

CHARACTERIZATION OF SUPEROXIDE DISMUTASE  
FROM *ACTINOMYCES*

by

Katherine Byer Barkley

Dissertation submitted to the Graduate Faculty of the  
Virginia Polytechnic Institute and State University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Nutrition

APPROVED:

\_\_\_\_\_  
E. M. Gregory, Chairman

\_\_\_\_\_  
R. E. Ebel

\_\_\_\_\_  
J. L. Hess

\_\_\_\_\_  
D. R. Bevan

\_\_\_\_\_  
L.V.H. Moore

October, 1988

Blacksburg, Virginia

CHARACTERIZATION OF SUPEROXIDE DISMUTASE  
FROM *ACTINOMYCES*

by

Katherine Byer Barkley

Committee Chairman: Eugene M. Gregory  
Biochemistry and Nutrition

(ABSTRACT)

The anaerobes *Actinomyces naeslundii*, *A. odontolyticus* and *Actinomyces* strain E1S.25D produce a Mn-containing superoxide dismutase (MnSOD). *Actinomyces*, once classified as yeast based on their morphology, are saprophytic organisms found among the normal flora of the mouth but can act as endogenous pathogens resulting in gingivitis and actinomycosis. The ability of *Actinomyces* to scavenge superoxide may increase survival of the cell from the  $O_2^-$ -dependent killing by polymorphonuclear leukocytes and also enable the organism to be transported through an oxygenated environment from one site to another. The MnSODs were purified 85-240 fold from crude extracts with 30-60% yield by two chemical fractionations and three chromatography steps. The enzymes,  $M_r$  96,000, were tetramers of equally sized, noncovalently associated subunits similar to the MnSOD found in *Saccharomyces cerevisiae*. Each of the *Actinomyces* MnSODs contained 0.5 g-atoms Mn/subunit and were stable in the presence of 1 mM NaCN, 1 mM  $NaN_3$  and 2.5 mM  $H_2O_2$ . The MnSODs from *Actinomyces* have isoelectric points of 4.2-4.6 and are negatively charged at physiological pH. Amino acid analyses of the high molecular weight MnSODs from *Actinomyces*, yeast, chicken liver, and *Thermus thermophilus* indicated similar composition of each subunit. The second order rate constants of each *Actinomyces* MnSOD were

measured at pH 7.8 and found to be in the range of  $0.9 - 2.8 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  as compared to the rate of  $1.8 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  for yeast MnSOD. Structural relatedness was evaluated by immunological studies. Rabbit antisera to each of the *Actinomyces* MnSODs were prepared. The MnSODs from *A. naeslundii* and *Actinomyces* strain E1S.25D both showed complete identity with their respective antibodies and partial identity with the antibody prepared against *A. odontolyticus* MnSOD. None of the antisera cross reacted with bovine Cu/Zn SOD, *Bacteroides* Fe- or MnSOD or MnSODs from either *Haemophilus influenzae*, *Deinococcus radiodurans*, or *S. cerevisiae*.

## ACKNOWLEDGEMENTS

I would especially like to thank Dr. E. M. Gregory for his patience, support, encouragement, and friendship. I feel very fortunate to have been able to work under his direction for these four years. Thanks to all the members of my committee, Drs. Hess, Bevin, Moore, and Ebel, who have been so helpful during my graduate studies. A special thanks is due to Dr. Ebel for his expert advice in several areas of my work. I would also like to thank Dr. D. F. Smith for his sense of humor that kept me smiling all the way. \_\_\_\_\_ also deserves a special kudos for teaching me the ropes.

I am grateful to my friends in our laboratory and in the Biochemistry Department who have helped make my time at Virginia Tech more enjoyable. I am also very appreciative of \_\_\_\_\_ and \_\_\_\_\_ for their technical assistance.

Finally, I would like to thank my family - my parents who always provided love and support; \_\_\_\_\_ for their encouragement; and my wonderful husband, \_\_\_\_\_, who believed in me and was always there.

## TABLE OF CONTENTS

	Page
ABSTRACT .....	ii
ACKNOWLEDGEMENTS .....	iv
TABLE OF CONTENTS .....	v
LIST OF TABLES .....	vi
LIST OF FIGURES .....	vii
LITERATURE REVIEW .....	1
EXPERIMENTAL PROCEDURE .....	11
a. Methods .....	11
b. Materials .....	18
RESULTS	
Characterization of Superoxide Dismutase from Anaero-bically Grown <i>Actinomyces</i> .....	21
Immunological Studies of Manganese Superoxide Dismutases from <i>Actinomyces</i> .....	60
Comparison of Manganese Superoxide Dismutases .....	69
DISCUSSION .....	77
REFERENCES .....	88
VITA .....	99

## LIST OF TABLES

		Page
TABLE 1	<i>Actinomyces</i> Strains . . . . .	20
TABLE 2	Purification Table for MnSOD from <i>Actinomyces naeslundii</i> (9985) . . . . .	26
TABLE 3	Purification Table for MnSOD from <i>Actinomyces</i> strain E1S.25D . . . . .	30
TABLE 4	Purification Table for MnSOD from <i>Actinomyces odontolyticus</i> (6962D) . . . . .	35
TABLE 5	Amino Acid Composition of the <i>Actinomyces</i> MnSODs . . . . .	38
TABLE 6	Inhibition and Inactivation of SODs . . . . .	42
TABLE 7	Metal Analysis of <i>Actinomyces</i> MnSODs . . . . .	44
TABLE 8	Catalytic Activity of <i>Actinomyces</i> MnSODs as a Function of pH . . . . .	53
TABLE 9	Reconstitution Results for MnSOD from <i>Actinomyces odontolyticus</i> (6962D) . . . . .	56
TABLE 10	Second Order Rate Constants (pH 7.8) for Reconstituted <i>A. odontolyticus</i> . . . . .	59
TABLE 11	Immunoblotting of <i>Actinomyces</i> MnSODs . . . . .	65
TABLE 12	Superoxide Dismutase Activity in <i>Actinomyces</i> Strains . . . . .	67
TABLE 13	Immunoblotting of <i>Actinomyces</i> Strains . . . . .	68
TABLE 14	Purification Table for MnSOD from <i>Saccharomyces cerevisiae</i> . . . . .	71
TABLE 15	Comparison of Physical Characteristics of MnSODs . . . . .	72

TABLE 16	Comparison of Amino Acid Analyses of several MnSODs . . . . .	74
TABLE 17	Cross-Reactivity of SODs on Ouchterlony Double Diffusion Gels . . . . .	75
TABLE 18	Characteristics of <i>Actinomyces</i> MnSODs . . . . .	79
TABLE 19	Catalytic Activity of SOD as Function of pH . . . . .	83

## LIST OF FIGURES

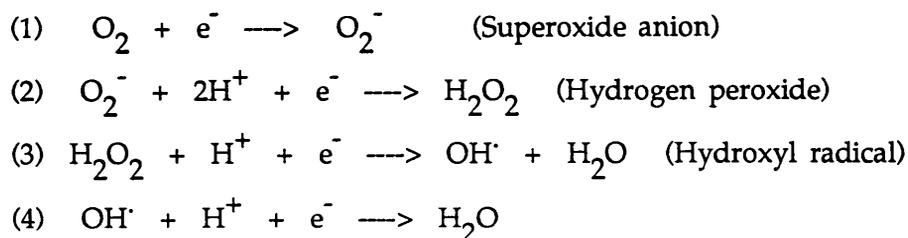
		Page
FIG. 1	Chromatography of MnSOD from <i>Actinomyces naeslundii</i> on Phenyl-sepharose: Elution with decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient . . . . .	22
FIG. 2	Chromatography of MnSOD from <i>Actinomyces naeslundii</i> on DE-53: Elution with KCl gradient . . . . .	23
FIG. 3	Chromatography of MnSOD from <i>Actinomyces naeslundii</i> on P100 gel exclusion column . . . . .	25
FIG. 4	Chromatography of MnSOD from <i>Actinomyces</i> strain E1S.25D on Phenyl-sepharose: Elution with decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient . . . . .	27
FIG. 5	Chromatography of MnSOD from <i>Actinomyces</i> strain E1S.25D on 2nd Phenyl-sepharose: Elution with decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient . . . . .	28
FIG. 6	Chromatography of MnSOD from <i>Actinomyces</i> strain E1S.25D on DE-53: Elution with KCl gradient . . . . .	29
FIG. 7	Chromatography of MnSOD from <i>Actinomyces odontolyticus</i> on Phenyl-sepharose: Elution with decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient . . . . .	32
FIG. 8	Chromatography of MnSOD from <i>Actinomyces odontolyticus</i> on P100 gel exclusion column . . . . .	33
FIG. 9	Chromatography of MnSOD from <i>Actinomyces odontolyticus</i> on DE-53: Elution with KCl gradient . . . . .	34
FIG. 10	Native Molecular Weight Determination of <i>Actinomyces</i> SODs by Gel Filtration Chromatography . . . . .	37
FIG. 11	Subunit Molecular Weight Determination of <i>Actinomyces</i> SODs by SDS-PAGE . . . . .	39
FIG. 12	Absorption Spectrum of <i>Actinomyces naeslundii</i> (9985) . . . . .	41

FIG. 13	Azide Inhibition of <i>Actinomyces</i> SODs . . . . .	43
FIG. 14	Temperature Stability of <i>Actinomyces</i> MnSODs . . . . .	46
FIG. 15	pH Profile of <i>Actinomcyes</i> SODs . . . . .	48
FIG. 16	Effect of Phosphate on the Activity of <i>Actinomyces</i> SODs . . . . .	49
FIG. 17	Effects of Increasing Ionic Strength on the SOD from <i>A. naeslundii</i> . . . . .	50
FIG. 18	Effects of Increasing Ionic Strength on the SOD from <i>Actinomyces</i> strain E1S.25D . . . . .	51
FIG. 19	Effects of Increasing Ionic Strength on the SOD from <i>A. odontolyticus</i> . . . . .	52
FIG. 20	Log $k_2$ of SOD versus pH . . . . .	54
FIG. 21	Azide Inhibition Studies of the Reconstituted versus Native MnSOD from <i>A. odontolyticus</i> . . . . .	57
FIG. 22	Ouchterlony Double Diffusion Plates for Cross-Reactivity between <i>Actinomyces</i> MnSODs . . . . .	61
FIG. 23	Antibody/Antigen Ratio versus % Activity of MnSOD isolated from <i>Actinomyces</i> . . . . .	663

## LITERATURE REVIEW

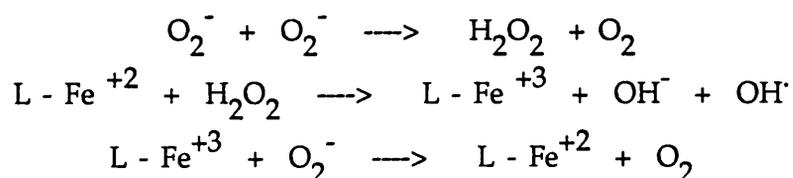
The utilization of molecular oxygen as an electron acceptor paradoxically sustains aerobic life but causes damage to these aerobic organisms. Oxygen is an excellent electron acceptor and reduction of  $O_2$  by NADH liberates 60 kcal of free energy. In organisms, this free energy change drives proton translocation and subsequently ADP phosphorylation. Thus, use of  $O_2$  as an electron acceptor is a benefit to the organism. However, partially reduced  $O_2$  metabolites have the potential to cause severe damage to the cells. Ground state oxygen exists as a diradical with the two unpaired electrons having parallel spins. Reduction of oxygen in the triplet ground state by a pair of electrons in the singlet state is spin-forbidden unless one of the electrons is inverted. This spin restriction can be overcome by one of several routes: (1) excitation of molecular oxygen to the singlet state involving inversion of one of the electrons in the oxidation/reduction couple; (2) ligation of oxygen to a transition metal, or (3) univalent reduction of the oxygen molecule. Biological oxidations use primarily the latter two routes for  $O_2$  reduction. Ligation of  $O_2$  to a metal ion increases the lifetime of the collisional complex to allow spin inversion. The stepwise reduction of molecular oxygen, (Scheme I), produces reactive intermediates which may be deleterious to biological systems (1).

### SCHEME I



The reduced metabolites, superoxide and hydrogen peroxide, are reactants in the catalyzed Haber-Weiss reaction (2) which forms hydroxyl radical (OH $\cdot$ ), a powerful oxidant [Scheme II]. Hydrogen peroxide, a product of the disproportionation of O $_2^-$  can oxidize liganded-Fe $^{+2}$  to form OH $\cdot$ . Iron chelated to ADP or to the plasma protein transferrin can act as a catalyst for the reaction (2). The cyclic mechanism is continued by the reduction of the liganded-Fe $^{+3}$  by O $_2^-$ .

#### SCHEME II

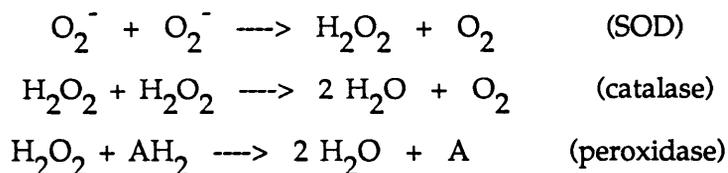


Each of these species, superoxide, H $_2$ O $_2$ , and OH $\cdot$  damage the cell. Superoxide was initially thought to exert its harmful effects indirectly through the Haber-Weiss catalyzed reaction. It has since been shown through the protective actions of the enzyme superoxide dismutase (SOD) that O $_2^-$  alone is toxic. Superoxide inactivates enzymes such as heme-catalase, the Mn-containing pseudocatalase of *Lactobacillus plantaru*, and glutathione peroxidase (3). Superoxide causes the release of Fe from the storage form ferritin (3). The released iron exacerbates the damage attributed to O $_2^-$  through a site-specific Haber-Weiss reaction. Chromosomal deletions and single stranded breaks in DNA, as well as membrane damage due to accumulation of lipid peroxides, have been attributed to the presence of hydrogen peroxide (4). Hydrogen peroxide can interact with reduced transition metals resulting in rapid metal oxidation and the production of hydroxyl radical. Hydroxyl radical will react with organic matter within 1 - 2

diffusion pathlengths from where it is formed. It was first argued that the OH $\cdot$  did not damage the cell because there was greater probability of it reacting with expendable molecules. It has since been shown that Fe $^{+2}$  may be bound selectively to negatively charged regions of nucleic acids, membranes, and proteins where the formation of OH $\cdot$  could produce the most drastic effects (3). The Haber - Weiss reaction is inhibited by removal of either O $_2^-$ , H $_2$ O $_2$ , or Fe. Scavengers of OH $\cdot$  will protect the cell against OH $\cdot$  but will not inhibit the Haber - Weiss cycle.

Organisms exposed to O $_2$  by design (aerobes) or by accident (anaerobes) must develop mechanisms to cope with the toxicity of partially reduced oxygen species. As a means of defense, organisms have developed several protective enzymes (1). These enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (Scheme III). These anti-oxidant enzymes decrease the amount of the active O $_2$  species within the cell and prevent cytotoxic reactions of the O $_2$  metabolites acting independently and in concert. Catalase and peroxidase scavenge H $_2$ O $_2$  whereas superoxide dismutase catalyzes the disproportionation of superoxide.

### SCHEME III



These enzymes inhibit the Haber - Weiss cycle by removing substrate and reductant rather than by reducing OH $\cdot$  directly.

There is good evidence that the physiological function of superoxide dismutase is that of an antioxidant. Although superoxide dismutation does occur spontaneously, it is a pH dependent reaction. At pH 7.7 the second order rate constant for spontaneous  $O_2^-$  dismutation is  $10^5 M^{-1} sec^{-1}$  (5). The slow reaction rate is due to the electrostatic repulsion of the two negatively charged reacting species,  $O_2^-$ . The second order rate constants for the SODs isolated thus far are approximately  $2 \times 10^9 M^{-1} sec^{-1}$  (6). SOD converts the damaging oxygen radical to a product which can in turn be removed by enzymes such as catalase and glutathione peroxidase. The role of SOD as a protection against  $O_2^-$  radical damage is supported by the finding of high levels of SOD in obligate aerobes but only very low quantities in anaerobes (7). In response to increasing concentrations of oxygen or superoxide, induction of SOD levels is a physiological response in many aerobes, microaerophiles, and facultative anaerobes (8,9). *Escherichia coli* can produce both an FeSOD and a MnSOD (10). An *E. coli* mutant lacking both SOD genes was unable to grow on minimal medium in the presence of oxygen and exhibited increased sensitivity to paraquat, which produces  $O_2^-$  through redox cycling, and to hydrogen peroxide. Restoration of aerobic growth on minimal media and increased resistance to the oxidizing agents was observed upon reintroducing SOD via a plasmid containing both SOD genes (11). This evidence gives further credence that the *in vivo* role of the protein is superoxide dismuting activity.

Another indication of the protective role of superoxide dismutase is the increased virulence of those strains of pathogenic bacteria which have increased levels of SOD. The normal host response to bacterial invasion includes the microbicidal activities of polymorphonuclear leukocytes (PMNLs). Once

phagocytized, PMNLs can destroy the invading bacteria by an oxygen-dependent mechanism. During this time, there is increased consumption of oxygen with concurrent increases in the production of superoxide and hydrogen peroxide (12). Virulent strains of *Nocardia asteroides* were shown to secrete SOD into the growth media where it became surface-associated (13). The amount of SOD secreted is associated with the ability of the organism to survive the phagocytic  $O_2^-$  radical production of the host. The correlation between increased levels of SOD and catalase and increased ability to survive the oxidative burst of PMNLs has been shown for in other bacteria including *Neisseria meningitidis* (14) and *Staphylococcus aureus* (15).

SODs are a family of metalloproteins having either Cu/Zn (CuZnSOD), Fe (FeSOD), or Mn (MnSOD) at the active site (16). Superoxide dismutases are differentiated directly by their metal content and indirectly by their characteristic reactivity with  $NaN_3$ , NaCN, and  $H_2O_2$ . The Cu/Zn enzyme is inhibited by 1mM NaCN. At this concentration of NaCN neither the Mn or Fe forms are inhibited (17). Low concentrations of sodium azide (1mM) inhibit the FeSOD, but concentrations as high as 20 mM are required for 50% inhibition of either the Cu/Zn or Mn proteins (18). The MnSODs are stable for up to 2 hours in 5 mM  $H_2O_2$  whereas the Cu/ZnSODs (19) and FeSODs (20) are inactivated under these conditions with half-lives of several minutes.

The types of SOD are characteristically distributed among the biota: Cu/Zn SOD is found in cytosols of eukaryotes, FeSOD in the procaryotes, and MnSOD in mitochondria and in some bacteria. Gram-negative bacteria generally contain only the FeSOD or both FeSODs and MnSODs whereas Gram-positive bacteria usually contain MnSOD. (21). There are exceptions to this distribution. For example,

FeSODs have been isolated from three families of plants - *Nuphar luteum* (22), *Ginkgo biloba* (23), and *Brassica campestris* (24). A Cu/ZnSOD has now been isolated from bacterial sources. These CuZnSODs (termed bacteriocupreins) are present in *Caulobacter crescentus* (25) and in two strains of *Pseudomonas* (26). The level of these enzymes is usually reflected by the contact of the organisms with an oxygen environment and sensitivity of the organisms to this exposure. Aerobic and microaerophilic organisms contain relatively high levels of SOD consistent with their niche in the biota, whereas anaerobic bacteria in general are devoid of the enzyme (7). The ability of some anaerobes to synthesize SOD greatly improves their resistance to oxygen toxicity (8).

#### **CuZn Superoxide Dismutase**

The CuZn form of superoxide dismutase was first identified by McCord and Fridovich (27) in 1969. Previously known as erythrocuprein, hepatocuprein, or hemocuprein, the activity was defined as an oxygen radical scavenger and the protein was officially named superoxide/ superoxide oxidoreductase (E.C. 1.15.1.1) (16). These enzymes are dimers with molecular weights of approximately 32,000. Each noncovalently associated subunit binds approximately 1.0 gram-atom of catalytically active copper and approximately 1.0 gram-atom of zinc which plays a structural role in the protein (21). The ligands binding the copper at the active site have been identified as four histidines with one of these histidines bridging the Cu and Zn. The Zn ligands are three histidine and one aspartic acid residue.

The UV spectrum of the purified CuZnSOD has no absorbance peak at 280 nm, which is consistent with a lack of aromatic amino acids. Amino acid analyses revealed 0 - 1 tryptophans and 0 - 2 tyrosines per mole enzyme (21).

Another constant and characteristic feature of the Cu/ZnSODs is the high degree of amino acid homology among the proteins (28). The greatest degree of homology is observed between bovine and porcine SODs with the lowest identity between the bacteriocupreins and eukaryotic SODs. When the entire amino acid sequences of the Cu/ZnSODs are compared, nearly 50% identity is found. This sequence conservation indicates a slow divergence of the Cu/Zn form of the enzyme from the original source.

