SODs. Since the SOD
peptides from *B. fragilis* and *B. gingivalis* were able to
incorporate either Fe or Mn to their active site as
demonstrated by Gregory et al. (48) and Amano et al. (60), the
peptide sequence of *B. gingivalis* was of special value in
examining the above amino acids.

Inspection of the peptide sequence of *B. gingivalis*
revealed that about one-third (36 residues) of the 115
conserved FeSOD amino acids were not conserved. Among them, Gly 78 and Gly 174 (shown in bold) were found to be changed from the conserved FeSOD amino acids Ala and Thr to the conserved MnSOD amino acids. It was also observed that Gly 78 and Gly 174 were one of the primary and one of the secondary candidate amino acids, respectively, that were proposed to distinguish between manganese and iron in SOD. Although several other secondary candidate amino acids in *B. gingivalis* were changed, none of them were changed to the conserved MnSOD amino acids. The other four primary candidate amino acids at positions 77, 86, 155, 156 in *B. gingivalis* were found to be invariant. The observations suggested Gly 78 and Gly 174 may be important for the cambialist effect of the SOD peptide, although the importance of other amino acids should not be understated.

Inspection of the *D. radiodurans* SOD peptide revealed that the amino acids of the primary and secondary candidate were typical of MnSODs, including Gly 78 and Gly 174. This suggested that the *D. radiodurans* SOD peptide could be a candidate for site-directed mutagenesis, where Gly 78 and Gly 174 could be changed to Ala 78 and Thr 174, respectively, without the complications of atypical potential discriminatory residues. If the two residues were sufficient to confer the difference between iron and manganese in SOD, we should be able to construct an FeSOD out of *D. radiodurans* MnSOD.
peptide. However, a prerequisite for the site-directed mutagenesis is the expression of active *D. radiodurans* MnSOD peptide.

The expression of *D. radiodurans* SOD gene in *E. coli* was achieved by simply inverting the *D. radiodurans* DNA insert in pJDS so that the direction of transcription is under the control of *E. coli* lac promoter. Figure 15 clearly shows the presence of SOD activity bands that migrate coincidentally with the purified *D. radiodurans* MnSOD. However, a number of SOD activity bands were also observed, which migrated differently from the expressed *D. radiodurans* SOD as well as the *E. coli* MnSOD, FeSOD and the SOD hybrid. The *E. coli* MnSOD and FeSOD had 42% sequence identity and they were able to form heterodimeric protein, therefore, the *E. coli* MnSOD should also be able to form heterodimeric protein with the *D. radiodurans* MnSOD with a sequence identity of 70%. The same should also applied to the *E. coli* FeSOD and the *D. radiodurans* MnSOD since there was 45% identity between the two peptides. The easiest way to demonstrate the validity of the above observation is by reconstitution. By denaturing the purified *E. coli* MnSOD and *D. radiodurans* MnSOD in guanidine-HCl followed by reconstitution, the two species of homodimers should be able to be regained along with the formation of an additional heterodimer. However, experiments needed to be performed in order to confirm the above observations.
Inspection of the 5'-untranslated region of the cloned gene, a sequence (TTAAAT) resembling the E. coli promoter -10 region (TATAAT) was identified (125). There was no sequence found to be homologous to the E. coli -35 promoter consensus sequence (TTGACA) 15-20 basepairs upstream from the TTAAAT region. This comparison suggested that the D. radiodurans MnSOD might not be expressed from its own promoter in E. coli. It was also concluded by Smith et al. that the D. radiodurans promoters often could not function as promoters in E. coli (81). Similar conclusions were drawn examining the expression of E. coli plasmid in D. radiodurans, where pBR322 derivatives (pKK232-8 and pKK175-6) were unable to express the drug-resistant genes in D. radiodurans unless a D. radiodurans promoter was provided (81). These observations suggest the presence of a different promoter system in D. radiodurans, which is recognized by a RNA polymerase different from the one in E. coli. However, the recognition consensus sequence of D. radiodurans promoters is yet to be identified.

Although the rRNA sequences and chain lengths in different organism varied significantly (126), the Shine-Delgarno sequence recognized by 16S rRNA seemed conserved among organisms. The putative ribosome binding site AGGAG was observed in the D. radiodurans superoxide dismutase gene, which is 11 basepairs upstream from the translational start site. Although the sequence was found to agree with the
ribosome binding site sequences commonly observed (127), it should be viewed with some caution since the nucleotide sequence of *D. radiodurans* 16S rRNA is not known.

Though superoxide dismutase is only one of many constituents of the defense system against oxygen toxicity, superoxide dismutase in *D. radiodurans* played another significant role in protecting the cells from radiation damage. The survey of superoxide dismutase activity in bacteria by McCord *et al.* (74) further associated the radiation resistance of *D. radiodurans* with an elevated level of superoxide dismutase activity in crude cell extracts. The similar specific activity among all of the purified superoxide dismutases suggested the presence of other control mechanisms in *D. radiodurans*, which lead to the constitutive production of the enzyme. The possible mechanisms may involve a stable mRNA or a powerful promoter.

Since not enough data are available to derive conclusions regarding the *D. radiodurans* SOD promoter, the discussion is now focused on the stability of the SOD mRNA. The turnover of mRNA has been demonstrated to affect gene expression in *E. coli*, where different mRNA species have very different half-lives and so produce very different yields of protein molecules per mRNA molecule (128). The presence of extragenic palindromic sequences (about 35 nucleotides in length) that are able to form secondary structures at the 3'
end of the operons and in intercistronic regions are known to control mRNA stability (129). Inspection of the 3' untranslated region of the D. radiodurans superoxide dismutase mRNA revealed a stem-loop structure. The structure had a stem of 12 basepairs and a loop of 5 nucleotides (Figure 17). The structure energy of -24.2 Kcal was sufficient to form stable secondary structures which might be able to protect the mRNA from exonuclease degradation (129). However, this structure may also serve as a rho-independent terminator based on the criteria of Brendel et al. (130, 131) for the signaling of the termination of transcription.

Examination of the preferred codon usage in D. radiodurans superoxide dismutase gene revealed an extreme bias for G or C in the third position of the codons. This actually accounted for the overall high GC content of the gene. A similar bias for codons ending with G or C was observed in the D. radiodurans phosphoglycerate kinase gene (Table 4) and Thermus thermophilus phosphoglycerate kinase gene (71% GC) (133). Comparison of the D. radiodurans and E. coli codon usages revealed several different preferences between the organisms. The CCC codon for proline, for example, which is strongly preferred by D. radiodurans, while the same codon only represents 1% of the codon for proline in strongly expressed E. coli genes. However, the codon bias should not be used for the prediction of the expression of high GC DNA in E.
Figure 17. The proposed mRNA stem-loop structures at the 3' noncoding region. The numbers (821 and 867) correspond to the number of nucleotides reported in Figure 14.
coli, since the *Bordetella pertussis* toxin subunit genes (60-70% GC) were able to be expressed to high level in *E. coli* (132).
Chapter 4.

Summary
Deinococcus radiodurans was of interest to our investigation because of its constitutive expression of abundant superoxide dismutase (74), its resistance to UV and ionizing radiations (94, 95), and its efficient DNA repair system (77, 78, 79). The large cellular content of superoxide dismutase and catalase plays an important role in protecting the cells from damages due to superoxide radicals and hydrogen peroxide generated from radiation. Our interest led to the study of the D. radiodurans superoxide dismutase from the aspects of protein structure as well as molecular regulation and expression of the gene. Two major goals of this study were (1) to characterize the superoxide dismutase of D. radiodurans and investigate the kinetics and metal specificity of reconstitution of the protein; and (2) to clone and express the structural gene encoding the protein for the examination of the putative control regions that regulate the expression of superoxide dismutase. The results of the investigation are summarized below.

The MnSOD from D. radiodurans was purified 350 fold to a specific activity of 3300 units/mg. The criterion of purity was determined by the comigration of protein bands on the nondenaturing gels stained with protein and activity staining. The native enzyme contained 1.5 gram-atom of manganese and had an apparent molecular mass of 43,000 as determined by Superose 6 gel filtration chromatography. The molecular mass of the
native enzyme was identical to that determined by sedimentation equilibrium performed in collaboration with W. F. Beyer at Duke University (data not shown). The subunit molecular mass determined by SDS-PAGE was 23,500, which was similar to other manganese-containing superoxide dismutases isolated from a number of prokaryotic cells (36). The UV and visible spectra of the protein revealed absorbance peaks at 280 nm and 410 nm, respectively. The absorbance at 280 nm corresponded to the presence of aromatic amino acids, especially tyrosine and tryptophan, and the absorbance at 410 nm was apparently generated from ligand-manganese interaction. The absorbance peak at 410 nm disappeared upon denaturation of the protein and removal of metal.