### **Fe Superoxide Dismutases**

The iron form of SOD was first isolated from *Escherichia coli* (29) and subsequently from other bacterial sources such as *Bacteroides fragilis* (30), *Methanobacterium bryantii* (31) and *Pseudomonas ovalis* (32). It was first thought that FeSODs were restricted to procaryotes but Salin and coworkers have isolated FeSOD from *Nuphar luteum* (water lily) (22) and *Ginkgo biloba* (ginkgo tree) (23). Generally these enzymes have a molecular weight of 40,000 and are dimeric in structure, although an 88,000 molecular weight tetramer has been isolated from *Mycobacterium tuberculosis* (33). The iron content ranges from 0.5 - 1.0 gram atom / mol subunit. Some of the isolated FeSODs have also been found to contain Zn (30), ranging from 0.2 gram-atoms/mol in *Bacteroides fragilis* to 1.7 gram-atoms/mol in *Methanobacterium bryantii*. The FeSODs are acidic proteins, pI 4 to 5, as has been characteristically found with few exceptions for all SODs (21).

### **Mn Superoxide Dismutases**

The first MnSOD isolated was from *E. coli* (34). Other Mn-containing enzymes have been found in *Streptococcus faecalis* (35), *S. mutans* (36), and *Thermus thermophilus* HB8 (37). Eukaryotic systems also produce constitutive MnSODs (80 - 100,000 molecular weight). These large molecular weight tetrameric

MnSODs are found in the mitochondria of *Saccharomyces cerevisiae* (38), bovine heart (39), and chicken liver (40). In some bacteria such as *E. coli*, *Bacteroides fragilis*, and *Propionibacterium freudenreichii* sp. *shermanii*, MnSOD is produced under special conditions. The synthesis of the Mn-containing protein in *E. coli* is repressed under anaerobic conditions (41), but when the cells were grown under 5 atm of O<sub>2</sub>, a 25-fold increase in the level of MnSOD was observed (8). The strict anaerobe, *Bacteroides fragilis*, will also synthesize a MnSOD as a response to oxidative stress, but under normal conditions contains an FeSOD (42). *Propionibacterium freudenreichii* sp. *shermanii* grown in Fe-restricted medium will produce a MnSOD instead of its native FeSOD (43).

The MnSODs vary in size from dimeric 40,000 to tetrameric 96,000 molecular weight proteins with an average subunit size of 23,000 daltons. Like the other SODs, they are an acidic group of enzymes with a pI of 4 to 5 (44). Several exceptions that have been reported are the MnSODs from *E. coli* B, pI = 6.6; from *Pleurotus olearius*, pI = 7.0 (21); and from rat liver, pI = 9.0 (45). The metal content of the purified MnSODs ranges from 0.5 - 1.0 gram atom Mn / subunit (44). Manganese-containing SODs in *Halobacterium halobium* (46) and in *Nocardia asteroides* (47) also incorporated 0.5 and 1.8 g atoms Zn / mol enzyme, respectively.

Structural and amino acid sequence data show distinct differences between the Cu/ZnSOD and the Fe or Mn forms of the enzyme. However, the Fe and MnSODs are structurally similar and show 30 - 50% sequence similarity (48). This strongly suggests the divergence of two families of SODs along separate evolutionary lines. It has also been proposed that an iron-containing SOD was the common ancestor of present-day Mn and Fe containing dismutases. This

proposal is based on the premise that anaerobes contain exclusively the Fe form of superoxide dismutase and because anaerobic organisms are believed to have preceded aerobic species in the evolution of life (21). The novel observation that superoxide dismutases in *B. fragilis* (42) and *B. thetaiotaomicron* (49) were O<sub>2</sub>-inducible and each organism apparently synthesizes an apoprotein capable of binding either Fe or Mn in a catalytically competent manner piqued our interest in superoxide dismutase from other anaerobes.

Three strains of *Actinomyces* produced 95,000 molecular weight MnSODs with specific activities of 4-8 units/mg protein measured in the cell extract. The tetrameric MnSODs have been found thus far in eukaryotes and in three aerobic bacteria. It is also another example of SOD being isolated from a facultative anaerobe. The novel physical properties of the antioxidant enzymes in these facultative anaerobes as well as the importance of *Actinomyces* in the medical community attracted our attention.

*Actinomycetaceae* is a family of gram positive bacteria which form branching filaments at some stage of their growth. It was this formation of filaments that was responsible for the initial classification of *Actinomyces* with the fungi (50). *Actinomyces* are found among the normal flora of the mouth and some species have been isolated in connection with gingivitis (51), an inflammation of the gums at the site of microbial plaque. The two *Actinomyces* species most frequently involved in oral colonization are *A. viscosus* and *A. naeslundii* (52). In addition to their importance in periodontal disease, *Actinomyces* also may be endogenous pathogens in compromised patients and cause actinomycosis (53,54). Actinomycosis is a chronic granulomatous disease marked by inflammatory lesions and can occur in cervicofacial, thoracic, or abdominal areas (50). *Actinomyces*

*naeslundii* was isolated in pure culture from several of the reported cases (54,55). *Actinomyces* play similar roles as endogenous pathogens in animal species and are a concern in veterinary medicine. *Actinomyces viscosus* which is a commensal in the oral cavity of dogs, has been reported in several cases of canine pyothorax (56). It is not apparent why this facultative anaerobe possesses SOD, but the ability of *Actinomyces* to scavenge superoxide may increase their chance of survival from the  $O_2^-$ -dependent killing mechanism of polymorphonuclear leukocytes (PMNLs) (12) in the body and also enable the organism to be transported through an oxygenated environment from one site to another.

## METHODS

### Bacterial Cultures

*Actinomyces* strains were grown anaerobically in prereduced peptone-yeast extract-glucose (PYG) media (5 g peptone, 5 g trypticase soy, 10 g yeast extract, 10 g glucose, 40 ml VPI salts solution per liter [57]) supplemented with hemin, vitamin K, 0.2% Tween 80, and 0.25% Na<sub>2</sub>CO<sub>3</sub>. The Na<sub>2</sub>CO<sub>3</sub> was added aseptically from filter-sterilized stocks to the autoclaved media. Stock cultures were maintained in chopped meat medium and were transferred aseptically under a stream of oxygen-free CO<sub>2</sub> (57). Original strains received from the VPI Anaerobe Laboratory were grown overnight in trypticase soy broth and stored at 0°C in 20% glycerol.

From 24 hr chopped meat cultures, a 2% inoculum (16 ml) was transferred to 800 ml of media which was incubated overnight at 37°C. These bacterial cultures were then transferred to 2 liters of PYG media, grown overnight, and were used to inoculate 18 liters of PYG. After 24 hours at 37°C, the cells were harvested using an Amicon Pellicon<sup>R</sup> Cell Harvester with a 0.5 um membrane. Cells were washed twice in 50 mM potassium phosphate, 1 mM EDTA, (pH 7.8) by repeated centrifugation (35,000 x g, 30 min, 4°C) and resuspension in 50 mM potassium phosphate buffer. Cells were lyophilized and stored at 0°C in a freezer.

### Molecular Weight Determination

The native molecular weight was determined chromatographically by using a reverse flow P-200 column (Biorad) calibrated with molecular weight standards. Blue dextran was used to determine the void volume.

The Laemmli method (58) of SDS polyacrylamide gel electrophoresis (15% acrylamide) was used to determine the subunit molecular weight. The samples were boiled for 10 minutes in SDS sample buffer with and without 2-mercaptoethanol. Standard molecular weight markers included bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and alpha-lactalbumin (14,200).

The molecular weights of the MnSODs from *A. naeslundii* and *Actinomyces* strain E1S.25D were verified by the method of low speed sedimentation equilibrium at Pennsylvania State University by Allan T. Phillips.

### **Metal Content**

Enzyme samples were dialyzed overnight in 20 mM chelex-treated Tris (pH 7.0). The metal content of the samples and a buffer blank was determined using a Perkin Elmer Model 560 atomic absorption spectrophotometer.

### **Enzyme Activity**

Superoxide dismutase activity was measured by the ability of SOD to inhibit the superoxide-dependent reduction of cytochrome *c* as described by McCord and Fridovich (27). The procedure was modified by the addition of 0.5 mM NaCN to each assay. When the conditions of the standard assay were altered by either change in pH, addition of  $\text{NaN}_3$ , or inclusion of different salts, the rate of cytochrome *c* reduction by superoxide was monitored under the conditions imposed and was maintained at the standard rate of 0.025 A/min by adding varying amounts of xanthine oxidase to the assay mixture.

SOD activity was visualized on native polyacrylamide gels with a modified method of Beauchamp and Fridovich (10). The gels were incubated for 30 min in a solution containing 16 mg nitrobluetetrazolium, 1 mg riboflavin and 0.2 ml TEMED (N,N,N',N'- tetramethylethylenediamine) in 80 ml of 50 mM potassium phosphate, 1 mM EDTA (pH 7.8). Upon exposure to light an insoluble blue formazan precipitate accumulated due to the superoxide-mediated reduction of nitrobluetetrazolium. Those areas of the gel containing superoxide dismutase activity appeared as achromatic bands. The SOD present in the gels scavenged the superoxide and prevented the reduction of nitrobluetetrazolium.

### **Amino Acid Analysis**

Samples were dialyzed overnight in distilled H<sub>2</sub>O and lyophilized. After vapor phase acid hydrolysis under N<sub>2</sub>, the samples were derivatized using phenylisothiocyanate and then analyzed for amino acid composition on a Waters Model 840 HPLC. Separate samples were reduced and alkylated for cysteine measurements. These assays were performed by the Protein and Nucleic Acid Sequencing Facilities at the University of Virginia. Tryptophan measurements on each purified enzyme was based on the spectrophotometric method of Edelhoch (59). Lysozyme and ribonuclease served as positive and negative control, respectively, for the tryptophan analyses.

### **Protein Determination**

The protein concentration of crude samples was estimated by measuring the absorbance at 280 nm and correcting the reading for contamination by nucleic

acids at 260 nm (60). Protein concentration of purified samples was measured by the Lowry method (61) using bovine serum albumin as the standard.

### **Second Order Rate Constant**

The second order rate constants of the SODs were measured by the procedure of Forman and Fridovich (62) based on the reaction of SOD with superoxide in competition with either cytochrome *c*. Rate constants for reaction of  $O_2^-$  with these scavengers are known.

### **Antibody Production**

Blood samples were taken from rabbits prior to immunization. Rabbits were injected with purified enzyme (100  $\mu$ g) in 1 ml of 0.9% NaCl emulsified with an equal volume of Freund's Complete Adjuvant. Three weeks later a 2 ml booster was given containing 100  $\mu$ g of the same purified protein mixed with 1 ml of Freund's Incomplete Adjuvant and 1 ml of sterile saline. This booster was given every three weeks. The serum was collected two weeks after each booster injection via ear vein venipuncture. Antibody against each of the purified SODs was obtained in individual animals by this method.

Immunoglobulin G from the rabbit serum was purified by addition of solid ammonium sulfate (243 g/l) to 40% saturation, centrifugation (35,000  $\times$  g, 15 min), and resuspension in 50 mM potassium phosphate, pH 7.4. This procedure was repeated until the ammonium sulfate precipitate was white. The white precipitate was resuspended in 20 mM potassium phosphate (pH 8.0), dialyzed in that buffer overnight and then loaded onto a DE-52 column (12  $\times$  1.5 cm) equilibrated in the same buffer. This column was then washed with the phosphate buffer. Two ml fractions were collected. The IgG fraction eluted isocratically and was monitored

by measuring the absorbance of each fraction at 280 nm. Fractions with  $A_{280} > 0.9$  were pooled and then concentrated under  $N_2$  over a YM10 ultrafilter. The protein concentration was measured by the Lowry method (61). Aliquots of 3 mls were frozen at  $0^\circ C$ .

### Ouchterlony Plates

Nine mls of 1% agarose in 10 mM sodium phosphate, 0.15 M NaCl, 0.1%  $NaN_3$ , pH 7.4 (PBS buffer) were poured into 9.5 x 4.5 cm forms and allowed to harden. The agarose was cut in the appropriate pattern with a standard double diffusion template from ICN. Wells were 4 mm in diameter. After pipetting antigen and antibody into their respective wells, the plates were incubated for 2 days at  $4^\circ C$ , washed with several changes of PBS buffer and then distilled  $H_2O$ , dried on a glass plate in a  $37^\circ C$  oven overnight, and stained with 0.5% Coomassie blue for protein (63).

### Western Blotting Technique

The immunoblotting technique developed by Towbin *et al.* (64) was used with several modifications. Proteins from the SDS gels were electrophoretically transferred to the nitrocellulose membrane using the GENIE transfer unit by Idea Scientific. The transfer buffer contained 25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS. Electrophoretic transfer was allowed to proceed for 2 hr at 24 volts. After the transfer was terminated, the SDS gels were stained with 0.2% Coomassie to monitor the completeness of the transfer. The nitrocellulose membrane was incubated at room temperature for 1 hr in 100 ml of 3% gelatin made in 10 mM Tris, 154 mM NaCl, pH 7.4 (TBS buffer). The membrane was

washed three times with TBS buffer containing Tween 20 (0.05%) for 5 minutes each. Tween 20 (0.05%) was used to decrease background reactivity. The membrane was incubated overnight with 1:100 dilution of antiserum or purified IgG (0.025 mg/ml) in TBS buffer containing 1% gelatin. The membrane was then washed three times with TBS buffer containing Tween 20 (0.05%) for 5 min each. A 1:10,000 dilution of Protein A-horseradish peroxidase (HRP) in TBS buffer containing 1% gelatin was incubated with the nitrocellulose membrane and shaken for 1 hr. After washing the membrane with TBS buffer containing Tween 20 (0.05%) three times for 5 min each, the HRP substrate mixture, 60 mg 4-chloro-1-naphthol in 20 ml cold methanol combined with 100 ml of 5 mM hydrogen peroxide in TBS buffer, was added, and the blue color development monitored. The membrane is then washed with water and dried between filter paper.

#### **Reconstitution Procedure**

The procedures used for reconstitution studies were modifications of the method by Yamakura (65) for reconstitution of FeSOD from *Pseudomonas ovalis* under alkaline conditions.

The purified MnSOD from *A. odontolyticus* was dialyzed overnight against 25 ml of 0.2M Na<sub>2</sub>CO<sub>3</sub>, 2 mM EDTA and 10 mM dithiothreitol (DTT) (pH 11.0). This reconstitution procedure was performed in Thunberg tubes and maintained under anaerobic conditions by evacuation and then flushing with N<sub>2</sub> gas. The dialysis bags were then transferred to 25 ml of 0.2 M Na<sub>2</sub>CO<sub>3</sub>, 1 mM EDTA and 1 mM DTT, dialyzed under anaerobic conditions for 8 - 10 hrs and then dialyzed overnight at 4<sup>o</sup>C against 2 L of 50 mM potassium phosphate (pH 7.8) containing 0.5 mM DTT. The procedure for reconstitution of the apoenzyme consisted of (1)

dialysis in 0.2 M  $\text{Na}_2\text{CO}_3$ , 10 mM DTT, and 12 mM  $\text{MnCl}_2$  (pH 11.0) for 2 hrs at  $25^\circ\text{C}$  and then (2) dialysis for 8-10 hours in 0.2 M  $\text{Na}_2\text{CO}_3$  containing 1 mM DTT and 1.2 mM  $\text{MnCl}_2$  (pH 9.2) at  $4^\circ\text{C}$ . These steps were carried out in Thunberg tubes under anaerobic conditions. Final steps involved dialysis in 50 mM potassium phosphate, 1 mM EDTA (pH 7.8) for 24 hrs followed by dialysis in 50 mM potassium phosphate with no chelator for 12 - 18 hrs.

## MATERIALS

The following products were purchased from Fisher Scientific: phenol reagent, sodium potassium tartrate, hydrogen peroxide, atomic absorption standards (Fe, Mn, Zn), sodium formate, Tween 80, Tween 20, glycerol, urea, ammonium sulfate, ammonium persulfate, and dialysis tubing. Sigma Chemical Company was the source of cytochrome *c* (type III), xanthine, riboflavin, EDTA, hemin, vitamin K<sub>1</sub>, cysteine, phenol red, bromphenol blue, blue dextran, nitroblue tetrazolium, protamine sulfate, phenyl-sepharose CL-4B resin, dithiothreitol, SDS-7 standards (Dalton Mark VII-L), SDS Molecular Weight Markers for Peptides, Wide Range Isoelectric Focusing Marker Kit, sodium azide, sodium cyanide, tetranitromethane, lipoxidase, aldolase, bovine serum albumin, ovalbumin, 4-chloro-1-naphthol and tris-hydroxymethyl-aminomethane (Tris). Acrylamide and Bis-acrylamide were purchased from Research Organics. Biorad was the source of sodium dodecylsulfate (SDS), Chelex 100, P-100 and P-200 gels, and protein A-horse radish peroxidase conjugate. DE-53 and DE-52 anion exchange resins are Whatman products. Nitrocellulose membranes and blotting paper were from Hoefer Scientific. Ultrapure guanidine hydrochloride was purchased from Heico. Resazurin, trypticase soy broth, yeast extract, tryptic peptone, dextrose, and gelatin were obtained from Difco. The ampholines (pH 3 - 10) were from LKB. Freund's Complete and Incomplete Adjuvants were purchased from ICN. Xanthine oxidase was purified from unpasteurized cream by the method of Waud *et al.* (66). VPI salts were prepared as described (57). Other chemicals were reagent grade and were used without further purification.

Three different strains of *Actinomyces* that contained superoxide dismutase were obtained from the Virginia Polytechnic Institute and State University (VPI) Anaerobe Laboratory. These organisms are *Actinomyces naeslundii* (VPI 9985), *Actinomyces* strain E1S.25D (VPI E1S.25D), and *Actinomyces odontolyticus* (VPI 6962D). The *Actinomyces* strain E1S.25D is a strain of *Actinomyces* that cross reacts with both *A. naeslundii* and *A. viscosus* antiserum provided by the California State Department of Health Services. Six other *Actinomyces* type strains used in these studies were also obtained from VPI Anaerobe Laboratory (Table 1).

Table 1.

## ACTINOMYCES STRAINS

VPI	Strain #s ATCC	Species	Source
1966	12102	<i>A. israelii</i> I	Human Brain Abscess
12594	23860	<i>A. israelii</i> II	Human Parotid Abscess
8617	35568	<i>A. meyeri</i>	Purulent Pleuresy
12571	15987	<i>A. viscosus</i>	Hamster periodontal disease
11468A	12104	<i>A. naeslundii</i>	Human sinus
1991-2	17929	<i>A. odontolyticus</i>	Deep carious lesions around the teeth
9985		<i>A. naeslundii</i>	Pleural fluid
E1S.25D		<i>Actinomyces</i> strain	Gingival crevice, ex- perimental gingivitis
6962D		<i>A. odontolyticus</i>	Acute respiratory infection

## RESULTS

### I. Characterization of Superoxide Dismutase from Anaerobically grown *Actinomyces*

#### A. Purification of *Actinomyces* Superoxide Dismutases

##### 1. *Actinomyces naeslundii* (9985):

Eleven grams of lyophilized cells were rehydrated in 110 mL of 50 mM potassium phosphate, 1 mM EDTA, (pH 7.8) and were sonicated for 3 five - minute bursts with the macrotip of a Branson Sonifier at 70 W input. These and subsequent operations were performed at 4<sup>o</sup> C. Cell debris was removed by centrifugation (35,000 x g, 30 min), and protamine sulfate added to the supernatant to a final concentration of 0.2% and stirred for 30 minutes. After clarification of the mixture by centrifugation (35,000 x g, 15 min), the supernatant was taken to 50% saturation by addition of solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (313 g/L). The solution was stirred for 1 hr, the mixture clarified by centrifugation (35,000 x g, 15 min) and the supernatant was loaded onto a phenyl-sepharose CL-4B column equilibrated with 30% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The column was washed with 30% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> until the effluent conductivity was < 60 mMHOs and fractions were eluted with a linear gradient of 600 ml of 30% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 600 ml of 25 mM potassium phosphate, 1 mM EDTA (pH 7.0), (Figure 1). Fractions with SOD activity were pooled, concentrated under N<sub>2</sub> over a YM10 Ultrafilter, dialyzed overnight in 25 mM potassium phosphate, 0.1 mM EDTA, (pH 7.0) and applied to a DE-53 column equilibrated with 25 mM phosphate buffer (pH 7.0). Fractions were eluted with a linear KCl gradient in 25 mM phosphate buffer (0.1 - 0.4 M KCl, 600 ml of each component) (Figure 2). Fractions with SOD activity were pooled, concentrated under N<sub>2</sub> over a YM10 Ultrafilter and applied to a P100 gel

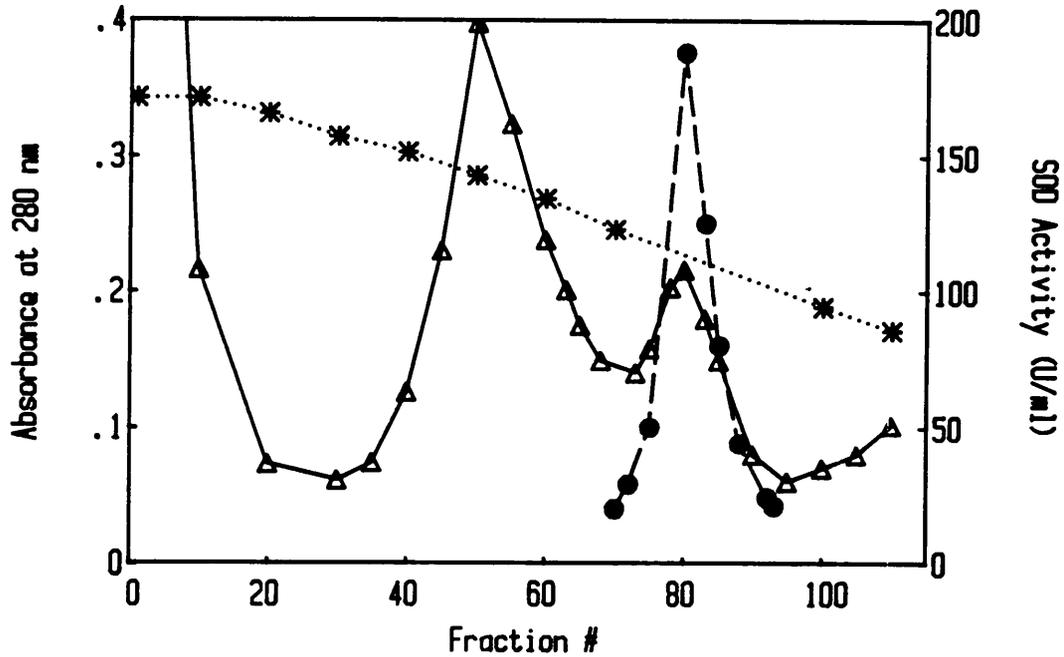


Figure 1: Chromatography of SOD from *A. naeslundii* on phenyl-sepharose CL-4B.