Superoxide dismutase was denatured by dialysis in 5 M guanidine hydrochloride containing chelating agent, 8-hydroxyquinoline. A stable metal-free apo-superoxide dismutase (renatured apo-superoxide dismutase) was produced upon dialysis to remove the denaturant. This renatured apoprotein assumed a gross conformation similar to the native protein as shown by fluorescence spectroscopy and circular dichroism spectroscopy (performed in collaboration with W. F. Beyer at Duke University [data not shown]). The renatured apoprotein was readily reconstituted by manganese in the absence of denaturant to regain full enzymatic activity and restore 1.7 gram-atom of manganese per mol dimer. Renatured apoproteins
prepared from SOD isolated from a number of sources were reported to be precipitated (32, 35) or unable to regain enzymatic activity (31 - 36) when the appropriate metal ions were added. This unique feature of the *D. radiodurans* SOD enabled us to study the kinetics of metal reconstitution. Our data showed that the kinetics of reconstitution was dependent on the pH of the reconstitution buffer, the relative molar ratio of the apoprotein to metal, and the presence of competing metals. Full enzymatic activity was observed in 20 minutes when the apoprotein was reconstituted at pH higher than 8.0; whereas full activity was reconstituted in three hours at pH 7.5. The pH dependency of reconstitution may be resulted from the deprotonation of the metal binding ligands or may be resulted from a minor conformational change of the protein upon metal binding. Manganese concentration relative to apoprotein concentration was also important. Excess manganese (at least 100 μM) was required to fully reconstitute 1.5 μM apo-superoxide dismutase, presumably because effective collisions between the protein and the manganese increased. The manganese-reconstituted SOD appeared to be stable in a buffer containing metal chelator such as EDTA. Binding of the metal to the renatured apoprotein might have induced local conformational change which in turn stabilized the metal-peptide structure.

Competing metal ions, iron and zinc, was found to
inhibit the manganese-dependent reconstitution of apoprotein. Zinc bound to the renatured apo-superoxide dismutase in a stoichiometric manner with 1.7 gram-atom of zinc per mol dimer. Iron, which was able to restore enzymatic activity in a number of iron-containing (47) and some manganese-containing superoxide dismutases (39, 48, 49, 59), did not reconstitute enzymatic activity of the *D. radiodurans* apc-superoxide dismutase. Moreover, it inhibited the manganese-dependent reconstitution and bound to the renatured apoprotein with a stoichiometry of 5–7 gram-atom iron per mol dimer. The inability of iron to reconstitute *D. radiodurans* apoprotein, however, triggered our interest to investigate the primary structure of *D. radiodurans* superoxide dismutase, and to compare the peptide sequence with other SODs whose activity were restored with either manganese or iron. Since there is a high degree of similarity between iron- and manganese-containing superoxide dismutase primary structures (50), we focused our investigation to the examination of potential residues that might be important in conferring the difference between iron and manganese. By cloning the *D. radiodurans* superoxide dismutase gene, we were not only able to deduce the peptide sequence and examine the residues that might be involved in metal specificity, we were also able to examine the control regions of the gene for clues about regulation of the SOD gene.
Anti-MnSOD antiserum was prepared for the purpose of cloning and for the examination of cross-reactive species in the cell extracts of other Deinococci. By use of western analysis, we were able to identify the MnSODs of *D. radiodurans* and *E. coli*, as well as a 23-kDa peptide in the cell extract of *D. radiopugnans*. Since *E. coli* FeSOD did not cross react with the anti-MnSOD antiserum, and all MnSODs had a molecular mass around 23-kDa, the cross-reactive species in *D. radiopugnans* cell extract might be a MnSOD. Another 42-kDa peptide presented in the purified *D. radiodurans* SOD preparation was also recognized by the anti-MnSOD antiserum. This peptide, however, was not recognized by the affinity-purified anti-MnSOD antibody, suggesting the presence of the anti-MnSOD and anti-42-kDa peptide antibodies in the polyclonal antiserum preparation. Presumably, this was due to the contamination of MnSOD preparation at the time of immunization. Molecular cloning of the MnSOD gene using the antiserum preadsorbed against *E. coli* cell extract resulted in the isolation of the gene encoding the 42-kDa peptide (Appendix). Nucleotide sequence determination and data base homology search coincided the nucleotide sequence with the 3-phosphoglycerate kinase (PGK) genes from a number of sources. The open reading frame of this *D. radiodurans* gene is composed of 1167 basepairs, which encodes a 40,448 dalton (deduced molecular weight) PGK peptide. Upon examination of the
antiserum screening that lead to the isolation of PGK gene instead of SOD gene, we concluded that the promotor of the D. radiodurans MnSOD gene was unable to be recognized by the E. coli RNA polymerase, therefore, no D. radiodurans MnSOD peptide was synthesized within E. coli. This view is supported by the findings of Smith et al. (81) who concluded that E. coli was unable to recognize the promotors of D. radiodurans and vice versa. This, however, may not always be true since the PGK gene of D. radiodurans was expressed in E. coli. Another speculation for the differential expression of the two genes in E. coli is the requirement for additional positive regulatory elements for the expression of D. radiodurans MnSOD gene in E. coli.

An alternative method for the screening of SOD clones was undertaken using radiolabeled probes generated by a polymerase chain reaction (PCR). A pair of primers was designed based on the N-terminal sequence of D. radiodurans MnSOD, and the homologous region shared by all SODs near the C-terminal end. The DNA fragment amplified by PCR had a size of about 500 basepairs, which was subsequently subcloned and sequenced. The nucleotide sequence and deduced peptide sequence of the PCR 500 bp DNA subclones confirmed that the DNA fragment amplified by PCR was that of D. radiodurans MnSOD. Full length SOD clones were obtained by screening of a genomic library, and the nucleotide sequence was determined.
The open reading frame contains 630 nucleotides, which encodes a peptide of 23,347 calculated molecular mass. The N-terminal sequence of the deduced peptide agreed with the N-terminal sequence obtained by chemical method; and the subunit molecular weight of the peptide agreed with that obtained from SDS-PAGE. The peptide sequence showed 70.6% identity with that of the *E. coli* MnSOD, and had a general homology with other MnSOD peptides. The gene contained 63% GC, which was reflected in the strong preference for G or C at the third positions of the codons. The sequence that could potentially form a hairpin structure at the 3′-untranslated region was observed. The structure has been speculated to be able to protect the mRNA from exonuclease degradation (128, 129). This finding might explain, in part, the ample production of the MnSOD in *D. radiodurans*. The SOD peptide sequence of *D. radiodurans* was also aligned with other SOD sequences, and the residues proposed by Parker and Blake (50) to be important in conferring the differences between iron and manganese were examined. Based on the conserved residues within MnSODs and FeSODs, and the residues of the cambialistic SOD peptide from *B. gingivalis*, I proposed that Gly 78 and Gly 174 might be important in distinguishing manganese and iron cofactors in SOD peptides. However, only by the tool of site-directed mutagenesis can I fully understand the residues involved in the process.
Ongoing effort has been concentrated on the expression of the *D. radiodurans* MnSOD clone in *E. coli*. Since I have pointed out that the promoters of *D. radiodurans* apparently were not recognized by the *E. coli* RNA polymerase, I decided to construct a clone that could make use of the *E. coli lac* promotor for the expression of the *D. radiodurans* SOD gene. Preliminary result suggested that the new construct expressed the *D. radiodurans* SOD gene in *E. coli* under induced condition (IPTG). Moreover, several SOD activity bands other than the *E. coli* MnSOD, FeSOD, and Mn/FeSOD hybrid were observed based on the result of the non-denaturing polyacrylamide gel stained for SOD activity. These extra bands were suspected to be the hybrid protein formed between *D. radiodurans* SOD monomer and *E. coli* MnSOD or FeSOD monomer. Since hybrid protein can be formed between *E. coli* MnSOD and FeSOD with an overall identity of 42%, the 70% identity between *D. radiodurans* MnSOD and *E. coli* MnSOD and 45% identity between *D. radiodurans* MnSOD and *E. coli* FeSOD should be able to form hybrid proteins as well. However, experiments are underway to test the hypothesis by performing the reconstitution in the presence of all three proteins.

Based on all of these findings, a model for the reconstitution of *D. radiodurans* MnSOD is summarized in the next page. The dimeric MnSOD of *D. radiodurans* can be denatured and the metal removed by exposure to guanidine
hydrochloride and 8-hydroxyquinoline. The denatured protein remains as monomeric peptide until it is freed of denaturant, and dimeric renatured apoprotein is generated. The renatured apoprotein assumes a gross conformation similar to the native protein, which can be readily reconstituted to regain full activity without changing the overall conformation. However, since the manganese-reconstituted protein is stable in the buffer containing EDTA, addition of manganese to the apoprotein might induce a local conformational change which could stabilize the metal-protein complex. Also if the hypothesized cross-species hybrid formations do occur, other SOD peptides present during the reconstitution process can be paired up with the D. radiodurans peptide, and a number of hybrid proteins can be formed. However, the model remains to be tested in the near future.
Chapter 5.

References


11. Misra, H. P., and Fridovich, I. (1972) The role of


55. Parker, M. W., and Blake, C. C. F. (1988) Crystal structure of manganese superoxide dismutase from *Bacillus*
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Biochem. Biophys. 181, 308-312.


algorithm for testing potential prokaryotic terminators. 


Chapter 6.

Appendix: Cloning and Sequencing of the Gene Encoding 3-Phosphoglycerate Kinase from *Deinococcus radiodurans*
INTRODUCTION

An open reading frame encoding the 3-phosphoglycerate kinase (PGK) in *D. radiodurans* was cloned during the course of the study. The enzyme, 3-phosphoglycerate kinase (EC 2.7.2.3), catalyzes the reversible transfer of phosphoryl group from 1,3-diphosphoglycerate to ADP with the formation of 3-phosphoglycerate. The consecutive action of glyceraldehyde-3-phosphate dehydrogenase (GAP) and 3-phosphoglycerate kinase catalyze the metabolically central oxidation of an aldehyde to an acid during the fermentation of glucose (1). A number of PGKs have been isolated from mammalian, yeast, and bacteria, all of which are monomers with a molecular weight of 40,000 to 45,000 dalton (1). The X-ray studies of the horse muscle (2) and yeast (3) enzymes have shown that they are structurally homologous with the principal structural feature being the occurrence of two widely separated domains of almost equal size. This biglobal feature of the enzyme together with the inferred binding position for the 3-phosphoglycerate has led to the suggestion that the ATP and 3-phosphoglycerate bind to different domains which move toward each other in order to create a water-free environment for catalysis (2).