Supernatant from the 50% ammonium sulfate (A.S.) fractionation (313 g/L) was adsorbed onto a phenyl-sepharose CL-4B column (2.5 × 20 cm) equilibrated with 30% A.S. and then washed with the A.S. solution to remove unbound protein until the conductivity was < 60 mMHOs. The fractions were eluted with a decreasing salt gradient (58 - 30 mMHOs) consisting of 600 mLs of 30% A.S. and 600 mL of 25 mM potassium phosphate, 1mM EDTA, (pH 7.0). Fractions (7.0 mL) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

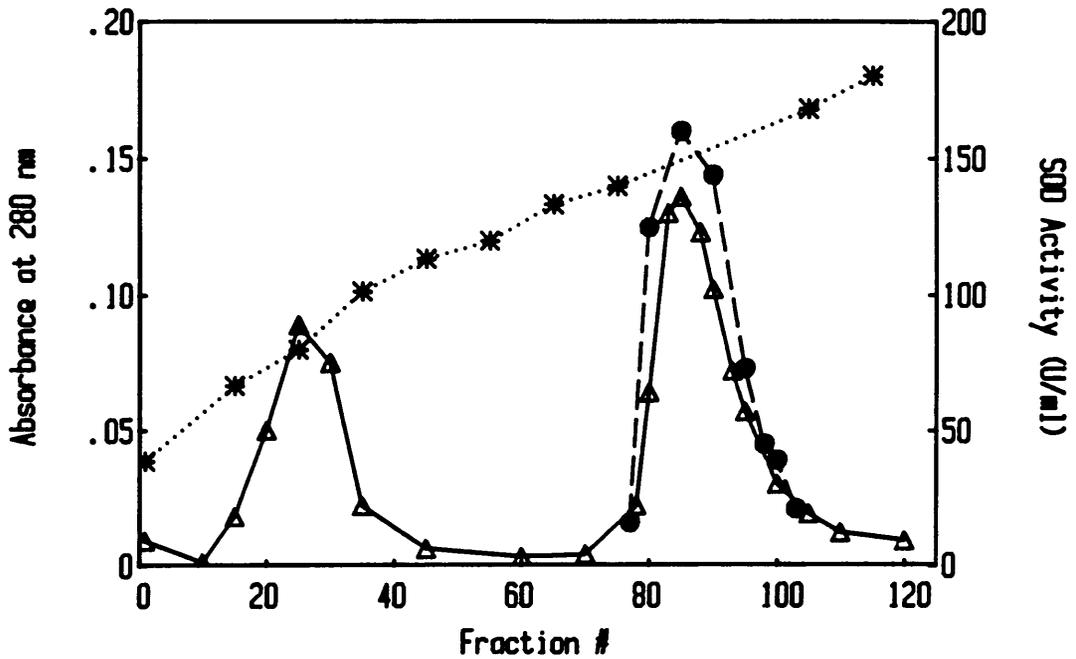


Figure 2: Chromatography of SOD from *A. naeslundii* on DE - 53.

The dialyzed sample was adsorbed onto the DE-53 column (2.5 x 16 cm) equilibrated with 25 mM potassium phosphate, 0.1 mM EDTA, (pH 7.0) and washed with buffer to remove unbound protein. The fractions were eluted with increasing salt gradient (1.5 - 14 mMHO) consisting of 600 mLs of 100 mM KCl and 600 mLs of 400 mM KCl in 25 mM potassium phosphate, 0.1 mM EDTA, (pH 7.0). Fractions (7.0 mL) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

exclusion column equilibrated in 50 mM potassium phosphate, (pH 7.0) (Figure 3). Fractions with SOD activity were pooled, concentrated, divided into 1 ml aliquots and stored at 0°C. The enzyme from *A. naeslundii* was isolated to a specific activity of 2200 U/mg with 240-fold purification and 57% yield (Table 2).

## 2. *Actinomyces* strain E1S.25D

Eleven grams of lyophilized cells were rehydrated in 110 mL of 25 mM potassium phosphate, 0.1 mM EDTA, (pH 6.5). All steps through the phenyl-sepharose CL-4B column (Figure 4) are identical to those used for isolation of *A. naeslundii* except the potassium phosphate buffer used was pH 6.5. Fractions with SOD activity were pooled and adjusted with solid  $(\text{NH}_4)_2\text{SO}_4$  (89 mg/L) to 30% saturation. This sample was rechromatographed on phenyl-sepharose CL-4B equilibrated with 30% ammonium sulfate. The column was washed with 30%  $(\text{NH}_4)_2\text{SO}_4$  and fractions were eluted with a linear gradient of 400 ml of 30%  $(\text{NH}_4)_2\text{SO}_4$  and 400 ml of 25 mM potassium phosphate, 1 mM EDTA, (pH 6.5) (Figure 5). Fractions with SOD activity were pooled, concentrated under  $\text{N}_2$  over a YM10 Ultrafilter and dialyzed overnight against 4 L of 25 mM potassium phosphate, 0.1 mM EDTA, (pH 6.5). The dialyzed sample was applied to a DE-53 column equilibrated with the dialysis buffer. This sample was eluted with a linear KCl gradient in 25 mM phosphate buffer (0.1 - 0.4 M KCl, 400 ml of each component) (Figure 6). Fractions with SOD activity were pooled, concentrated under  $\text{N}_2$  over a YM10 Ultrafilter, divided into 1 ml aliquots, and stored at 0°C. The enzyme isolated from *Actinomyces* strain E1S.25D had a specific activity of 1300 U/mg (Table 3).

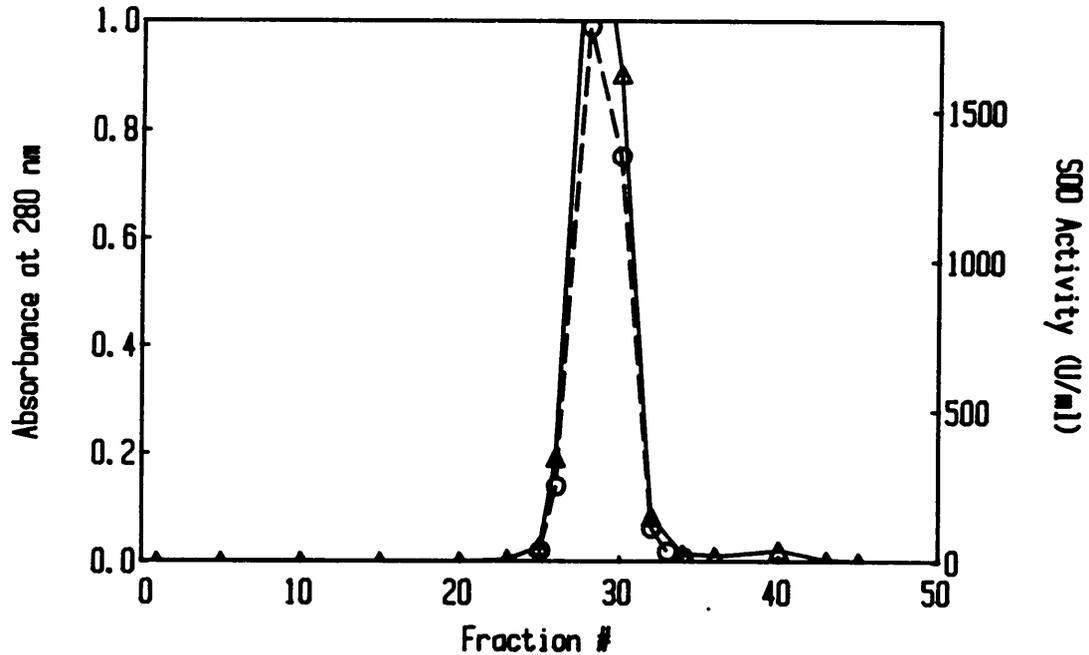


Figure 3: Gel filtration chromatography of SOD from *A. naeslundii* on Biogel P100.

The fractions with SOD activity from the DE-53 column were concentrated and applied to a Biogel P100 column (1.5 x 90 cm) equilibrated in 50 mM potassium phosphate, pH 7.0. Fractions (2 mL) were assayed for SOD activity (U/mL) [●—●] and the absorbance at 280 nm [Δ—Δ] was measured.

Table 2.

Isolation of SOD from Actinomyces naeslundii

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity	Fold Purification	% Yield
Crude Extract	102	21,000	2280	9.3	1	100
Protamine Sulfate	110	26,000	880	29.5	3.2	100
50% Ammonium Sulfate	122	33,200	650	51.3	5.5	100
Phenylsepharose (Pooled, conc. & dialyzed)	86	15,000	30	500	54.0	71
DE-53, pH 7.0 (pooled)	174	19,000	13	1460	157.0	89
P - 100 (pooled)	20	12,200	5.5	2200	240.0	57

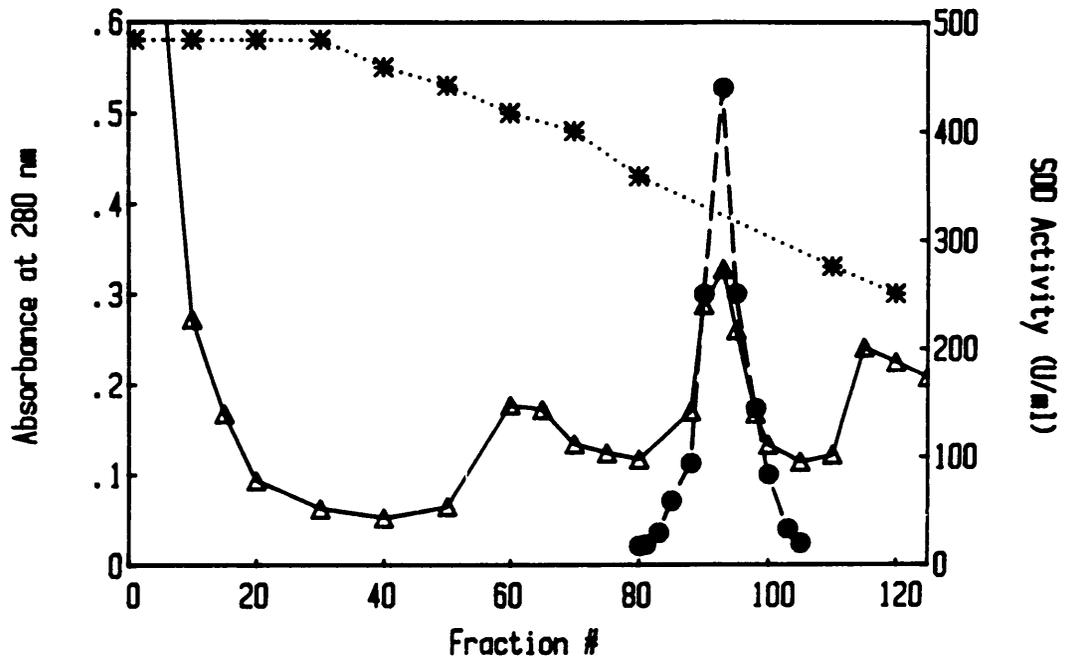


Figure 4: Chromatography of SOD from *Actinomyces* strain E1S.25D on phenyl-sepharose CL-4B.

Supernatant from the 50% ammonium sulfate (A.S.) fractionation (313 g/L) was adsorbed onto the phenyl-sepharose CL-4B column (2.5 x 20 cm) equilibrated with 30 % ammonium sulfate and then washed with the A.S. solution to remove unbound protein until the conductivity was 60 mMHOs. The fractions were eluted with a decreasing salt gradient (60 - 30 mMHOs) consisting of 600 mLs 30% A.S. and 600 mLs of 25 mM potassium phosphate, 1mM EDTA, (pH 6.5). Fractions (7.0 mL) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

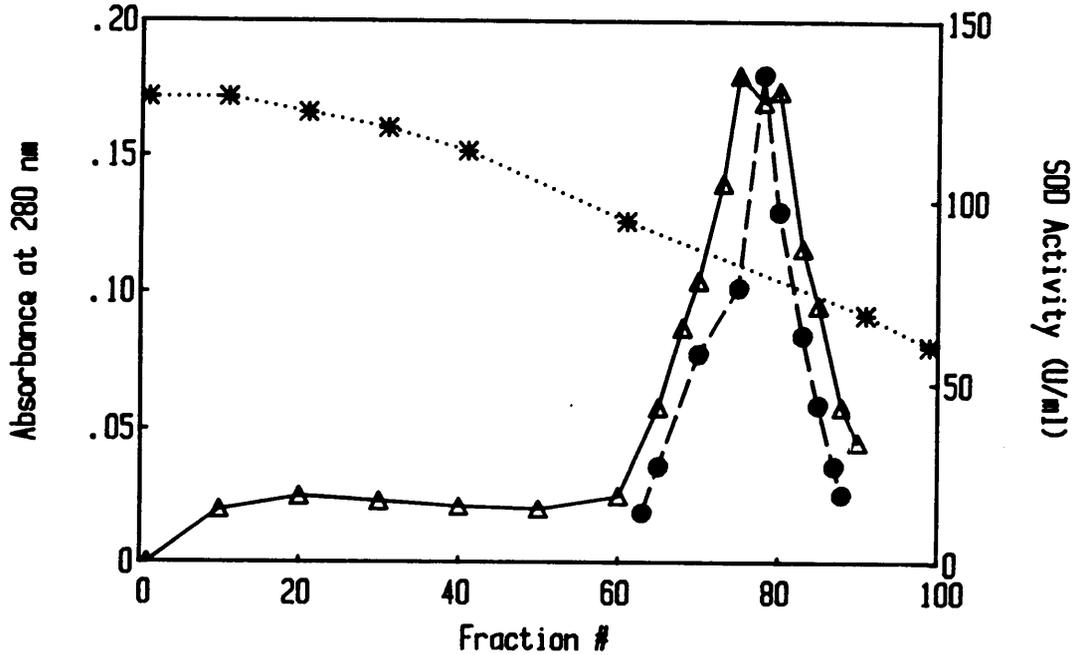


Figure 5: Chromatography of SOD from *Actinomyces* strain E1S.25D on phenyl-sepharose CL-4B (2).

The sample, readjusted to 30% ammonium sulfate, was adsorbed onto the phenyl-sepharose CL-4B column (2.5 x 16 cm) equilibrated with 30% A.S. and then washed with the A.S. solution. The fractions were eluted with a decreasing salt gradient (60 - 28 mMHOs) consisting of 400 mLs of 30% A.S. and 400 mLs of 25 mM potassium phosphate, 1 mM EDTA, (pH 6.5). Fractions (6.5 mLs) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

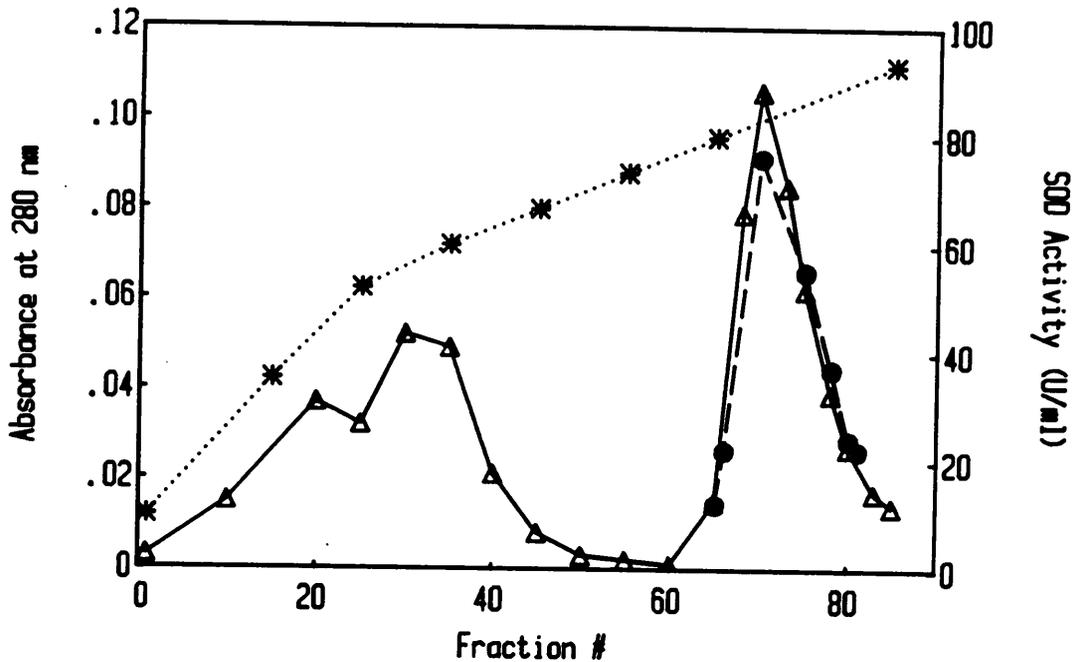


Figure 6: Chromatography of SOD from *Actinomyces* strain E1S.25D on DE-53.

The dialyzed sample was adsorbed onto the DE-53 column (1.5 x 15 cm) equilibrated with 25 mM potassium phosphate, 0.1 mM EDTA, (pH 6.5) and then washed with the same buffer to remove unbound protein. Fractions were eluted with increasing salt gradient (1.5 - 15 mMHOs) consisting of 400 mLs of 100 mM KCl and 400 mLs of 400 mM KCl in 25 mM potassium phosphate, 0.1 mM EDTA, (pH 6.5). Fractions (6.5 mLs) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

Table 3.

Isolation of SOD from Actinomyces strain E1S.25D

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity	Fold Purification	% Yield
Crude Extract	90	17,300	1390	12.5	1	100
Protamine Sulfate	96	15,200	710	21.3	1.7	87
50% Ammonium Sulfate	102	17,700	450	39.5	3.1	100
Phenylsepharose (1) (pooled)	198	10,300	13	830	66	59
Phenylsepharose (2) (pooled & conc.)	52	9,000	12	800	63	52
Post DE-53, pH 6.5 (pooled & conc.)	8	5,400	4	1300	104	31

### 3. *Actinomyces odontolyticus* (6962D)

Eleven grams of lyophilized cells were rehydrated in 110 mL of 25 mM potassium phosphate, 1 mM EDTA, (pH 7.0). All steps preceding the phenyl-sepharose CL-4B column are identical to the procedure used for *A. naeslundii* except the potassium phosphate buffer is pH 7.0. The clarified 50%  $(\text{NH}_4)_2\text{SO}_4$ -saturated supernatant was loaded onto a phenyl-sepharose CL-4B column equilibrated with 20%  $(\text{NH}_4)_2\text{SO}_4$ . The column was washed with 20%  $(\text{NH}_4)_2\text{SO}_4$  until the conductivity was < 45 mMHOS and fractions were eluted with a non-linear gradient of 450 ml of 20%  $(\text{NH}_4)_2\text{SO}_4$  and 830 ml of 5 mM potassium phosphate, 0.1 mM EDTA, (pH 7.0) (Figure 7). Fractions with SOD activity were pooled and concentrated under  $\text{N}_2$  over a YM10 Ultrafilter to 2 - 3 mL. This sample was applied to a P100 gel exclusion column equilibrated in 50 mM potassium phosphate, (pH 7.0) (Figure 8). Fractions with SOD activity were pooled and loaded onto a DE-53 column equilibrated with 25 mM phosphate, (pH 7.0). Fractions were eluted with a linear KCl gradient in 25 mM phosphate buffer (0.1 - 0.4 M KCl, 600 ml of each component) (Figure 9). Fractions with SOD activity were pooled, concentrated under  $\text{N}_2$  over a YM10 Ultrafilter, divided into 1 ml aliquots, and stored frozen. The enzyme from *A. odonto lyticus* was isolated with 85-fold purification and 38% yield (Table 4). The specific activity of the isolated enzyme was 720 U/mg.