The genes encoding the PGK from a number of sources have been sequenced. The amino acid sequence homology between human, horse muscle and mouse PGKs is greater than 96% over
the entire 416 amino acid residues, and the homology between yeast and mammalian PGKs is around 65% (4). Although the amino acid sequence identity between yeast and Thermus thermophilus PGKs is only 41%, most of the differences represent conservative amino acid substitutions (5). High resolution X-ray crystal studies as well as the homologous primary structures of PGKs suggest that the enzyme has been highly conserved during the course of evolution. Indeed, the only significant difference in the results obtained from structural studies of the yeast and horse enzymes concerns the binding of substrates (6).

The cloning of the PGK gene took place during the screening of the D. radiodurans superoxide dismutase (SOD) gene using partially purified antiserum raised against the purified MnSOD. The MnSOD preparation used as antigen, however, also contained PGK polypeptide. Although present in minute amount, PGK turned out to be very antigenic. The polyclonal antiserum, therefore, contained both anti-MnSOD and anti-PGK antibodies. The two groups of polyclonal antibodies do not cross-react with each other’s antigen as demonstrated in Chapter 2. Due to the differential expression of the SOD gene and PGK gene in E. coli, only the clones containing the PGK gene were selected during the antibody screening. We report here the cloning, sequencing and expression of the PGK gene from D. radiodurans. The primary structure of PGK from
D. radiodurans will be compared with that from other sources, and the structural feature will also be discussed based on homology analysis.
EXPERIMENTAL PROCEDURE

Most of the materials and experimental procedures are identical with those described in the previous two chapters. The purification of the antiserum, the library screening with antibody and the preparation of single-stranded DNA template are described below:

Antiserum preadsorption: Antiserum was preadsorbed against E. coli strain DH5α cell extract to decrease the background interferences. The CNBr-activated Sepharose 4H (1 gram dry weight, from Bio-Rad Laboratory) was used for the coupling of E. coli cell extract, which was activated by washing with 1 mM HCl followed by coupling buffer (0.1 M NaHCO₃, pH 8.3, 0.5 M NaCl). E. coli cell extract (20 mg) was coupled to the activated Sepharose 4H by incubation at 7°C overnight with shaking. The resin was collected by centrifugation, and the free binding sites were blocked with 1 M ethanolamine, pH 8.0. The resin was then repeatedly washed in coupling buffer and washing buffer (0.1 M sodium acetate pH 4.0, 0.5 M NaCl), and finally resuspended in PBS (phosphate-buffered saline, 150 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 1.75 mM KH₂PO₄, pH 7.4). The resin was packed into a column, and antiserum (5 ml) was passed through the column. The flow-through of the antiserum
was collected and was used for the screening of the *D. radiodurans* genomic library.

**Screening of the genomic library:** *D. radiodurans* lambda EMBL3 genomic library was plated with *E. coli* strain LE392. Duplicate membranes (Millipore HATF) prepared from each plate were blocked in 5% non-fat dry milk (prepared in PBS) then were incubated with primary antibody. Protein A horseradish peroxidase (HRPO)-conjugate (Sigma Chemical Co.) was used instead of secondary antibody, and diaminobenzidine (DAB) plus hydrogen peroxide were used as the colorimetric substrates for peroxidase. After positive clones were identified and selected, subsequent screenings were carried out to obtain homogeneous clones.

**Subcloning of the gene:** Homogeneous lambda clones were propagated, and DNA from each clone was extracted and purified. The DNA was partially digested with restriction endonuclease Sau3AI, and the resulting DNA fragments were ligated to BamHI digested pBluescript II KS vector. After transformation, the colonies were replicated onto nitrocellulose membranes, and the cells were lysed by exposing to chloroform vapor. The cell debris were washed off the membranes, and the colonies were screened for antibody positive clones as described above.
Single-stranded DNA preparation: Replicated forms (RFs) of M13mp18 and mp19 obtained from New England Biolabs were used for the generation of single-stranded template for DNA sequence determination. RF DNA digested with restriction endonucleases EcoRI and XbaI was ligated to insert DNA EcoRI-XbaI fragment. The ligated DNA was used to transform E. coli strain JM101 (Δ(lac-proAB) supE thi/F′ traD36 proAB′ lacIqZΔM15). Since M13 only suppressed the growth of the host cells, the resulting infected cells were indicated by the presence of turbid plaques. Recombinant colorless plaques were selected and grown in YT medium (1.6% w/v tryptone, 1% w/v yeast extract, 0.5% w/v NaCl) containing JM101 (OD600 of 0.1). The culture was grown for 5 hours and cells were pelleted by centrifugation (16,000 × g, 20 min). The supernatant containing the single-stranded M13 was collected, and 20% PEG (in 3.5 M ammonium acetate) was added to one-fifth of the culture volume. The mixture was incubated on ice for 30 min and centrifuged. The pellet was resuspended in TE buffer and was extracted three times with phenol/chloroform. The DNA was precipitated with ethanol in the presence of 2.5 M ammonium acetate, which was used for DNA sequence determination.
RESULTS AND DISCUSSION

1. Antiserum Preadsorption

The antiserum raised against *D. radiodurans* superoxide dismutase reacted strongly with SOD of *D. radiodurans* as well as an unknown 42 kDa protein that was also present in the SOD preparation (Figure 1, lane 1). It also cross-reacted with a number of proteins in *E. coli* cell extract as shown in Figure 1, lane 2. Since the cross-reacting species would give rise to high background when screening the lambda EMBL3 library (propagated in *E. coli*), the antiserum was preadsorbed with *E. coli* cell extract. After the adsorption, the reactivities of the specific antibodies with the SOD and 42 kDa protein were not changed, but the overall cross-reactivity with the *E. coli* crude extract was greatly diminished (Figure 1, lane 3 and lane 4, respectively). However, the preadsorbed antiserum still reacted strongly with the MnSOD in *E. coli* cell extract. The preadsorbed antiserum thus prepared was used for the screening of the genomic library without further purification.

2. Genomic Library Screening and Subcloning

*D. radiodurans* lambda EMBL3 genomic library was constructed as described in the previous chapter. Six plates representing approximately 50 libraries were prepared, and duplicate nitrocellulose membranes against each plate were
Figure 1. Preadsorption of anti-MnSOD antiserum. Antiserum raised against *D. radiodurans* purified MnSOD was preadsorbed with Superose 4H coupled *E. coli* cell extract. The flow-through containing the antibody against MnSOD was collected and was used for western analysis. Lane 1 and lane 2 contained the purified *D. radiodurans* MnSOD and *E. coli* cell extract, respectively, which were incubated with the antiserum before adsorption. Lane 3 and lane 4 contained the same antigens as lane 1 and lane 2, respectively, which were incubated with the preadsorbed antiserum.
also prepared as described in the previously section. The membranes were blocked, incubated with preadsorbed antiserum followed by protein A HRPO-conjugate, and finally developed with DAB plus hydrogen peroxide.

Four independently isolated lambda clones were selected and the DNA from each clone was purified. To determine if the four clones were identical, the DNA were either doubly digested with SalI and SmaI, or triply digested with ApaI, SalI and SmaI, and the resulting DNA fragments were analyzed on agarose gel. Since the clones displayed different patterns on the triple-digested DNA, two clones were randomly selected for further studies.

The DNA isolated from the two lambda clones were partially digested with Sau3AI, which was ligated with BamHI digested pBluescript II KS vector. After transformation, the colonies were repeatedly screened with preadsorbed antiserum until homogenous culture was obtained. Two positive subclones were selected from each lambda clone, and were characterized by western blotting analysis. The cell extracts of the four subclones and the host harboring the vector (E. coli strain DH5α), as well as the SODs isolated from E. coli and D. radiodurans were subjected to western analysis. Figure 2 shows that the preadsorbed antiserum recognized the MnSODs of D. radiodurans and E. coli (lane 2 and lane 8, respectively) as well as the MnSOD in the host cell extract (lane 1). This
Figure 2. Western analysis of the cloned genes. Protein samples separated on 12.5% SDS gel were transferred to nitrocellulose membrane. The membrane was incubated with partially purified anti-MnSOD antiserum followed by protein A HRPO-conjugate, which was developed with the addition of DAB plus hydrogen peroxide. Lane 1: host *E. coli* cell extract. Lane 2: purified *D. radiodurans* SOD. Lane 3 to lane 6: transformed *E. coli* cell extracts from four independently isolated clones. Lane 7 and lane 8: purified *E. coli* FeSOD and MnSOD, respectively. Lane 9: prestained protein subunit marker.
antiserum did not cross-react with the *E. coli* FeSOD as shown in lane 7. However, the antiserum also recognized the 42-kDa peptide present in the *D. radiodurans* SOD preparation (lane 2, upper band), as well as a 39-kDa protein in three of the four subclones (lanes 3, 4, and 6). Since the 39-kDa peptide was clearly expressed from the recombinant plasmid (comparing to lane 1), and the size of the peptide as almost twice as large as expected for the superoxide dismutase peptide, it was suspected that the cloned gene might not be of SOD related. Rather, it was more likely that the cloned gene was related to the 42-kDa peptide. Also, since the expressed peptide was smaller than the 42-kDa peptide, the genes might represent the truncated species of the 42-kDa peptide in all three clones. To determine the nature of these clones, DNA sequencing of the genes was necessary. The clone that generated a minor western-positive band (lane 5) was not further investigated since it could be another truncated 42-kDa peptide clone.