### B. Characterization of *Actinomyces* Superoxide Dismutases

Homogeneity of the samples was demonstrated by electrophoresis on 10% native polyacrylamide gels and in polyacrylamide gels containing SDS. Each of the purified samples migrated as a single band on gels stained either for SOD

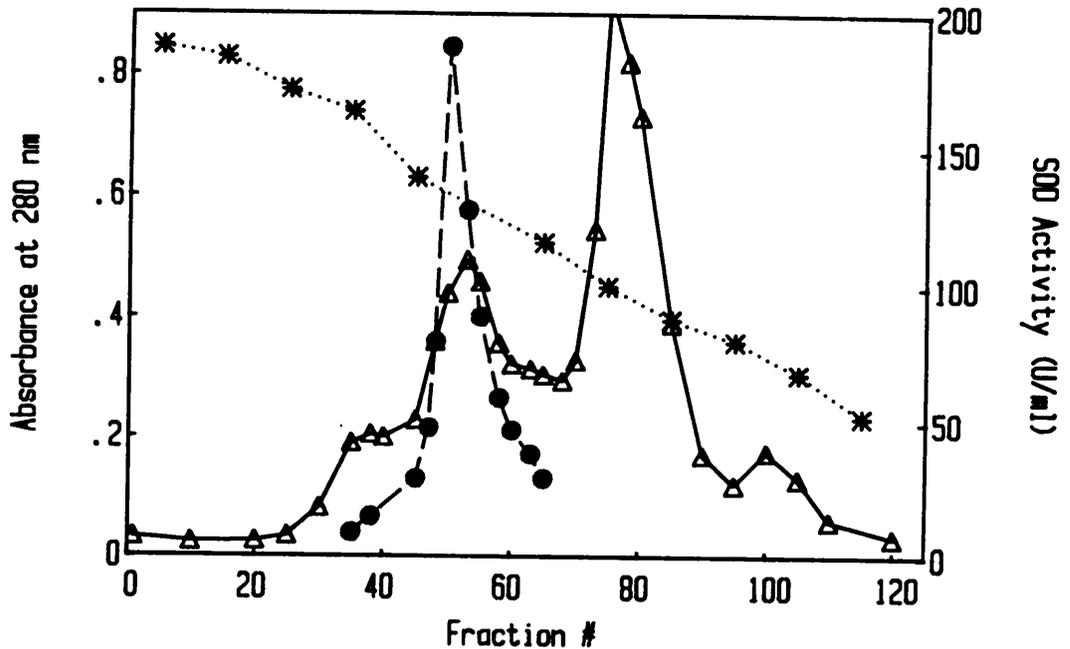


Figure 7: Chromatography of SOD from *Actinomyces odontolyticus* on phenylsepharose CL-4B.

Supernatant from the 50% ammonium sulfate (A.S.) fractionation (313 g/l) was absorbed onto the phenyl-sepharose CL-4B column (2.5 x 20 cm) equilibrated with 20 % ammonium sulfate and then washed with the same solution to remove unbound protein until the conductivity was <50 mMHOs. The fractions were eluted with a decreasing salt gradient (44 - 13 mMHOs) consisting of 450 mLs of 20% A.S. and 830 mLs of 25 mM potassium phosphate, 0.1 mM EDTA, (pH 7.0). Fractions (7.0 mL) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

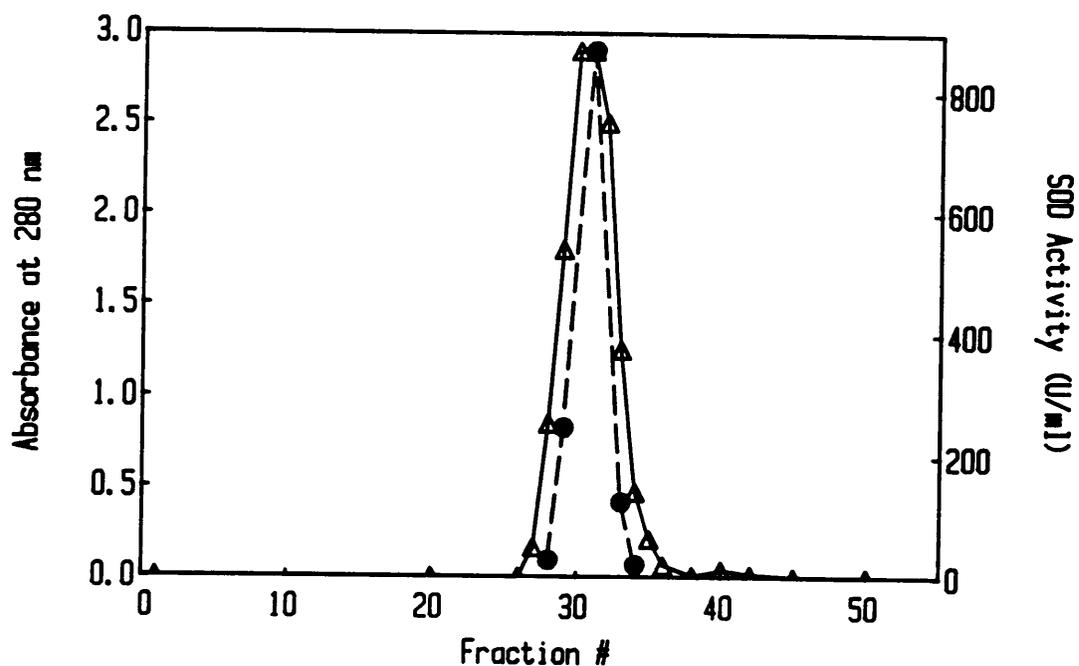


Figure 8: Gel filtration chromatography of SOD from *A. odontolyticus* on Biogel P100.

The fractions with SOD activity from phenyl-sepharose CL-4B were concentrated and applied to a Biogel P100 column. Conditions for chromatography were identical to those described in Figure 3. Fractions (2 mL) were assayed for SOD activity (U/mL) [●—●] and the absorbance at 280 nm [Δ—Δ] was measured.

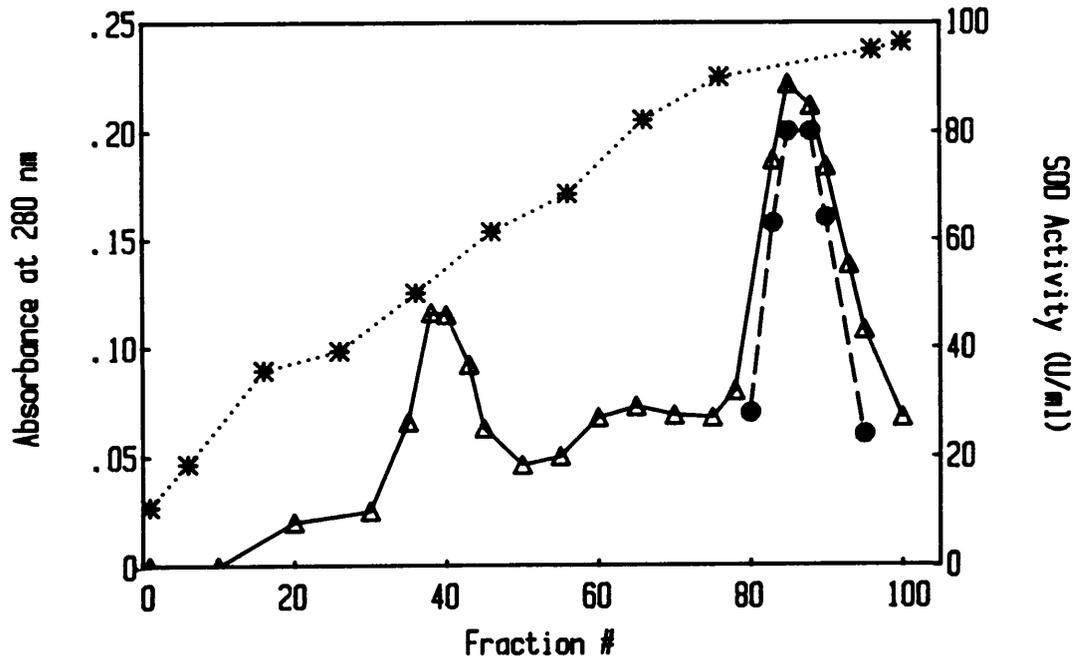


Figure 9: Chromatography of SOD from *A. odontolyticus* on DE-53.

The sample was adsorbed onto DE-53 column (1.5 x 15 cm) equilibrated with 25 mM potassium phosphate, pH 7.0 and then washed with the same buffer to remove any unbound protein. The fractions were eluted with an increasing salt gradient (1.5 - 14 mMHOs) consisting of 400 mLs of 100 mM KCl and 400 mLs of 400 mM KCl in 25 mM potassium phosphate, (pH 7.0). Fractions (6.5 mLs) were assayed for SOD activity (U/mL) [●—●], and the absorbance at 280 nm [Δ—Δ] and conductivity (mMHOs) [\*.....\*] were measured.

Table 4.

Isolation of SOD from Actinomyces odontolyticus

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity	Fold Purification	% Yield
Crude Extract	120	16,500	1960	8.4	1	100
Protamine Sulfate	130	13,000	730	17.9	2.1	80
50% Ammonium Sulfate	146	20,100	540	37.3	4.4	100
Phenylsepharose (pooled & conc.)	118	11,300	45	250	30	69
P - 100 (pooled)	12	6,200	17	370	45	38
Post DE-53, pH 7.0 (pooled & conc.)	30	6,200	9	720	85	38

activity (5 units applied) or for protein (50 ug of protein applied) in the native gel or a single band in the denaturing gel. If a contaminant was present, it was < 0.1% of the total protein.

#### *Molecular Weight*

The molecular weights of *Actinomyces* superoxide dismutases were determined in a calibrated P-200 polyacrylamide column. The native molecular weights were 109,000, 110,000, and 106,000 for the enzymes from *A. naeslundii*, *Actinomyces* strain E1S.25D, and *A. odontolyticus*, respectively (Figure 10). The native molecular weight of the enzymes from *A. naeslundii* and *Actinomyces* strain E1S.25D were also determined by low speed sedimentation equilibrium method. That method yielded molecular weights of 98,000 and 93,000 daltons for the proteins, respectively. Molecular weights of 95,000 were calculated for SODs from *A. naeslundii* and *A. odontolyticus*, and 98,500 for *Actinomyces* strain E1S.25D MnSOD based on amino acid analyses (Table 5), in agreement with the values determined from the other methods.

#### *Subunit Molecular Weight*

The subunit molecular weight was determined on SDS-containing gels as described by Laemmli (58). The SOD samples were denatured in the presence of SDS with or without 2-mercaptoethanol. The subunit molecular weights are: *A. naeslundii*, 23,000; *Actinomyces* strain E1S.25D, 24,000; and *A. odontolyticus*, 26,000 (Figure 11). Identical molecular weights were obtained with or without 2-mercaptoethanol. Thus, these proteins are tetramers with equally sized, noncovalently associated subunits.

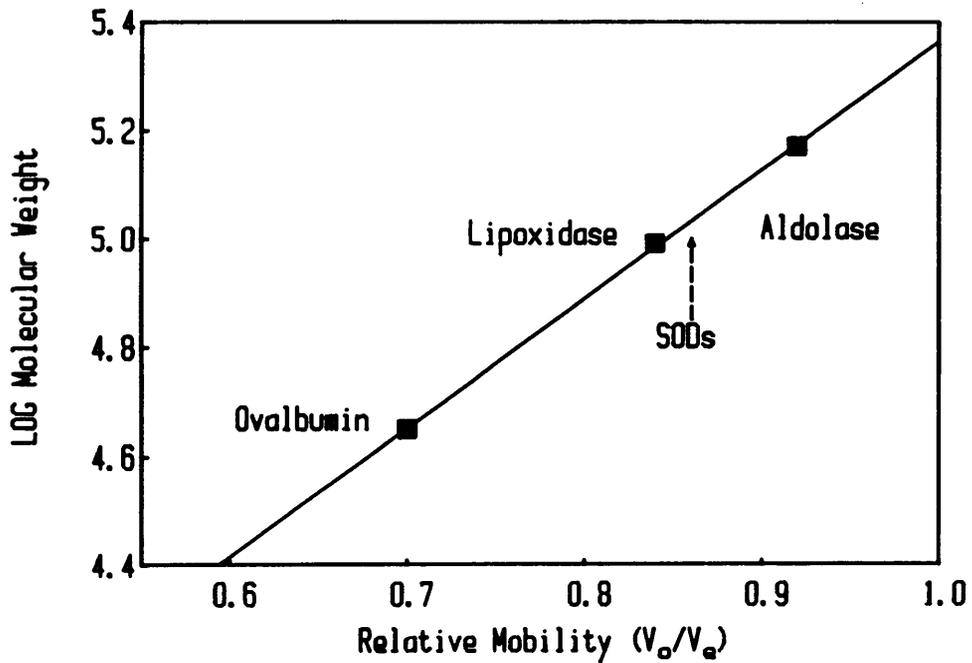


Figure 10: Native molecular weight determination of *Actinomyces* SODs by gel filtration chromatography.

Biogel P200 (1.5 × 90 cm) reverse flow column was equilibrated with 50 mM potassium phosphate, 1 mM EDTA, 100 mM KCl (pH 7.0) and calibrated with the standards indicated. SOD (0.5 mL, 0.6 mg/mL) was loaded onto the column. Fractions (1 mL) were assayed for SOD activity to determine elution volume.

Table 5.

AMINO ACID ANALYSIS *ACTINOMYCES* MnSODs

(residues/molecule)

Amino Acid	<i>A. naeslundii</i> (9985)	<i>Actinomyces</i> E1S.25D	<i>A. odontolyticus</i> (6962D)
Lys	40.0	27.2	30.0
His	31.5	32.6	32.0
Arg	17.0	17.0	34.0
Asp	90.0	95.1	87.1
Thr	31.8	30.0	49.0
Ser	40.0	48.0	53.3
Glu	94.0	103.0	93.3
Pro	35.0	35.0	27.0
Gly	87.0	83.0	71.0
Ala	140.0	106.4	136.0
Val	57.7	58.0	56.0
Ile	37.1	48.0	37.3
Leu	90.5	102.0	101.3
Tyr	44.7	37.1	28.3
Phe	42.3	45.1	38.4
Met	20.0	15.2	17.0
Cys	0	0	0
Try	23.4	26.1	34.4

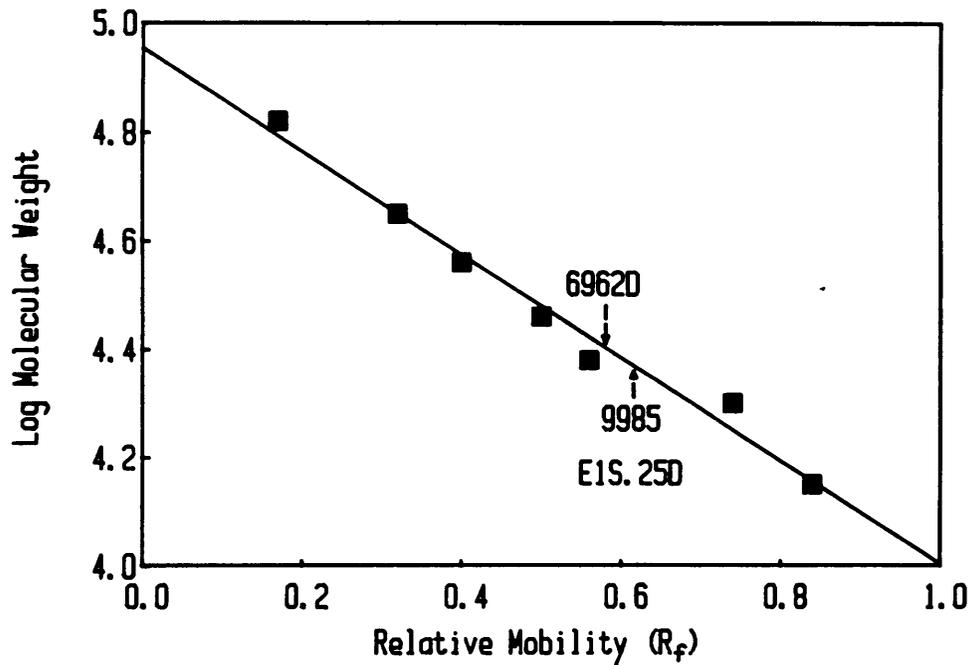


Figure 11: Subunit molecular weight determination of *Actinomyces* SODs by SDS-PAGE.

Subunit molecular weight was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate as described by Laemmli (58). The standards used were bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and alpha-lactalbumin (14,200).

### UV Spectra

Each of the three *Actinomyces* SODs exhibits a single absorbance peak with a maximum at 280 nm and a clearly discernible shoulder at 288 nm (Figure 12, Panel A). These spectral characteristics are consistent with the presence of tryptophan. There are 23, 26, and 34 tryptophan residues per mol enzyme in the purified SODs from *A. naeslundii*, *Actinomyces* strains E1S.25D, and *A. odontolyticus*, respectively (Table 5). The extinction coefficients at 280 nm are 1.85 ml·mg<sup>-1</sup>·cm<sup>-1</sup> for *A. naeslundii* SOD, 1.95 ml·mg<sup>-1</sup>·cm<sup>-1</sup> for *Actinomyces* strain E1S.25D SOD, and 2.4 ml·mg<sup>-1</sup>·cm<sup>-1</sup> for *A. odontolyticus* SOD. The values were calculated from samples whose protein content was determined by the Lowry method (61). Bovine serum albumin was used as the standard. The visible spectrum for *A. naeslundii* is shown in Figure 12, Panel B, and is similar to that of many MnSODs which have a broad absorption maximum around 480 nm and a shoulder near 600 nm (37,67).

### Metal Content

The effect of NaCN, NaN<sub>3</sub>, and H<sub>2</sub>O<sub>2</sub> on *Actinomyces* SOD was determined. *Actinomyces* SODs are stable in 2.5 mM hydrogen peroxide for up to 2 hrs and are not inhibited by 1 mM NaCN. These characteristics are similar to those of the known MnSODs, but differ from those of the Cu/Zn or FeSODs (Table 6). High azide levels (20 mM) are necessary for 20-30% inhibition of the *Actinomyces* MnSODs (Figure 13). The metal content of the *Actinomyces* SODs was measured by atomic absorption spectrophotometry. Each subunit contains 0.4 - 0.6 gram-atoms Mn and 0.4 gram-atoms Zn. Iron, if present, was below the limits of detection (< 0.1 gram-atoms Fe) (Table 7).

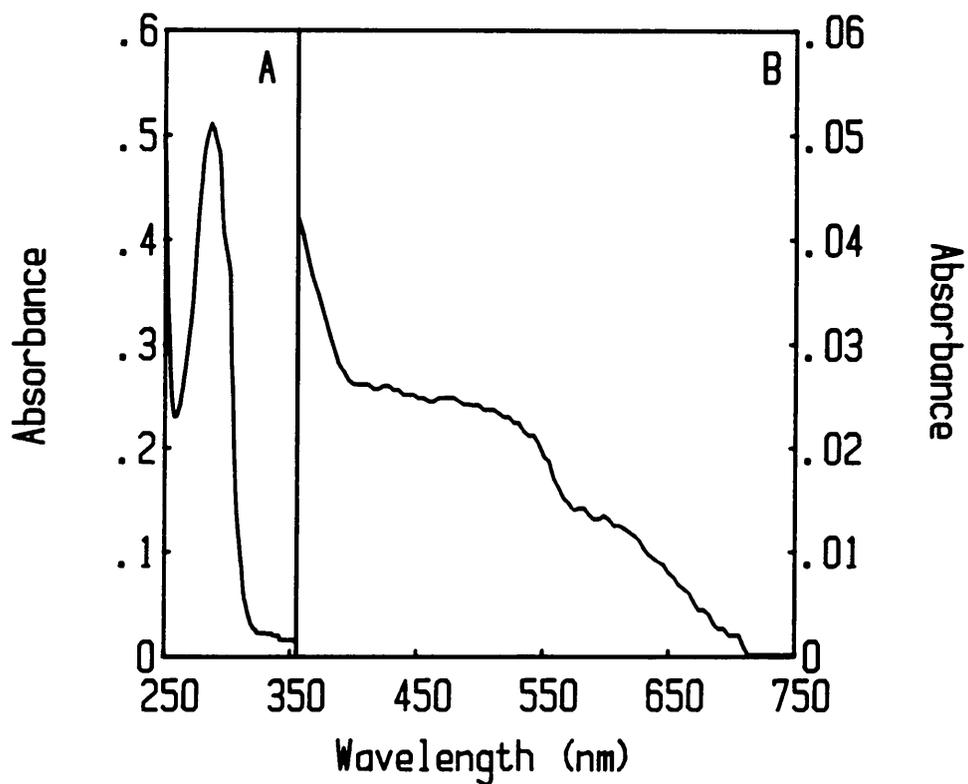


Figure 12: Absorption spectrum of *A. naeslundii* (9985) MnSOD.