3. DNA Sequencing

Sequence determination of the cloned gene was initiated by subcloning the gene from pBluescript II KS to M13mp18 and M13mp19. M13 is a filamentous bacteriophage which packages its single-stranded viral DNA and releases into the culture media without lysing the host cells. The M13mp18 and mp19 were constructed so that the multiple cloning sites of the two
vectors were in reverse orientation to each other. The single-stranded DNAs isolated from the two M13 vectors were used as the templates for sequencing reactions.

Two of the clones which encoded the 39-kDa peptide were used for the DNA sequencing determination. The recombinant plasmids of the clones were randomly assigned A1 and B1, and the plasmids were digested with restriction endonuclease EcoRI and XbaI. The digestion of the plasmids liberated the insert DNAs which were around 1.4 Kb and 1.0 Kb for A1 and B1 clones, respectively. Replicated forms (RF) of M13mp18 and mp19 DNA were also digested with EcoRI and XbaI, which were ligated to the gel-purified EcoRI-XbaI fragments of A1 and B1. E. coli strain JM101 was used as the host for transformation, and colorless turbid plaques were isolated. The recombinant M13 clones were propagated and single-stranded DNAs were isolated as described.

Since D. radiodurans DNA contains 60%-70% GC, DNA sequencing at 37°C using Sequenase® resulted in faint and compressed DNA sequences. However, by using the reaction components of TAQuence™ Version 2.0 DNA sequencing kit in conjunction with the KT5 Taq DNA polymerase, the DNA sequencing was devoid of any compression. The initial sequence information was obtained by using M13 forward primer, and as sequencing progressed, oligonucleotide primers were synthesized. There were 10 oligonucleotide primers synthesized
for both strands of the A1 clone (1.4 Kb), and the resulting DNA sequence was found to overlap the entire B1 sequence (1.0 Kb). To determine the nature of the clone, the nucleotide sequence of the A1 clone was input to the database to search for homologous sequences in GenBank. A high GC DNA which encoded 3-phosphoglycerate kinase (PGK) in *Thermus thermophilus* was found to share 62.8% identity with 1060 bases of the A1 clone at the 3' end; and another high GC DNA which encoded the glyceraldehyde 3-phosphate dehydrogenase (GAP) in *Thermus aquaticus* share 64.5% identity with the first 110 nucleotides of the A1 clone. Translation of the 1060 nucleotide sequence that was homologous to the *T. thermophilus* PGK gene, we found that the two peptides shared around 60% identity and an overall 70% similarity. However, the peptide sequence of the *T. thermophilus* PGK contained an additional of 23 amino acids at the C-terminal, which happened to be highly homologous in PGKs. The absence of the stop codon at the 3' end of the 1060 nucleotide sequence further sustained the presence of an incomplete coding region. Molecular weight calculation of the partial gene indicated a 36-kDa peptide, which was in agreement with the 39-kDa peptide obtained from western blotting analysis.

To obtain full length PGK nucleotide sequence, direct lambda DNA sequencing by dsDNA Cycle Sequencing System (from BRL) was employed. Oligonucleotide near the 3'-end of the 1060
nucleotide sequence was synthesized as primer, which was end labeled with [γ-^{32}P]ATP for sequencing reaction. The sequencing strategy of the full length PGK gene is shown in Figure 3, and the resulting PGK nucleotide and the translated peptide sequences are shown in Figure 4. The sequence at the -7 region from the start codon, CCGAG, strongly resembles the Shine-Dalgarno sequence of *E. coli* (AGGAG). The two underlined sequences at the 5' untranscribed region resemble the *E. coli* promoter -35 sequence (TTGACA), however, the relative -10 TATAAT consensus sequence is not identified. The open reading frame contained 1167 nucleotides, which encoded a PGK peptide of 40,450 dalton. The molecular weight is in agreement with the 42-kDa peptide seen on the western blotting analysis. The calculated isoelectric point of the peptide is 4.99 with a net charge of -12 at pH 7.0. The codon usage of PGK gene is presented in Figure 4 of the previous chapter, which also shows strong bias for G and C at the third position as does the SOD gene.