The spectrum in the ultraviolet (Panel A) and visible (Panel B) regions was obtained with solution containing 0.27 mg/mL and 1.8 mg/mL respectively of the purified enzyme.

Table 6.

## INHIBITION AND INACTIVATION OF SODS

Enzyme	1 mM NaCN	1 mM NaN <sup>3</sup>	$t^{1/2}$ 5 mM H <sup>2</sup> O <sup>2</sup>
Cu/Zn	92% <sup>a</sup>	1% <sup>b</sup>	< 5 min <sup>c</sup>
Fe <sup>d</sup>	0%	89%	4 min
<i>Actinomyces</i> <sup>e</sup>	0%	0%	> 2 hrs
Yeast MnSOD <sup>e</sup>	0%	0%	> 2 hrs

a - Haffner and Coleman (17)

b - Misra and Fridovich (18)

c - Hodgeson and Fridovich (19)

d - Gregory and Dapper (30)

e - This work

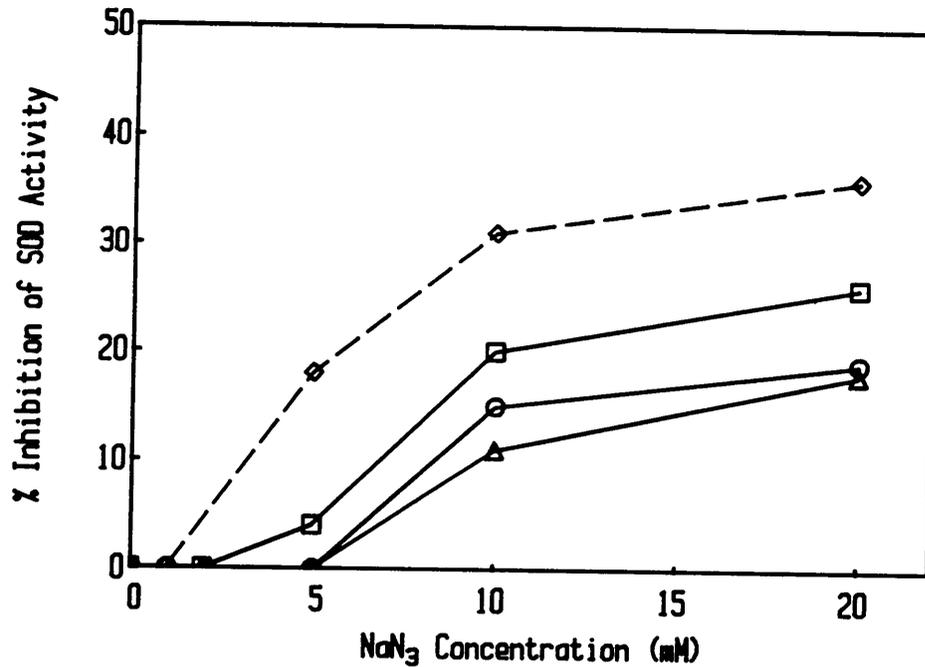


Figure 13: Azide inhibition of *Actinomyces* SODs.

The appropriate concentration of azide was added to each assay mixture and SOD activity was measured. At each concentration of azide, the amount of xanthine oxidase was adjusted to maintain an uninhibited slope of 0.025 A/min. Samples were the MnSODs from *A. naeslundii* (9985) [ $\square$ — $\square$ ]; *Actinomyces* strain E1S.25D [ $\Delta$ — $\Delta$ ]; *A. odontolyticus* (6962D) [ $\circ$ — $\circ$ ]; and *S. cerevisiae* [ $\diamond$ — $\diamond$ ].

Table 7.

METAL ANALYSES OF *ACTINOMYCES* MnSOD

Sample	Metal Content (gram-atoms/mol enzyme) <sup>a</sup>		
	Mn <sup>b</sup>	Zn <sup>b</sup>	Totals
<i>A. naeslundii</i> <sup>c</sup> (9985)	2.3	1.4	3.7
<i>Actinomyces</i> strain E1S.25D <sup>d</sup>	1.8	1.2	3.0
<i>A. odontolyticus</i> <sup>e</sup> (6962D)	1.4	1.8	3.2

a - Iron, if present, was below the detection limit  
(0.4 g-atoms / mol) for that element.

b - values are reported as mean + range

c n = 4

d n = 3

e n = 2

### *Isoelectric Points*

Each of the SODs was focused to its isoelectric point in acrylamide gels containing ampholytes. The pH gradient of isoelectric focusing gels loaded with each of the *Actinomyces* SODs formed during electrophoresis was measured directly by a surface electrode and indirectly using control gels cut into 0.5 cm pieces, extracted in 1 ml of 0.1 M KCl and measured with a standard pH electrode. The isoelectric points of each of the *Actinomyces* SODs were found to be in the range of 4.3 - 4.6. Enzymes from *A. naeslundii* and *Actinomyces* strain E1S.25D are more acidic with values of 4.3 and 4.4, respectively. The isoelectric point for *A. odontolyticus* MnSOD is 4.6. In native gel electrophoresis, *A. naeslundii* and *Actinomyces* strain E1S.25D MnSODs migrate more rapidly toward the anode ( $R_m = .45$  and  $.47$ ) than does the *A. odontolyticus* enzyme ( $R_m = .35$ ).

### *Temperature Stability*

The *Actinomyces* MnSODs and the MnSOD from *S. cerevisiae* (0.26 mg/ml) were incubated at 75<sup>o</sup> and 100<sup>o</sup>C. Aliquots (2 -5 uL) were removed periodically, diluted into 2.5 mls of 50 mM phosphate buffer, 1 mM EDTA, (pH 7.8) at room temperature, and assayed for SOD activity. Control samples incubated at room temperature and diluted appropriately showed no loss of activity for the time periods used. *Actinomyces odontolyticus* MnSOD and the MnSOD from yeast lose 100% activity after 10 minutes at 100<sup>o</sup>C. The enzymes from *A. naeslundii* and *Actinomyces* strain E1S.25D retain 20 - 30 % activity after 10 minutes incubation at 100<sup>o</sup>C but lose all activity after 40 minutes at that temperature (Figure 14A). *Actinomyces odontolyticus*, *A. naeslundii*, *Actinomyces* strain E1S.25D MnSODs retain 20, 39, and 46% of their SOD activity respectively, after 40 minutes incubation at

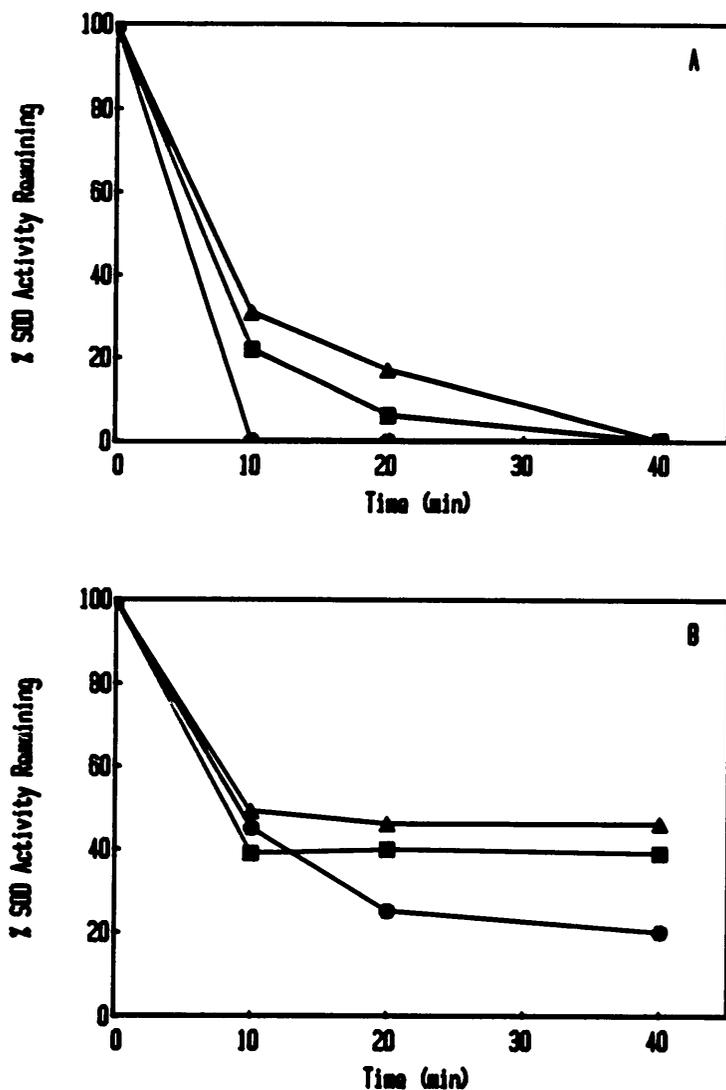


Figure 14: Temperature Stability of *Actinomyces* MnSODs.

The purified MnSODs from *Actinomyces* (0.26 mg/mL) were incubated in sealed microcentrifuge tubes at either 100°C (Figure 14A) or 75°C (Figure 14B) and aliquots were removed at the specified times and assayed for SOD activity. Control samples incubated at room temperature for the time periods used showed no loss of activity. Samples were *A. naeslundii* (9985) [■—■]; *Actinomyces* strain E1S.25D [▲—▲]; and *A. odontolyticus* (6962D) [●—●]

at 75°C (Figure 14B). The MnSOD from *S. cerevisiae* had 60 % activity remaining after 40 minutes at 75°C.

#### *Effect of pH*

At pH 8.0 for the enzymes isolated from *A. naeslundii* and *Actinomyces* strain E1S.25D and pH 7.0 for the MnSOD from *A. odontolyticus*, the enzymes showed maximal activity in 100 mM sodium phosphate buffer (Figure 15). The activity of the MnSODs from *A. naeslundii* and *Actinomyces* strain E1S.25D rapidly decrease above pH 8.0 whereas the activity of *A. odontolyticus* diminishes gradually as the pH increased from 7.0 to 9.0.

#### *Effect of Ionic Strength*

The activity of each *Actinomyces* MnSOD was measured in the presence of increasing ionic strength with several different salts. There was no inhibition of SOD scavenging activity with phosphate concentrations up to 100 mM (Figure 16). Potassium chloride, NaCl, and NaClO<sub>4</sub> diminished the SOD activity as a function of increasing ionic strength (Figure 17, 18, and 19). This dependence of activity on ionic strength also reflects a dependence on the salt used. A large monovalent salt, NaClO<sub>4</sub>, was a more effective inhibitor than the small monovalent NaCl ions. Na<sub>2</sub>SO<sub>4</sub>, a divalent salt, did not inhibit SOD activity.

#### *Second Order Rate Constants*

The second order rate constants for the *Actinomyces* MnSODs measured at pH 7.8 (Table 8, Figure 20) are 0.9 to 2.8 x 10<sup>9</sup> M<sup>-1</sup>sec<sup>-1</sup>. The rate constants for the Cu/ZnSOD from bovine liver were measured as a positive control and were

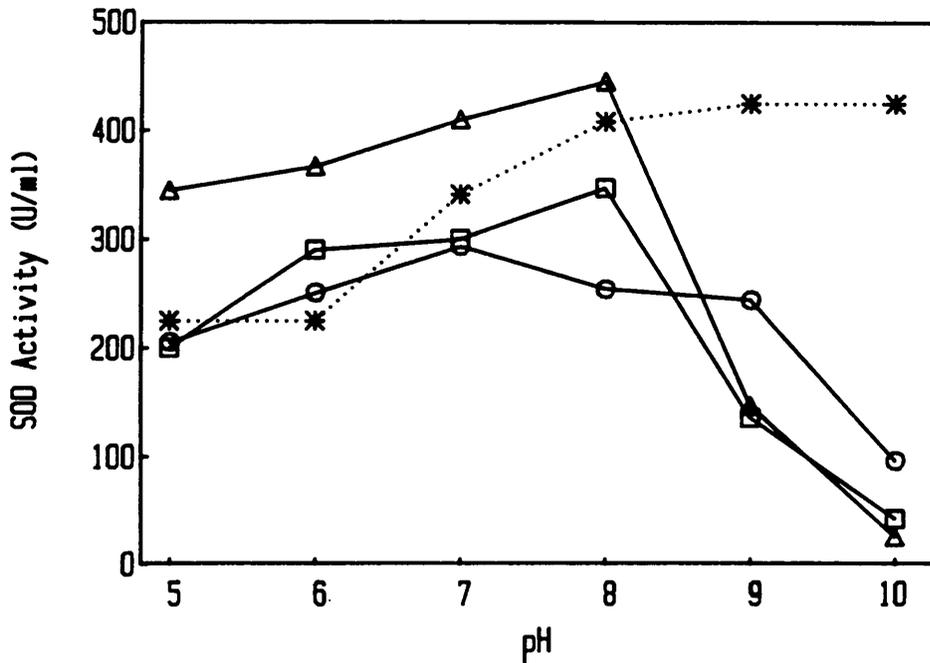


Figure 15: pH Profile of *Actinomyces* SODs.

Each solution contained 100 mM sodium phosphate, 10 mM sodium formate, 0.1 mM EDTA at the appropriate pH. At each pH value, the ability to inhibit completely the reduction of cytochrome *c* was determined by adding 5 units of SOD. SOD samples were: *A. naeslundii* (9985) [△—△]; *Actinomyces* strain E1S.25D [□—□]; and *A. odontolyticus* (6962D) [○—○]. The conductivity (mMHOs) [\*.....\*] for each pH value was determined.

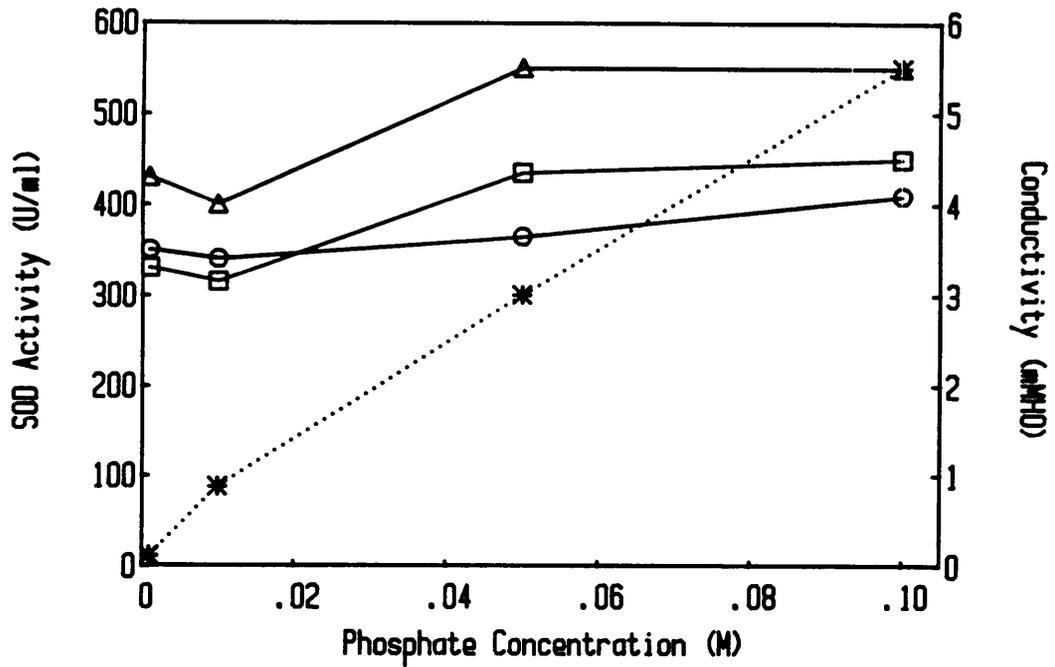


Figure 16: Effect of phosphate on the activity of *Actinomyces* SODs.

SOD activity was measured in 0.05 mM xanthine,  $1 \times 10^{-2}$  mM cytochrome *c*, and the indicated concentration of phosphate. The pH was adjusted to 7.8. SOD samples were: *A. naeslundii* (9985) [ $\Delta$ — $\Delta$ ]; *Actinomyces* strain E1S.25D [ $\square$ — $\square$ ]; and *A. odontolyticus* (6962D) [ $\circ$ — $\circ$ ]. The conductivity (mMHOs) [ $*$ ..... $*$ ] for each pH value was determined.

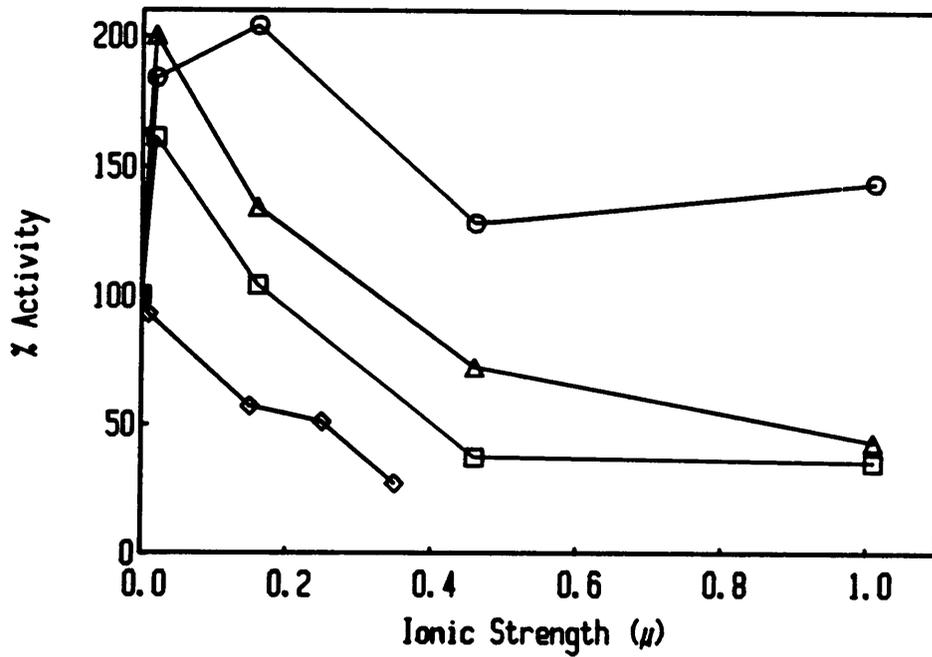


Figure 17: Effects of increasing ionic strength on the SOD from *A. naeslundii* (9985).

Salt solutions were made in 10 mM sodium formate, 0.1 mM EDTA (pH 6.5). 100% activity for each sample was measured under standard assay conditions of 50 mM potassium phosphate, 1 mM EDTA, (pH 7.8). Salts used were:  $\text{Na}_2\text{SO}_4$  ( $\circ$ ),  $\text{NaCl}$  ( $\triangle$ ),  $\text{KCl}$  ( $\square$ ), and  $\text{NaClO}_4$  ( $\diamond$ ).

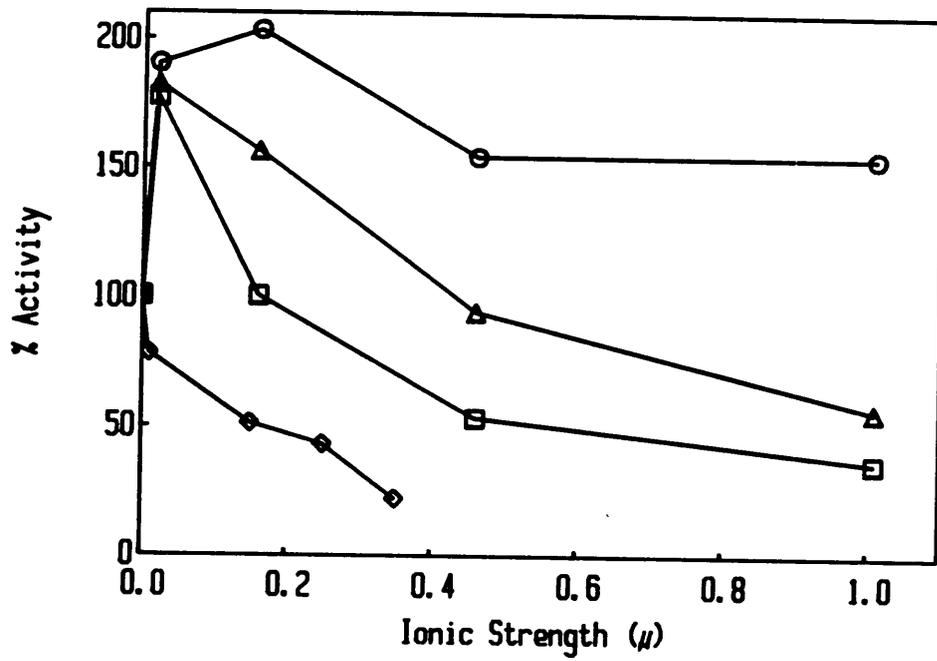


Figure 18: Effects of increasing ionic strength on the SOD from *Actinomyces* strain E1S.25D.

Conditions were as described in Figure 17.

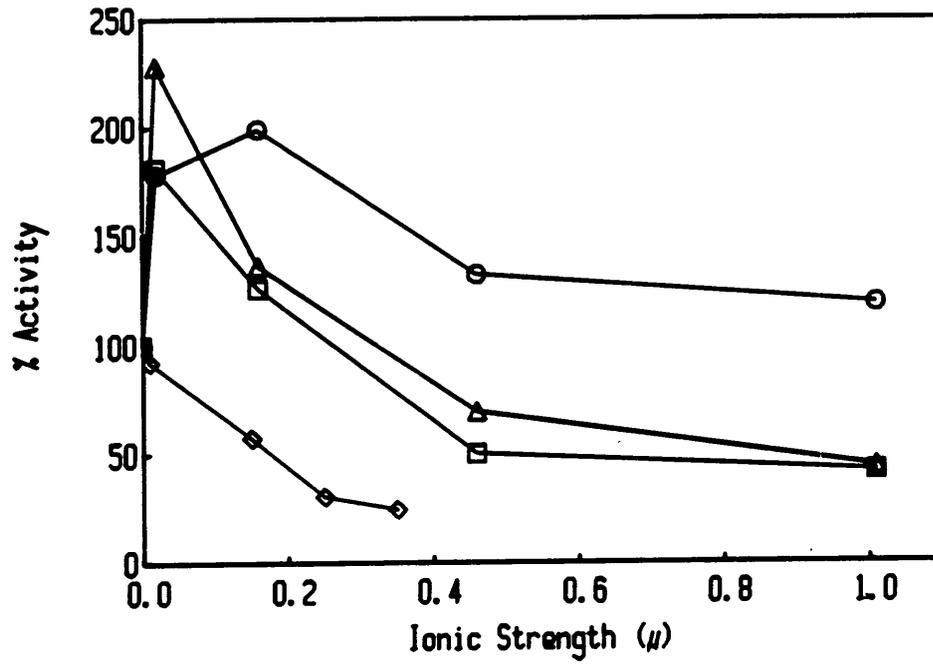


Figure 19: Effects of increasing ionic strength on the SOD from *A. odontolyticus*. Conditions were as described in Figure 17.

Table 8.

## CATALYTIC ACTIVITY OF SOD AS A FUNCTION OF pH

Enzyme Source	pH			
	6.0	7.8	8.5	10.2
	(k x 10 <sup>-9</sup> M <sup>-1</sup> sec <sup>-1</sup> )			
<i>A. odontolyticus</i> (6962D)	2.1	.91	.24	.0015
<i>Actinomyces</i> strain E1S.25D	3.1	2.7	.19	.0074
<i>A. naeslundii</i> (9985)	3.1	2.8	.23	.00046

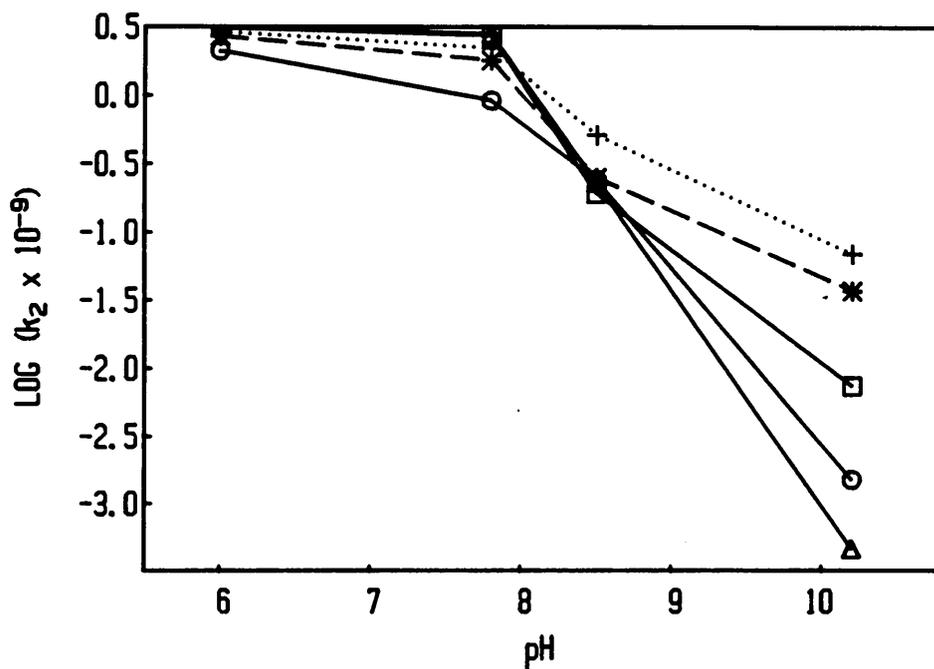


Figure 20.  $\text{Log } (k_2 \times 10^{-9} \text{ M}^{-1} \text{ sec}^{-1})$  of SOD versus pH.

The second order rate constants were measured according to the method of Forman and Fridovich (62). The samples were *A. naeslundii* (9985) MnSOD ( $\Delta$ ); *Actinomyces* strain E1S.25D MnSOD ( $\square$ ); *A. odontolyticus* (6962D) MnSOD ( $\circ$ ); Yeast MnSOD (—\*—); and Bovine liver Cu/ZnSOD (.....+.....)

used for comparison with the established values of Cu/ZnSOD. The values measured are in good agreement with the reported values for these enzymes except those at pH 10.2. As the pH was increased from 7.8 to 8.5, the rate constants for the MnSODs isolated from *Actinomyces* decreased 10 - 15 fold to average rates of  $2.2 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$ . At pH 10.2, these Mn-containing enzymes have greatly reduced second order rate constants of  $1.5 \times 10^6$  to  $4.6 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$ .

### C. Reconstitution of *Actinomyces odontolyticus* MnSOD

When the modified procedure of Yamikura (65) was used, *A. odontolyticus* SOD reconstituted with Mn had a 1.9 - 3.5 fold increase in specific activity over that of the native sample (Table 9). Attempts to reconstitute *A. naeslundii* and *Actinomyces* strain E1S.25D MnSODs were unsuccessful. Other methods utilizing urea or guanidine hydrochloride as the denaturant and EDTA or 8-hydroxy-quinoline, respectively, as the chelator did not result in appreciable recovery of MnSOD activity from *A. odontolyticus*. Reconstitution of the *Actinomyces* apoenzymes with  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  by several different methods failed to reconstitute enzymatic activity.

The isoelectric points of the native, apo-, and Mn-reconstituted SODs were measured by isoelectric focusing and were found to be identical,  $\text{pI} = 4.6$ , and there was no difference in migration of each sample in native polyacrylamide gels. The azide inhibition pattern of the reconstituted versus the native *A. odontolyticus* MnSOD (Figure 21) were very similar, with 20 mM  $\text{NaN}_3$  inhibiting the SOD activity only 33 and 22 % respectively.

Table 9.

RECONSTITUTION RESULTS FOR MnSOD  
FROM *ACTINOMYCES ODONTOLYTICUS*

	<u>Exp 1</u>	<u>Exp2</u>
Initial Specific Activity (Native - U/mg)	540	480
Final Specific Activity (Reconstituted - U/mg)	1040	1680
[Mn] concentration (Reconstituted g-atoms/mol)	2.1	1.9
Fold Increase [Mn] over Native (Native = 1.4 g-atoms/mol)	1.5	1.7
% Recovery of Total Units	128	158
% Recovery of Total Protein	67	45

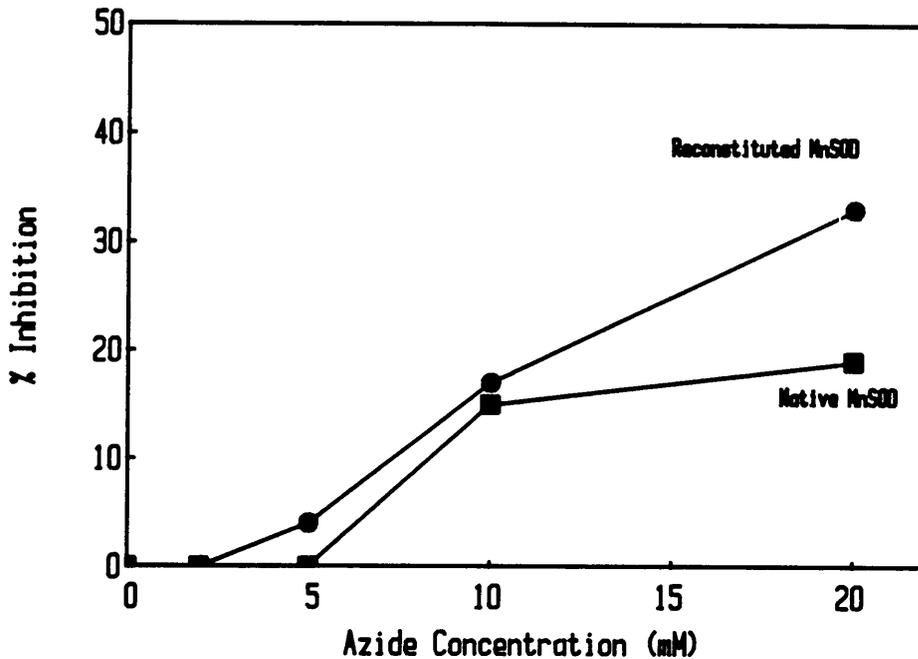


Figure 21. Azide Inhibition Studies of the Reconstituted versus Native MnSOD from *A. odontolyticus*.

The appropriate concentration of  $\text{NaN}_3$  was included in each assay mixture before the addition of the SOD sample. The slight inhibition of  $\text{NaN}_3$  on the rate of cytochrome *c* reduction was corrected to the standard rate of 0.025 A/min by adjusting the amount of xanthine oxidase added to the assay.

The calculated  $k_2$  for *A. odontolyticus* MnSOD with a specific activity of 500 U/mg was  $0.84 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  (Table 10). After reconstitution, the 2nd order rate constant based on protein concentration was increased 2-3 fold, concomitant with a 2-3 fold increase in specific activity and a 1.5-fold increase in mol Mn/mol enzyme over that of the native MnSOD (Table 9).

Table 10.

## SECOND ORDER RATE CONSTANTS (pH 7.8)

Enzyme	$M \times 10^9$	$k \times 10^{-9} M^{-1} \text{sec}^{-1}$	U/mg
<i>A. odontolyticus</i>			
Native	6.5	0.91	540
Reconstituted	3.4	1.8	1040
<hr/>			
MnSOD ( <i>E. coli</i> ) <sup>a</sup>	3.25	1.8	3800
MitoSOD (chicken liver) <sup>a</sup>	1.25	4.7	3400

a - from (62) Forman and Fridovich

## RESULTS

### II. Immunological Studies of Manganese Superoxide Dismutases from *Actinomyces*

#### A. Antibody to the *Actinomyces* MnSODs

The antibodies raised against the *Actinomyces* MnSODs reacted with the homologous antigen in Ouchterlony double diffusion gels. A single precipitin line was observed between the antiserum and the purified *Actinomyces* SOD used as the antigen. No precipitation band was found when the preimmune serum was tested against the same antigen.

#### B. Cross Reactivity between the Antibodies for *Actinomyces* MnSODs

The antibodies raised to the MnSODs from *A. naeslundii* (9985) or *Actinomyces* strain E1S.25D cross-reacted with complete identity on Ouchterlony plates with either of these two antigens, but reacted with the enzyme from *A. odontolyticus* (6962D) with only partial identity (Figure 22). In contrast, immunodiffusion analysis of the antibody prepared from rabbits injected with purified MnSOD from *A. odontolyticus* (6962D) developed spurs with the proteins from *A. naeslundii* and *Actinomyces* strain E1S.25D.

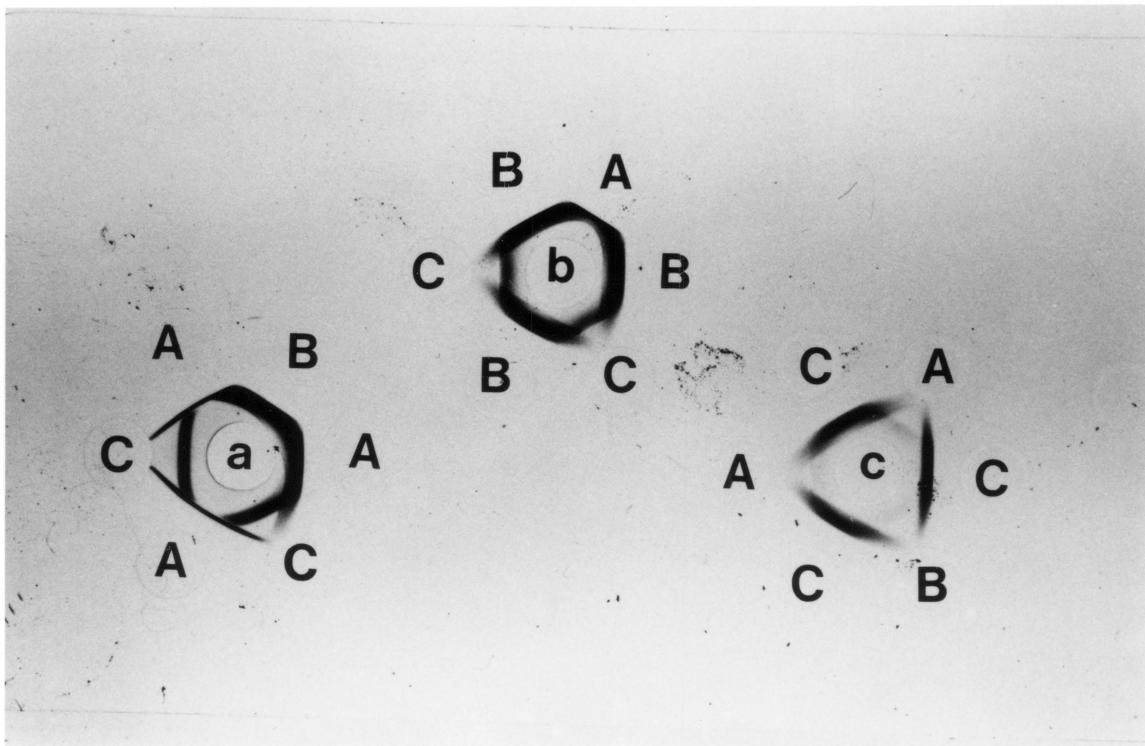
#### C. Antibody Inhibition of Superoxide Dismutase Activity

The antibody raised to *A. naeslundii* (9985) and *Actinomyces* E1S.25D inhibited SOD activity of their respective antigens (Figure 23). The enzyme from *A. naeslundii* (250 U/mL, 26 ug) retained only 13% activity after incubation with 1.35 mg of homologous antibody (52:1 ration of antibody to antigen). The same amount of anti-*A. naeslundii* MnSOD IgG (1.35 mg) inhibited the MnSODs from *A.*

Figure 22. Ouchterlony double diffusion plates for cross-reactivity between *Actinomyces* MnSODs.

Thirty uL of the isolated IgG from antisera raised to each of the *Actinomyces* MnSODs were placed in each corresponding center well. In the surrounding wells, 2.5 ug of each purified MnSOD was added. These plates were incubated at 4°C for 2 days.

- A *A. naeslundii* MnSOD
- B *Actinomyces* strain E1S.25D MnSOD
- C *A. odontolyticus* MnSOD
- a anti-*A. naeslundii* MnSOD
- b anti-*Actinomyces* strain E1S.25D MnSOD
- c anti-*A. odontolyticus* MnSOD



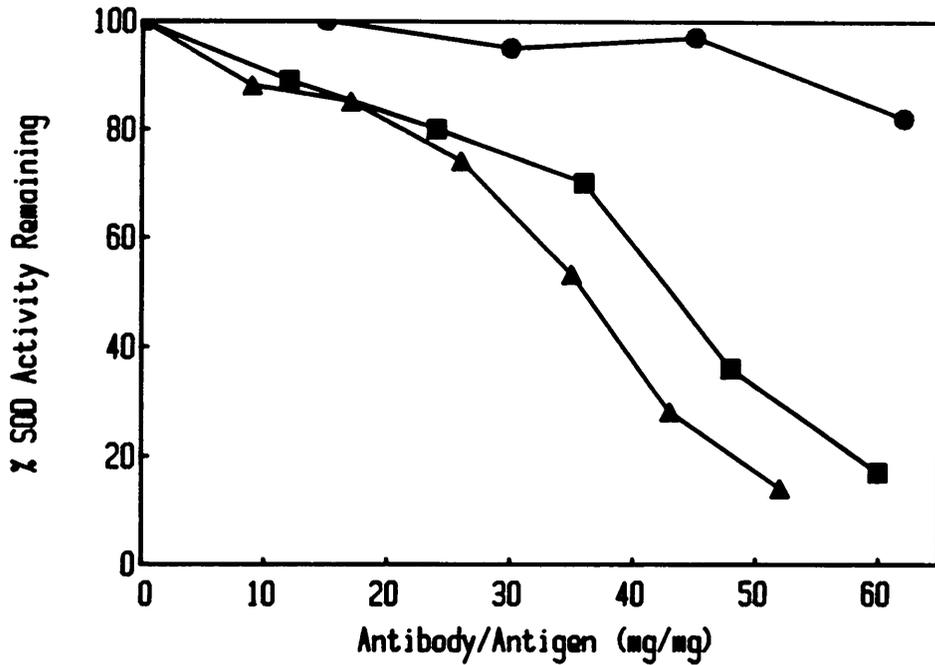


Figure 23. Antibody/Antigen Ratio versus % Activity of MnSOD isolated from *Actinomyces*.

Purified MnSODs (21 - 26  $\mu$ g) from *Actinomyces* were incubated with the corresponding amount of isolated IgG (0.21 - 1.35 mg) from the antisera against the *Actinomyces* MnSODs. The samples were incubated at 4°C for 2-4 hrs, mixed, and the remaining activity was measured. Controls included a sample with no antibody (100% activity) and a sample with no antigen (no activity). Samples were [●—●] *A. odonotolyticus* (6962D), [■—■] *Actinomyces* strain E1S.25D, and [▲—▲] *A. naeslundii* (9985).

*odontolyticus* (21 ug) and *Actinomyces* strain E1S.25D (26 ug), 89% and 83%, respectively. The enzyme from *Actinomyces* strain E1S.25D (160 U/mL, 26 ug) was inhibited 83% by 1.25 mg of isolated IgG from antisera raised against the *Actinomyces* strain E1S.25D MnSOD. Although the three antibodies raised against the *Actinomyces* MnSODs were precipitating antibodies, only *A. naeslundii* and *Actinomyces* strain E1S.25D were also inhibitory of SOD activity. The antibody to *A. odontolyticus* only inhibited the *A. odontolyticus* MnSOD 18% at a 62:1 ratio of antibody to antigen (1.35 mg:0.021mg). Neither of the antibodies against the enzymes from *A. odontolyticus* or *Actinomyces* strain E1S.25D inhibited the activity of the SOD from *A. naeslundii* (9985).

#### D. Western Blots

##### 1. *Actinomyces* strains 9985, E1S.25D, and 6962D

The purified MnSODs from the three *Actinomyces* strains and the corresponding crude extracts from these organisms were denatured in SDS with 2-mercaptoethanol, loaded onto 13.5% SDS gels, and electrophoresed. The proteins were transferred to nitrocellulose membranes and incubated with either the isolated IgG or the antiserum against one of the MnSODs from *Actinomyces* (Table 11). The antiserum from rabbits immunized against *A. naeslundii* (9985) MnSOD bound to purified *A. naeslundii* and *Actinomyces* strain E1S.25D enzymes, but did not bind to SOD from *A. odontolyticus* (6962D) in the crude or purified samples. There were two protein bands, molecular weights 23,000 and 44,000, that reacted with anti-*A. naeslundii* (9985) MnSOD in each of the crude samples of 9985 and *Actinomyces* strain E1S.25D. The antiserum from *Actinomyces* strain E1S.25D MnSOD immunized animals reacted with a 25,000 molecular weight

Table 11.