Comparison of the peptide sequences of the *D. radiodurans* and *T. thermophilus* PGK indicated a 61.6% identity and 77.7% similarity; whereas the identity between *D. radiodurans* and *E. coli* PGKs, and *D. radiodurans* and yeast PGKs were 45.1% and 46.8%, respectively. The aligned PGK peptide sequences of horse, yeast, *T. thermophilus* and *D. radiodurans* are shown in Figure 4.
Radiodurans is shown in Figure 5, where the asterisks represent the invariant amino acid residues. The twelve β-sheets and adjacent peptides that form the inner loops in the substrate-binding cleft are indicated by double and single underlines, respectively, according to the crystal structure of horse PGK (2). It is clear that the residues surrounding the substrate-binding cleft are highly conserved during
Figure 3. Sequencing strategy of the *D. radiodurans* PGK gene. Sequencing of the PGK gene was performed as described in the text. The short bar (−) represents the oligonucleotide primers, and the arrow lines represent the lengths and the directions of sequencing.
186

10  30  50
GGGGGATCTGGGGCGCGACGTGACTGTGAGGAAGTCAACAACGTCTTCCGTGAAAGCCG

70  90  110
CGAACGGCAAGTACAAGGCATCATGGCCTAACCAGAAAGCCCAATTGTGCTGACCACAA

130  150  170
TTCAAGGCGACCGCAACAGGGCGATCATTTGACCGCGCCTGACCAGTGGGCTAGGGGACC

190  210  230
TCGTCAAGTTCTTTCTGTGTACGAACAACGAGTGGGCTACAGAAACCACATCGCGGACC

250  270  290
TGGTGCAAGCTCGTTCAAGAACAAGCAAGCTAAAGCTGAGAGTGATGTTGATGTTGACGG

310  330  350
GTGTGTGCCCCGCTCCTCAACCACATCACCTCTTTGACCATCCACATCCCGAGAAACACC

S.D.  M

370  390  410
GCAAAACCTCGTCAGTCAATTGGATGTCAAGGGCAAGCGGTCCCTGTGGCGGCGTGGACTACA

QNLSSLVDVNGKRVRVVRVDYN

430  450  470
CGTGCGGGTTTGAGGGCGCGTGGTACAGGGACGACCGCCGTCATCGCGGCGTCGGAC

VPVGVDGVVQDIDTRITASVPT

490  510  530
CATCAAGAACGTGTGGACCGCGCGCGCGTCGTCGTGATGAGGACACTTCCGACGGCC

IKKLDDGGASVVLMSHFGRP

550  570  590
CAAGAATGGCCCCGAGGACAAGTACAACCCTGGAACGGGTGGCTGAGGGCGTCAGCCGGCGC

KNGPDEKYSLSKPVBAEAVSRA
Figure 4. The *D. radiodurans* PGK nucleotide and deduced peptide sequences. The putative ribosome binding site is as indicated (S.D.). The underlined regions are sequences homologous to the *E. coli* -35 promoter consensus sequence.
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<th>T. ther</th>
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**Figure 5.** The aligned PGK peptide sequences. PGK peptide sequences from horse muscle (2), yeast (6), D. radiodurans (D. radi), T. thermophilus (T. ther; ref. 5), and E. coli (E. coli; ref. 9) were aligned. The asterisks (*) represent the invariant residues, and the double and single underlines represent the 12 β-sheets and the adjacent residues that are present in the substrate-binding cleft.
evolution (7).

In summary, the antibody screening of the *D. radiodurans* genomic library enabled the selection of the PGK clones, but not the SOD clones. This might due to the lack of expression of SOD gene in *E. coli*, since Smith et. al. (81) also reported the lack of expression of the cat gene in *E. coli* using *D. radiodurans* plasmid pUE10 as expression vector. However, not all of the *D. radiodurans* genes were unable to express in *E. coli*, since the PGK gene of *D. radiodurans* was expressed. The PGK gene was subcloned into expression vector pBluescript II KS, and at least three out of four subclones contained the truncated gene. It was observed during the process of screening that more than 85% of the positive subclones were unstable. The pBluescript subclones lysed easily when propagated in either rich or minimum media, or under induced or uninduced conditions. It was possible that the enzymatic activity exerted by the full length PGK gene introduced some biochemical imbalance to the cells, therefore, only truncated PGK clones were able to survive. However, we were able to obtain full length PGK DNA sequence without the risk of subcloning by direct sequencing of the lambda clones using dsDNA Cycle Sequencing System. The resulting nucleotide and deduced peptide sequences have greater than 60% identity with the *T. thermophilus* 3-phosphoglycerate kinase gene and gene product as isolated by Bowen et. al. (5) and Nojima et. al.
(8). However, Bowen et al. also identified the gene immediately preceeding the PGK gene being the gene encoding for GAP in *T. thermophilus* (5). The same two genes were also found to be closely linked in *E. coli*, where the GAP gene was preceded by the PGK gene (9). We suspect the same type of genomic organization was also present in *D. radiodurans*, since the nucleotide sequence preceeding the PGK gene has 65% identity with the 3′-end of the *T. aquaticus* GAP gene. However, more sequencing information has to be obtained in order to confirm the nature of the gene.
REFERENCES


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