IMMUNOBLOTTING OF *ACTINOMYCES* MnSODs

Samples:	Molecular Weight Protein reacting with:		
	anti- 9985	anti- E1S.25D	anti- 6962D
<u>Purified MnSODs</u>			
<i>A. naeslundii</i> (9985)	23,000	22,100	ND
<i>Actinomyces</i> strain E1S.25D	25,200	25,200	ND
<i>A. odontolyticus</i> (6962D)	ND	25,200	27,000
<u>Crude Extracts</u>			
<i>A. naeslundii</i> (9985) 23,000	44,000	24,000	49,000
<i>Actinomyces</i> strain E1S.25D	44,000 23,000	39,000 23,000	49,000
<i>A. odontolyticus</i> (6962D)	ND 25,200	38,000	27,000

ND - No Band Detected

protein found in purified MnSOD samples and crude extracts of the three *Actinomyces* MnSODs. Anti-*Actinomyces* MnSOD from *A. odontolyticus* (6962D) bound to a 50,000 molecular weight protein in the crude extracts from *A. naeslundii* and *Actinomyces* strain E1S.25D. There was no reaction of the *A. odontolyticus* (6962D) antibody with the purified MnSODs from these same strains of *Actinomyces* (9985 and E1S.25D). The crude extract and the purified MnSOD from *A. odontolyticus* (6962D) exhibited a strongly reacting band with the antibody against 6962D MnSOD at a molecular weight of 27,000.

## 2. *Actinomyces* Type Strains

The crude extracts of several *Actinomyces* type or reference strains were measured for SOD activity (Table 12). Only one strain, *Actinomyces odontolyticus* (VPI 1991-2), had levels greater than 1 U/mg. These same samples were denatured in SDS with 2-mercaptoethanol, electrophoresed, transferred to nitrocellulose, and probed with each of the antibodies to the purified MnSOD from *Actinomyces* strains 9985, E1S.25D, and 6962D. The results (Table 13) show that each of the type strains except *A. meyeri* has a protein that reacts with at least one of the anti-*Actinomyces* MnSOD antibodies.

Table 12.

SUPEROXIDE DISMUTASE ACTIVITY IN *ACTINOMYCES*

VPI Strain #	Species	Specific Activity U/mg	Protein mg/mL
1966 <sup>T</sup>	<i>A. israelii</i> I	ND*	6.4
12594	<i>A. israelii</i> II	ND*	5.3
8617	<i>A. meyeri</i>	0.3	8.8
12571 <sup>T</sup>	<i>A. viscosus</i>	0.2	7.9
11468A <sup>T</sup>	<i>A. naeslundii</i>	0.1	11.2
1991-2 <sup>T</sup>	<i>A. odontolyticus</i>	6.9	7.7
9985	<i>A. naeslundii</i>	6.2	13.3
E1S.25D	<i>Actinomyces</i> strain	5.4	8.5
6962D	<i>A. odontolyticus</i>	4.7	13.3

\*ND- None Detected (<0.1 U/mg)

<sup>T</sup> - Type strain

*Actinomyces* strains obtained from Virginia Tech Department of Anaerobic Microbiology were grown anaerobically in 700 mL of Peptone - Yeast - Glucose media supplemented with 0.2% Tween 80 and 0.25% Na<sub>2</sub>CO<sub>3</sub>. Cells were harvested by centrifugation and lyophilized.<sup>2</sup> Crude extracts of the rehydrated cells were opened by sonication followed by centrifugation to remove cell debris. SOD activity and protein content were measured in those crude cell extracts.

Table 13.

## IMMUNOBLOTTING OF ACTINOMYCES STRAINS

Protein Reacting With:		Molecular Weight		
VPI Strain#	Sample	anti- 9985	anti- E1S.25D	anti- 6962D
1966	<i>A. israelii</i> I	41,000	ND	50,000
12594	<i>A. israelii</i> II	ND	ND	50,000
8617	<i>A. meyeri</i>	ND	ND	ND
12571	<i>A. viscosus</i>	42,000 22,000	26,000	50,000
11468A	<i>A. naeslundii</i>	42,000 23,000	24,000	50,000
1991-2	<i>A. odontolyticus</i>	ND	28,000	ND

ND - No Band Detected

## RESULTS

### III. Comparison of Manganese Superoxide Dismutases

The tetrameric MnSODs isolated from the three *Actinomyces* strains were a unique discovery in anaerobic organisms. Other anaerobes studied synthesize either FeSODs or low molecular weight MnSODs. *Actinomyces* are filamentous rods and were first classified with the yeast on the basis of similarities in morphological characteristics (filaments). *Saccharomyces cerevisiae*, or yeast, has a mitochondrial MnSOD which is also a high molecular weight tetramer (97,000). Because of the similarities between morphology and enzyme physical characteristics, the MnSOD from *S. cerevisiae* was studied in parallel with the *Actinomyces* MnSODs. The purification of yeast MnSOD was a significant modification to the published procedures (38,68) and is reported below.

#### Purification of MnSOD from Yeast

Eighteen grams of commercial dried baker's yeast (Eats, Inc., Blacksburg, Va.) were rehydrated in 60 mL of distilled water and incubated at 35°C for 90 minutes in a shaking water bath. The sample was centrifuged (35,000 × g, 15 min) and the pellet resuspended in 45 mL of 25 mM potassium phosphate, 0.1 mM EDTA, pH 7.4. These cells were disrupted with two passes through a French press operated at 22,000 p.s.i. Cell debris was removed by ultracentrifugation (100,000 × g, 60 min). The supernatant was divided into four equal portions and heated to 70°C for 5 minutes. The samples were immediately cooled in an ice bath. After centrifugation (35,000 × g, 20 min), protamine sulfate was added to

the supernatant to a final concentration of 0.2% and stirred for 30 minutes. This and subsequent operations were performed at 4°C. The mixture was centrifuged (35,000 × g, 15 min) and then taken to 50% saturation with  $(\text{NH}_4)_2\text{SO}_4$  by the addition of solid  $(\text{NH}_4)_2\text{SO}_4$  (313 g/L). The solution was stirred for 1 hr, the mixture was clarified by centrifugation (35,000 × g, 15 min) and the supernatant was loaded onto a phenyl-sepharose CL-4B column (2.5 × 20 cm) equilibrated with 30%  $(\text{NH}_4)_2\text{SO}_4$ . The column was washed with 30%  $(\text{NH}_4)_2\text{SO}_4$  and fractions were eluted with a linear gradient of 600 ml of 30%  $(\text{NH}_4)_2\text{SO}_4$  and 600 ml of 25 mM potassium phosphate, 1 mM EDTA, (pH 7.0). Fractions with SOD activity were pooled, concentrated under  $\text{N}_2$  using a YM10 Ultrafilter, dialyzed overnight against 25 mM potassium phosphate, 0.1 mM EDTA, (pH 7.4) and applied to a DE-53 column (2.5 × 16 cm). Fractions were eluted with a linear KCl gradient in 25 mM phosphate buffer (0.025 - 0.2 M KCl, 600 ml of each component). Fractions with SOD activity were pooled, concentrated, divided into aliquots and stored at 0°C. The enzyme from *S. cerevisiae* was isolated to a specific activity of 2200 U/mg with 280-fold purification and 31% yield (Table 14).

The resulting purified MnSOD from *S. cerevisiae* migrated as a single band on 10% polyacrylamide gels stained for protein (50 ug applied) coincident with a single band of activity (5 Units applied) on duplicate gels stained for SOD activity.

#### Characterization of Yeast MnSOD (Table 15)

The subunit molecular weight, metal content, and specific activity of the MnSOD isolated from *S. cerevisiae* have been reported (38). Slight differences were found in the enzyme Mn content and specific activity when isolated from

Table 14.

Isolation of SOD from Saccharomyces cerevisiae

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity	Fold Purification	% Yield
Crude Extract	95	22,200	2700	8.2	1	100
Heat to 70°C	86	17,500	1040	17.0	2.1	79
Protamine Sulfate	92	16,700	820	20.4	2.5	75
50% Ammonium Sulfate	100	19,500	710	27.0	3.3	88
Phenylsepharose (Pooled, conc. & dialyzed)	153	15,300	27	590	72	69
DE-53, pH 7.4 (pooled & conc.)	7	6,900	3	2300	280	31

Table 15.  
Comparison of MnSODs

Source	9985 <sup>c</sup>	E1S.25D <sup>c</sup>	6962D <sup>c</sup>	Yeast <sup>a</sup> MnSOD	<u>Thermus<sup>b</sup></u> thermophilus
Native MW	109,000	110,000	106,000	97,000	84,000
Subunit MW	23,000	24,000	26,000	26,000 <sup>c</sup> 25,000	25,000
Mn Conc. g-atoms/mol	2-3	1.6	1.4	2.3 <sup>c</sup> 4.0	2
pI	4.3	4.4	4.6	5.1 <sup>c</sup>	6.0
E <sub>280</sub> (ml/mg cm)	1.85	1.95	2.4	2.0	1.6
R <sub>m</sub> (10% gels)	.45	.47	.36	.23 <sup>c</sup>	ND
Specific Activity (U/mg)	2200	1300	700	2300 <sup>c</sup>	14,000 3000
2nd Order Rate constant (k x 10 <sup>-9</sup> M <sup>-1</sup> sec <sup>-1</sup> )	2.8	2.7	0.91	1.8 <sup>c</sup>	ND

ND - Not Determined      a - Ravindranath and Fridovich (38)      b - Sato and Nakazawa (37)      c - This work

yeast by our modified procedure. Although the metal content of the MnSOD from yeast was reported as 1 gram-atom Mn/subunit (38), the manganese content of the enzyme purified by our procedure was 0.6 gram-atoms per mol subunit. Purified yeast MnSOD isolated by this alternative procedure had specific activities of 2000 - 2600 U/mg, similar to 3000 U/mg reported by Ravindranath and Fridovich (38).

The second order rate constant at pH 7.8 for yeast MnSOD was  $1.8 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  and decreased rapidly as the pH increased to 10 as had been reported by Ravindranath and Fridovich (38) (Table 8, Figure 20). The isoelectric point for the MnSOD from yeast was higher (pI = 5.1) than that determined for the *Actinomyces* MnSODs, but was within the range of 4 - 6 found for other MnSODs.

#### **Comparison of Actinomyces MnSODs and other SODs**

The comparison between the reported amino acid analyses of yeast MnSOD (38) and that measured for the MnSOD from *Actinomyces naeslundii* is seen in Table 16. Also included is the reported compositional analysis of the tetrameric MnSODs from *T. thermophilus* (37) and chicken liver mitochondria (40). They contain similar number of residues per subunit for each amino acid.

Utilizing Ouchterlony double diffusion gels, other SODs were tested with *Actinomyces* MnSOD antisera for common antigenic determinants. The results (Table 17) of these incubations of antigen with antiserum showed no cross reactivity of the anti - *Actinomyces* MnSODs with Cu/Zn SOD from bovine heart, Mn- and FeSOD from *Bacteroides fragilis*, or MnSODs from yeast, *Deinococcus radiodurans*, and *Haemophilus influenzae*.

Table 16.  
AMINO ACID ANALYSES

(residues/subunit)

Amino Acid	9985 <sup>a</sup>	Yeast MnSOD <sup>b</sup>	Chicken liver mitochondria <sup>c</sup>	<i>Thermus thermophilus</i> <sup>d</sup>
Lys	10	19	12	12
His	8	7	7	8
Arg	4	4	5	5
Asp	23	28	19	16
Thr	8	11	10	7
Ser	10	8	10	3
Glu	24	28	19	20
Pro	9	10	8	12
Gly	22	19	16	16
Ala	35	21	13	18
Val	14	14	11	12
Ile	9	12	8	6
Leu	23	20	17	20
Tyr	11	9	8	9
Phe	11	11	5	8
Try	6	6	5	6
Met	5	1	3	3
Cys	0	1	2	0

a - This work (*A. naeslundii*)

b - (38)

c - (40)

d - (37)

Table 17.

CROSS REACTIVITY -  
OUCHTERLONY DOUBLE DIFFUSION PLATES

Antigen	Anti-9985	Anti-E1S.25D	anti-6962D
<i>A. naeslundii</i> (9985)	C	C	P
<i>Actinomyces</i> strain E1S.25D	C	C	P
<i>A. odontolyticus</i> (6962D)	P	P	C
<i>S. cerevisiae</i> MnSOD	N	N	N
Bovine Heart Cu/Zn	N	N	N
<i>B. fragilis</i> FeSOD	N	N	N
MnSOD	N	N	N
<i>H. influenzae</i> MnSOD	N	N	N
<i>D. radiodurans</i> MnSOD	N	N	N

C - COMPLETE IDENTITY  
P - PARTIAL IDENTITY  
N - NO CROSS-REACTIVITY

Purified yeast MnSOD (0.4 mg/mL) was denatured in 1% SDS with 2-mercaptoethanol and then electrophoresis on 13.5% polyacrylamide gels was performed. After the protein was transferred to nitrocellulose membranes, the membranes were incubated with the purified IgG or the antiserum from rabbits immunized against one of the three purified MnSODs from *Actinomyces*. None of the three antibodies bound with the 26,000 molecular weight, yeast MnSOD.

## DISCUSSION

Although all Mn-containing SODs isolated from eukaryotic sources are tetramers, only a few prokaryotes such as *Thermus aquaticus* (69), *T. thermophilus* (37), and *Mycobacterium phlei* (70) produce 80 - 100 kilodalton MnSODs. Three strains of *Actinomyces*, grown under anaerobic conditions, produce a tetrameric SOD composed of non-covalently associated 24,000 molecular weight subunits. Superoxide dismutase found in the anaerobes *Bacteroides fragilis* (30), *Chlorobium thiosulphatophilum* (21), and *Desulfovibrio desulfuricans* (21) produce dimeric FeSODs. The unique appearance of a tetrameric MnSOD in a facultative anaerobe raised questions as to the organism's phylogenetic relationship to the eukaryotic and prokaryotic kingdoms. Ribosomal RNA oligonucleotide cataloging is not consistent with a strong relationship between *Actinomyces* and any of the other organisms synthesizing tetrameric MnSODs (71,72). The only obvious relationship was based entirely on morphological similarities and historical association of yeast and *Actinomyces*.

The MnSODs from *Actinomyces* were purified by slight modifications of the same procedure. These purification procedures were repeated ten times with consistently high yields of enzymes. It was noted that the total number of SOD units measured in crude extracts of *A. naeslundii* (9985) and *A. odontolyticus* (6962D) increased after protamine sulfate and ammonium sulfate fractionation. This anomalous increase in total units may have been the result of the removal of an inhibitor of SOD activity or a component interfering with the enzyme assay. Although this occurred each time during purification, the phenomenon was not investigated.

The method of purification of MnSOD from *A. naeslundii* was successfully applied to the isolation of *S. cerevisiae* MnSOD, with the exception of using a French press to open the yeast cells. Initial attempts were made to use a combination of the two reported purification schemes for yeast MnSOD (38,68) but resulted in samples containing multiple protein bands without corresponding SOD activity bands on duplicate 10% polyacrylamide gels. Comparisons of MnSODs isolated from *Actinomyces* and *S. cerevisiae* showed that each enzyme is a high molecular weight tetrameric protein with a pIs between 4 - 6. They are antigenically distinct in the native and denatured forms, and comparison of the number of amino acid residues per subunit for each amino acid does not indicate homologous proteins.

Upon comparison of the three *Actinomyces* MnSODs (Table 18), very few differences are seen between the two proteins isolated from *A. naeslundii* and *Actinomyces* strain E1S.25D. The cross-reactivity of *Actinomyces* strain E1S.25D with the whole cell antisera against *A. viscosus* and *A. naeslundii* indicates a close relationship between this strain and *A. naeslundii*. In the native form, the MnSODs from *A. naeslundii* and *Actinomyces* strain E1S.25D are identical in immunological cross-reactivity but have antigenic differences from *A. odontolyticus* MnSOD. *Actinomyces naeslundii* (9985) and *Actinomyces* strain E1S.25D contained all the epitopes to produce single precipitin lines of complete identity with the antibodies to either protein. Conversely, the enzyme purified from strain *A. odontolyticus* contained some but not all of the epitopes and lines of partial identity formed with antibody to *A. naeslundii* and *Actinomyces* strain E1S.25D MnSODs. The same results were seen when the anti-*A. odontolyticus* MnSOD was used with the three proteins, i.e. complete identity with MnSOD from *A.*

Table 18.

Characteristics of ACTINOMYCES MnSODS

Enzyme Source	Specific Activity	Native MW	Subunit MW	Rf Native Gels	pI	$k_2 \times 10^{-9} \text{ M}^{-1}\text{sec}^{-1}$ (pH 7.8)
<u>A. naeslundii</u> (9985)	2200	109,000	23,000	.45	4.2	2.7
<u>Actinomyces</u> strain E1S.25D	1300	110,000	24,000	.47	4.3	2.8
<u>A. odontolyticus</u> (6962D)	720	106,000	26,000	.35	4.6	0.9

*odontolyticus* and partial identity with *A. naeslundii* and *Actinomyces* strain E1S.25D MnSODs.

Superoxide dismutase activity was measured in the presence of antibody. The antibodies against MnSODs from *A. naeslundii* and *Actinomyces* strain E1S.25D inhibited the activity of their respective enzymes, whereas anti-*A. odontolyticus* MnSOD at the same concentrations did not inhibit the *A. odontolyticus* SOD. The antibody against *A. odontolyticus* (6962D) is apparently binding to the enzyme at a site which does not interfere with the enzymatic activity. The antibody to *A. naeslundii* (9985) inhibits activity of all three purified *Actinomyces* MnSODs. Differences in antigenic determinants as suggested by antigen-antibody cross-reactivity studies may reflect an alteration in active site conformation or sequence between *A. odontolyticus* MnSOD and the MnSODs of *A. naeslundii* and *Actinomyces* strain E1S.25D.

When the *Actinomyces* MnSOD were denatured and transferred to nitrocellulose from SDS gels, the reactivity of antibody and antigen differed from those results from Ouchterlony double diffusion gels. The antibody to *Actinomyces* strain E1S.25D MnSOD gave bands in all the purified samples and crude extracts of the three strains of *Actinomyces* used for Western blotting technique, but the antibody against *A. naeslundii* MnSOD did not react with purified MnSOD or crude extracts of *A. odontolyticus* (6962D). Anti-*A. odontolyticus* MnSOD (6962D) reacted with purified SOD from *A. odontolyticus*, but not with the other purified *Actinomyces* MnSODs. This difference in immunological cross-reactivity is probably due to the differences in the conformation of the native versus denatured protein. This difference may be a result of amino acids located at a distance in the linear sequence which are

brought together in the native structure to form an epitope. Denaturation of the protein then destroys the epitope. A 50,000 molecular weight protein detected in the crude extracts reacted with the antibodies. Some of the tetrameric MnSODs are probably not completely denatured to monomeric form in the samples with higher protein concentrations. However, boiling the samples for 10 or 30 minutes did not destroy the 50,000 molecular weight band, and it could not be ruled out as adventitious binding to a non-related protein.

*Actinomyces odontolyticus* MnSOD is electrophoretically distinct from the other two *Actinomyces* MnSODs and has a slightly higher pI indicating less negative charge at neutral pH. This is consistent with the presence of twice as many arginine residues in this MnSOD compared to the other two. All three of the MnSODs have an overall negative charge at physiological pH as does the substrate superoxide. At very low ionic strength, the full potential of positive and negative charges in the active site are expressed. Increasing ionic strength slightly shields both positive and negative charges, but the effect is apparently that of shielding negative charges to a greater degree than positive charges. The enzyme activity as a result increases. As the ionic strength increases, the shielding of positive charges becomes more pronounced resulting in inhibition of the enzyme activity. This decrease in activity may be due to the loss of positive charges that facilitate funneling of the negatively charged substrate to the active site. In each case, the inhibition by chloride salts were similar, suggesting that the effect was due to the chloride anion rather than the cation. Larger monovalent anions have been suggested to partition into a low dielectric active site and pair with monovalent cations such as positively charged lysine and arginine residues. Perchlorate, a larger monovalent anion was a more effective

inhibitor than either chloride or divalent sulfate anions. Similar results were observed with the FeSOD in *E. coli* (74) and the Cu/Zn SOD from bovine erythrocytes (73).

These results also correlate well with the rapid decrease of the *Actinomyces* MnSODs 2nd order rate constants as the pH is increased. These results are shown in Table 19 with the reported 2nd order rate constants for other SODs. Titration of lysyl residues would result in fewer positive charges at the active site. The decrease in rate constant may also be due to competition between superoxide and the of hydroxyl anion or may be due to instability of the *Actinomyces* MnSODs at alkaline pH. However, the SOD assays at the more basic pHs were linear for two minutes. Differences in the measured 2nd-order rate constants at pH 8.5 and 10.2 between the Cu/ZnSOD from bovine liver used as a control and the values reported for Cu/ZnSOD from bovine erythrocytes could be due to the differences in the enzymes.

The metal analysis of the purified enzymes indicated approximately 0.5 gram atoms of Mn per mol subunit. Other MnSODs have been reported to contain 0.5 - 1.0 gram atoms Mn per subunit although it is believed that lower than stoichiometric amounts of metal are due to loss during purification or failure to incorporate the full complement of metal during synthesis of the protein (44). Purification of the yeast MnSOD by a procedure similar to the ones used for *Actinomyces* MnSODs resulted in metal concentration of 2.3 g-atoms per mol tetramer for the yeast enzyme instead of the reported value of 4 g-atoms per mol enzyme. In this case, the Mn is probably lost during the purification procedure.

The total amount of manganese plus zinc in the *Actinomyces* SODs approaches 4 mol metal per mol tetramer. This is consistent with 1 metal binding

Table 19.

## CATALYTIC ACTIVITY OF SOD AS A FUNCTION OF pH

Enzyme Source	pH			
	6.0	7.8	8.5	10.2
	(k x 10 <sup>-9</sup> M <sup>-1</sup> sec <sup>-1</sup> )			
<i>A. odontolyticus</i> <sup>a</sup> (6962D)	2.1	.91	.24	.0015
<i>Actinomyces</i> strain E1S.25D <sup>a</sup>	3.1	2.7	.19	.0074
<i>A. naeslundii</i> <sup>a</sup> (9985)	3.1	2.8	.23	.00046
Yeast MnSOD <sup>a</sup>	3.7	1.8	.25	.037
Cu/Zn <sup>a</sup> Bovine liver	2.9	2.2	.51	.069
Cu/Zn <sup>b</sup> Bovine erythrocytes	3.4	1.9	1.8	1.6
<i>E. coli</i> MnSOD <sup>b</sup>	2.0	1.8	.65	.33
<i>E. coli</i> FeSOD <sup>b</sup>	1.9	1.6	.30	.38
Chicken liver mitochondria MnSOD <sup>b</sup>	7.3	4.7	1.0	1.8

a - This work

b - Forman and Fridovich (62)

site per subunit. Zinc found in the Cu/ZnSOD binds to a specific ligand site different from the copper ligand site. However for these *Actinomyces* SODs, zinc is presumed to bind to a Mn binding site. This has been observed in other Fe- and MnSODs (30,46,47).

The importance of metal for catalytic activity has been shown in resolution-reconstitution studies where removal of the intrinsic metal abolishes enzyme activity. There are three basic classes of reconstitutable proteins: 1) reconstitution of denatured apoprotein with intrinsic metal, 2) reconstitution of the denatured apoprotein with a different metal and 3) reconstitution of the renatured apoprotein with the intrinsic metal. Most of the enzymes thus far studied are reconstituted from the denatured apoprotein with the metal found in the native form. In a few cases, SODs were reconstituted with a different metal. The FeSOD from *B. fragilis* is a 42,000 molecular weight dimer and was reconstituted with either Mn or Fe (30). Denatured MnSOD apoprotein from *Deinococcus radiodurans* can be renatured in the absence of metal and then reconstituted with Mn to form the holoenzyme (75). The MnSOD isolated from *A. odontolyticus* was denatured and then the denatured apoprotein reconstituted by a modified procedure of Yamikura (65). The resulting renatured holoenzyme had increased specific activity and greater total units than the initial sample. This increased activity was accompanied by a 1.5-fold increase in Mn concentration in the reconstituted versus native enzyme. The MnSOD purified from *A. odontolyticus* had a lower specific activity and a slightly lower Mn concentration than the other two MnSODs from *Actinomyces*. Whether the lower specific activity of the purified protein is due to the purification technique used or a characteristic of the protein *in vivo* is not certain, but under reconstitution conditions the specific activity and

the Mn concentration of the protein is increased to levels equalling the other *Actinomyces* MnSODs. We were unable to reconstitute the denatured apoenzyme from any of the *Actinomyces* MnSODs with iron. Several other reconstitution methods using urea or guanidine hydrochloride as the denaturing agent were tried, but the Yamikura method as modified gave the best recovery of activity without significant loss of protein. Although the MnSODs from *A. naeslundii* and *Actinomyces* strain E1S.25D were used in similar studies, all attempts to reconstitute the enzymes with Mn or Fe were unsuccessful.

There were differences in the temperature stability of the three *Actinomyces* MnSODs. *Actinomyces naeslundii* and *Actinomyces* strain E1S.25D MnSODs were more stable at 75<sup>o</sup> and 100<sup>o</sup>C than was the *A. odontolyticus* enzyme. The reconstituted enzyme from *A. odontolyticus* with higher specific activity and greater manganese content than the native enzyme did not exhibit increased heat stability.

Because SOD activity was found in these three strains of *Actinomyces*, other strains were examined for SOD activity and probed with antibody in the more sensitive Western blotting procedures to determine whether low concentrations of SOD or SOD apoprotein were present. Only one strain, *A. odontolyticus* (VPI 1991-2), contained levels of SOD activity comparable to that of the three strains used in this study. The antibodies produced against the purified MnSODs from *Actinomyces* were used to detect five other strains of *Actinomyces* (1 each of *A. israelii* I & II, *A. viscosus*, *A. naeslundii*, and *A. odontolyticus*) that contain an anti-MnSOD reacting protein(s).

Superoxide dismutase is a ubiquitous enzyme found among aerobic organisms and is an important line of defense against cellular damage from partially reduced oxygen species (6). The discovery of SOD in *Actinomyces* raises

questions as to the advantages of this facultative anaerobe to spend energy to maintain the genetic integrity and regulatory systems to produce SOD. Eight of the nine strains of *Actinomyces* used for this study were isolated from human sources: three from lung infections, two from dental caries, and one each from the sinus, ear, and brain. Four of the nine strains contained relatively high levels of SOD (4-7 U/mg), and only one of the nine strains did not react with antibody against purified *Actinomyces* MnSOD. Attempts to induce the SOD levels of *A. naeslundii* (9985), *Actinomyces* strain E1S.25D, and *A. odontolyticus* (6962D) by increasing levels of O<sub>2</sub> to 2 or 20% were unsuccessful. This failure may be due to the inability of these organisms to grow under the imposed conditions or maybe inability to transport manganese for holoenzyme synthesis. Anaerobic bacteria are generally devoid of SOD (7), but the ability of *Actinomyces* to synthesize the antioxidant enzyme may offer protection during exposure to an oxygen environment. This protection would be a significant physiological advantage to the invading organism while undergoing transport or while under attack by the body's polymorphonuclear leukocytes oxidative defense mechanism.

The relationships of the Fe-, Mn-, and the Cu/Zn - containing SODs have been studied and questions have been raised about their evolutionary divergence. It seems clear that the Fe- and MnSODs developed along a separate line from the Cu/ZnSODs (48). Those anaerobic organisms that have SOD generally produce the FeSOD and it is thought to be the oldest form of the enzyme (21). At some point the dimeric iron enzyme has evolved into a tetrameric MnSOD found in mitochondria and a few prokaryotes. In order to establish the evolutionary relationship between the *Actinomyces* Mn-containing SOD and MnSODs from other

organisms, it will be necessary to analyze the amino acid sequence of SOD from *Actinomyces*.

## REFERENCES

- (1) Byczkowski, J. Z., and T. Gessner. 1988. Minireview: Biological role of superoxide ion-radical. *Int. J. Biochem.* 20: 569 - 580.
- (2) McCord, J.M., and E. D. Day. 1978. Superoxide-dependent production of hydroxyl radical catalyzed by an iron-EDTA complex. *FEBS. Letts.* 86: 139-142.
- (3) Fridovich, I. 1986. Biological effects of the superoxide radical. *Arch. Biochem. Biophys.* 247: 1-11.
- (4) Inlay, J. A., and S. Linn. 1988. DNA damage and oxygen radical toxicity. *Science* 240: 1302-1309.
- (5) Behar, D., G. Czapski, J. Rabini, L. M. Dorfman, and H. A. Schwarz. 1970. The acid dissociation constant and decay kinetics of the perhydroxy radical. *J. Phys. Chem.* 74: 3209-3212.
- (6) Fridovich, I. 1974. Superoxide dismutase. *Adv. Enzymol.* 41: 35-97.
- (7) McCord, J. M., B. B. Keele, Jr., and I. Fridovich. 1971. An enzyme-based theory of obligate anaerobiosis: The physiological function of superoxide dismutase. *Proc. Nat. Acad. Sci. U.S.A* 68: 1024-1027.

- (8) Gregory, E. M., and I. Fridovich. 1973. Induction of superoxide dismutase by molecular oxygen. *J. Bacteriol.* 114: 543-548.
- (9) Hassan, H. M., and I. Fridovich. 1977. Regulation of the synthesis of superoxide dismutase in *Escherichia coli*. *J. Biol. Chem.* 252: 7667-7672.
- (10) Beauchamp, C., and I. Fridovich. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44: 276-287.
- (11) Natvig, D. O., K. Imlay, D. Touati, and R. A. Hallewell. 1987. Human copper-zinc superoxide dismutase complements superoxide dismutase-deficient *Escherichia coli* mutants. *J. Biol. Chem.* 262: 14697-14701.
- (12) Root, R. K., and M. S. Cohen. 1981. The microbicidal mechanisms of human neutrophils and eosinophils. *Rev. Infect. Diseases* 3: 565-598.
- (13) Beaman, B. L., C. M. Black, F. Doughty, and L. Beaman. 1985. Role of superoxide dismutase and catalase as determinants of pathogenicity of *Nocardia asteroides*: Importance in resistance to microbicidal activities of human polymorphonuclear neutrophils. *Infect. Immun.* 47: 135-141.
- (14) Hassan, H. M., A. R. Bhatti, and L. A. White. 1984. Superoxide dismutase, catalase and peroxidase in four strains of *Neisseria meningitidis* of different virulence. *FEMS Micro. Lettr.* 25: 71-74.

- (15) Kanafani, H., and S. E. Martin. 1985. catalase and superoxide dismutase activities in virulent and nonvirulent *Staphylococcus aureus* isolates. *J. Clin. Micro.* 21: 607-610.
- (16) Fridovich, I. 1986. Superoxide dismutases. *Advances in Enzymology* 58: 61-97.
- (17) Haffner, P. H., and J. E. Coleman. 1973. Cu(II)-carbon bonding in cyanide complexes of copper enzymes. *J. Biol. Chem.* 248: 6626-6628.
- (18) Misra, H. P., and I. Fridovich. 1978. Inhibition of superoxide dismutases by azide. *Arch. Biochem. Biophys.* 189: 317-322.
- (19) Hodgeson, E. K., and I. Fridovich. 1975. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide. Inactivation of the enzyme. *Biochem.* 14: 5294-5299.
- (20) Kanematsu, S., and K. Asada. 1978. Superoxide dismutase from an anaerobic photosynthetic bacterium, *Chromatium vinosum*. *Arch. Biochem. Biophys.* 185: 473-482.
- (21) Steinman, H. M. Superoxide dismutases: Protein chemistry and structure - function relationships. In: Oberley, Larry W., ed. *Superoxide Dismutase*, Vol I. Florida: CRC Press, Inc.; 11 - 68.

- (22) Salin, M. L., and S. M. Bridges. 1982. Isolation and characterization of an iron-containing superoxide dismutase from the water lily, *Nuphar luteum*. *Plant. Physiol.* 69: 161-165.
- (23) Duke, M. V., and M. L. Salin. 1985. Purification and characterization of an iron-containing superoxide dismutase from a eucaryote, *Ginkgo biloba*. *Arch. Biochem. Biophys.* 243: 305-314.
- (24) Salin, M. L., and S. M. Bridges. 1980. Isolation and characterization of an iron-containing superoxide dismutase from a eukaryote, *Brassica campestris*. *Arch. Biochem. Biophys.* 201: 369.
- (25) Steinman, H. M. 1982. Copper-zinc superoxide dismutase from *Caulobacter crescentus* CB15. A novel bacteriocuprein form of the enzyme. *J. Biol. Chem.* 257: 10283-10293.
- (26) Steinman, H. M. 1982. Bacteriocuprein superoxide dismutases in pseudomonads. *J. Bacteriol.* 162: 1255-1260.
- (27) McCord J. M., and I. Fridovich. 1969. Superoxide dismutase. An enzymatic function for erythrocyte hemocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049-6055.
- (28) Bannister, J. V., W. H. Bannister, and G. Rotilio. 1987. Aspects of the structure, function, and applications of superoxide dismutase. In: *CRC Critical Reviews of Biochemistry.* 22: 111-180.

- (29) Yost, F. J., Jr., and I. Fridovich. 1973. An iron-containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem.* 248: 4905-4908.
- (30) Gregory, E. M., and C. H. Dapper. 1983. Isolation of iron-containing superoxide dismutase from *Bacteroides fragilis*: reconstitution as a Mn-containing enzyme. *Arch. Biochem. Biophys.* 220: 293-300.
- (31) Kirby, T. W., J. R. Lancaster, Jr., and I. Fridovich. 1981. Isolation and characterization of the iron-containing superoxide dismutase of *Methanobacterium bryantii*. *Arch. Biochem. Biophys.* 210: 140-148.
- (32) Yamikura, F. 1976. Purification, crystallization and properties of iron-containing superoxide dismutase from *Pseudomonas ovalis*. *Biochem. Biophys. Acta.* 422: 280-294.
- (33) Kusunose, E., K. Ichihara, Y. Noda, and M. Kusunose. 1976. Superoxide dismutase from *Mycobacterium tuberculosis*. *J. Biochem.* 80: 1343-1352.
- (34) Keele, B.B., Jr., J.M. McCord, and I. Fridovich. 1970. Superoxide dismutase from *Escherichia coli* B. A new manganese-containing enzyme. *J. Biol. Chem.* 245: 6176-6181.

- (35) Britton, L., D. P. Malinowski, and I. Fridovich. 1978. Superoxide dismutase and oxygen metabolism in *Streptococcus faecalis* and comparisons with other organisms. *J. Bacteriol.* 134: 229-236.
- (36) Vance, P. G., B. B. Keele, Jr., and K. V. Rajagopalan. 1972. Superoxide dismutase from *Streptococcus mutans*. Isolation and characterization of two forms of the enzyme. *J. Biol. Chem.* 247: 4782-4786.
- (37) Sato, S., and K. Nakazawa. 1978. Purification and properties of superoxide dismutase from *Thermus thermophilus* HB8. *J. Biochem.* 83: 1165-1171.
- (38) Ravindranath, S. D., and I. Fridovich. 1975. Isolation and characterization of a manganese-containing superoxide dismutase from yeast. *J. Biol. Chem.* 250: 6107-6112.
- (39) Marklund, S. 1978. Purification and characterization of a manganese containing superoxide dismutase from bovine heart mitochondria. *Int. J. Biochem.* 9: 299-306.
- (40) Weisiger, R. A., and I. Fridovich. 1973. Superoxide dismutase: Organelle specificity. *J. Biol. Chem.* 248: 3582-3592.
- (41) Hassan, H. M., and I. Fridovich. 1977. Enzymatic defenses against the toxicity of oxygen and of streptonigrin in *Escherichia coli*. *J. Bacteriol.* 129: 1574-1583.

- (42) Gregory, E. M. 1985. Characterization of the O<sub>2</sub>-induced manganese-containing superoxide dismutase from *Bacteroides fragilis*. *Arch. Biochem. Biophys.* 238: 83-89.
- (43) Meier, B., D. Barra, F. Bossa, L. Calabrese, and G. Rotilio. 1982. Synthesis of either Fe- or Mn-superoxide dismutase with an apparently identical protein moiety by an anaerobic bacterium dependent on the metal supplied. *J. Biol. Chem.* 257: 13977-13980.
- (44) Ludwig, M. L., K. A. Patridge, and W. C. Stallings. 1986. Manganese superoxide dismutase: structure and properties. In: Schramm, V.L.; Wedler, F.C., editors. *Manganese in Metabolism and Enzyme Function*. New York: Academic Press, Inc.; 405-430.
- (45) Ishikawa, T., A. R. Hunaiti, G. Piechot, and B. Wolf. 1987. Isolation and characterization of basic superoxide dismutase consisting of M<sub>r</sub>-25,000 subunits in rat liver. *FEBS.* 317-323.
- (46) Salin, M. L., and D. Oesterhelt. 1988. Purification of a Mn-containing superoxide dismutase from *Halobacterium halobium*. *Arch. Biochem. Biophys.* 260: 806-810.

- (47) Beaman, B. L., S. M. Scates, S. E. Moring, R. Deam, and H. P. Misra. 1981. Purification and properties of a unique superoxide from *Nocardia asteroides*. *J. Biol. Chem.* 258: 91-96.
- (48) Harris, J. I., A. D. Auffret, F. D. Northrop, and J. E. Walker. 1980. Structural comparisons of superoxide dismutases. *Eur. J. Biochem.* 106: 297-303.
- (49) Pennington, C. D., and E. M. Gregory. 1986. Isolation and reconstitution of iron- and manganese-containing superoxide dismutases from *Bacteroides thetaiotaomicron*. *J. Bacteriol.* 166: 528-532.
- (50) Slack, J.M., and M.A. Gerencser. 1975. *Actinomyces, Filamentous Bacteria. Biology and Pathogenicity*. Minneapolis, Mn.: Burgess Publishing Co.
- (51) Page, R.C. 1986. Gingivitis. *J. Clin. Periodontol.* 13: 345-355.
- (52) Ellen, R. P. 1982. Oral colonization by gram-positive bacteria significant to periodontal disease. In: *Host Parasite Interactions in Periodontal Diseases*, Genko, R. J., and W. E. Mergenhagen, Eds. Washington, D.C.: American Society for Microbiology, 98-111.
- (53) Dobson and Edwards. 1987 Extensive *Actinomyces naeslundii* infection in a child. *J. Clin. Micro.* 25: 1327-1329.

- (54) Freland, C., B. Massoubre, J. M. Horeau, J. Caillon, and H. B. Drugeon. 1987. Actinomycosis of the gallbladder due to *Actinomyces naeslundii*. *J. Infect.* 15: 251-257.
- (55) Bonnez, W., G. Lattimer, N. A. C. Mohanraj, and T. H. Johnson. 1985. *Actinomyces naeslundii* as an agent of pelvic actinomycosis with presence of an intrauterine device. *J. Clin. Microbiol.* 21: 273-275.
- (56) Turner, W. D., and E. M. Breznock. 1988. Continuous suction drainage for management of canine pyothorax - A retrospective study. *J. Amer. Hosp. Assoc.* 24: 485-494.
- (57) Holdeman, L. V., E. P. Cato, and W. E. C. Moore, eds. 1977. *Anaerobe Laboratory Manual, 4th Edition*. Blacksburg, Va.: VPI Anaerobe Laboratory
- (58) L  emmler, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- (59) Edelhoch, H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochem.* 6: 1948-1954.
- (60) Walberg, O., and W. Christian. 1941. Isolierung und kristallisation des garung fermentes enolase. *Biochem. Z.* 310: 384-421.

- (61) Lowry, O. A., N. J. Rosebrough, L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- (62) Forman, H.J., and I. Fridovich. 1973. Superoxide dismutase: A comparison of rate constants. *Arch. Biochem. Biophys.* 158: 396-400.
- (63) Ouchterlony, O. 1968. *Handbook of Immunodiffusion and Immunelectrophoresis*. Ann Arbor Sci. Publi.: Ann Arbor, Michigan.
- (64) Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4354.
- (65) Yamakura, F. 1978. A study on the reconstitution of iron-superoxide dismutase from *Pseudomonas ovalis*. *J. Biochem.* 83: 849-857.
- (66) Waud, W. R., R. O. Brady, R. D. Wiley, and K. V. Rajagopalan. 1975. A new purification procedure for bovine milk xanthine oxidase: Effect of proteolysis on the subunit structure. *Arch. Biochem. Biophys.* 169: 695-701.
- (67) Bjerrum, M. J. 1987. Structural and spectroscopic comparison of manganese-containing superoxide dismutases. *Biochem. Biophys. Acta* 915: 225-237.
- (68) Ditlow, C., and J. T. Johansen. 1982. Isolation of manganese-superoxide dismutase from *Saccharomyces cerevisiae*. *Carlsberg Res. Commun.* 47: 71-79.

- (69) Sato, S., and J. I. Harris. 1977. Superoxide dismutase from *Thermus aquaticus*. Isolation and characterization of manganese and apo enzymes. *Eur. J. Biochem.* 73: 373-381.
- (70) Chikata, Y., E. Kusunose, K. Ichihara, and M. Kusunose. 1975. Purification of superoxide dismutases from *Mycobacterium phlei*. *Osaka City Med. J.* 21: 127.
- (71) Stackebrandt, E., and C. R. Woese. 1981. Towards a phylogeny of the actinomycetes and related organisms. *Curr. Microbiol.* 5: 197-202.
- (72) Woese, C. L. 1987. Bacterial evolution. *Microbiol. Rev.* 51: 221-271.
- (73) Cudd, A., and I. Fridovich. 1982. Electrostatic interactions in the reaction mechanism of bovine erythrocyte superoxide dismutase. *J. Biol. Chem.* 257: 11443-11447.
- (74) Benovic, J., T. Tillman, A. Cudd, and I. Fridovich. 1983. Electrostatic facilitation of the reaction catalyzed by the manganese-containing and the iron-containing superoxide dismutases. *Arch. Biochem. Biophys.* 221: 329-332.
- (75) Personal Communication, E. M. Gregory 1988.

**The vita has been removed from  
the scanned document**