In Vivo Metal Substitution in Bacteroides

Superoxide Dismutase

by

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Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biochemistry and Nutrition

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December 8, 1989

Blacksburg, Virginia

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(ABSTRACT)

The effect of various growth conditions on the type of superoxide dismutase (SOD) formed anaerobically in three *Bacteriodes* species was studied. *B. fragilis*, *B. distasonis*, and *B. thetaiotaomicron* were grown in iron-restricted media with or without manganese supplementation. Iron availability was decreased by treatment of the media with chelex-100, a metal-chelating resin, and addition of desferrioxamine mesylate (desferal, Ciba-Geigy), an iron chelator. Mn-containing (MnSOD) and Fe-containing superoxide dismutase (FeSOD) activities in cell extracts were differentiated by inhibition with azide and inactivation by H_2O_2. The amount of Mn-containing superoxide dismutase was estimated by the fraction of azide- and H_2O_2 -resistant activity. Cells grown in untreated media contained approximatelt 90% FeSOD and 10% MnSOD. Cells grown in Fe-restricted media supplemented with graded amounts of manganese synthesized a progressively larger fraction of MnSOD. Hemin, added to the Fe-restricted media, did not serve as an iron source for FeSOD formation. Superoxide dismutase

specific activities varied (3-6 U/mg) in each extract but not as a function of manganese concentration.

Superoxide dimutase was isolated from *B. distasonis,* grown in untreated or in Fe-restricted media supplemented with 1mM $MnCl_2$. SOD from cells grown in untreated media contained 1.5 gm-atoms Fe, <0.05 gm-atoms Mn and 0.16 gm-atoms Zn per mole dimer. The SOD, specific activity 2300 U/mg, was inhibited 90% by 2mM azide and was 90% inactivated upon incubation for 10 min. in 2.5mM H_2O_2. SOD, specific activity 2400 U/mg, isolated from cells grown with 1mM Mn contained 1.05 gm-atoms Fe, 1.16 gm-atoms Mn and 0.2 gm-atoms Zn per mole dimer. The enzymatic activity was inhibited about 50% by 2mM azide and was about 50% inactivated upon incubation for 10 min. in 2.5mM H_2O_2 . Electrophoretic separation of the native SODs revealed a single band of protein and one major band of activity. The proteins focussed to approximately pH 5.3.

Metal reconstitution experiments demonstrated that apoprotein from *Bacteroides* regained superoxide dismutase activity on addition of either Fe or Mn. Denatured crude samples of *Bacteroides* SOD regained 60-80% specific activity recovery with 1mM $FeSO_4$ or 90-100% specific activity with 1mM $MnCl_2$. Denatured apoprotein from *B. distasonis* grown in untreated or Fe-restricted, mn-supplemented media were similarly reconstituted. SOD of

B.distasonis rebained 70% specific activity with 1mM $FeSO_4$ or over 90% specific activity with 1 mM $MnCl_2$.

The data suggest that the *Bacteroides* species produce a single apoprotein which can have either Mn or Fe at its catalytic site, depending on the growth conditions.

Acknowledgements

The author wishes to express her gratitute and appreciation to Dr. E. M. Gregory for his patience, support and encouragement during her graduate studies. She feels very lucky to have the opportunity to work under his direction and supervision.

Grateful acknowledgements are also made to Dr. D. R. Bevan and Dr. J. L. Hess for their valuable advice and discussions.

The author is grateful to her friends who have helped her in her laboratory and in the biochemistry department.

Finally, support for this project by the Grants Al 15250 from the National Institutes of Health and J 104 from the Jeffress Trust was gratefully appreciated.

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REVIEW

The discovery of superoxide dismutase (SOD) in 1969 by McCord and Fridovich initiated research into the role of superoxide radical (O_2^-), and SOD in biological systems [1]. A reasonable viewpoint is that oxygen is toxic not because of its own reactivity, which is rather feeble, but because its reduction by a series of single electron transfers generates exceedingly reactive intermediates. Oxygen metabolites (O_2^-, $OH\cdot$ and H_2O_2), that cause oxygen toxicity are chemically unstable and very reactive. Organisms which utilize oxygen must minimize the production of these intermediates or have efficient scavengers when production of the metabolits can't be avoided [2].

Ground state molecular oxygen contains two unpaired electrons, each located in a different antibonding orbital. The two electrons have parallel spins which forbid the direct entry of singlet paired electrons. This spin restriction makes molecular oxygen much less reactive as a oxidant. Thus when adding spin-paired

electrons to ground state molecular oxygen, it is obvious that one electronic spin would have to be inverted in order to avoid parallel spins in one orbital. There are three ways to avoid the spin restriction. 1) One electron transfer to O_2 to form O_2^- and related species. 2) Metallocomplexes to stabilize the collision complex, allowing spin inversion, 3) Molecular oxygen excited to singlet oxygen. Most biological systems use 1) and 2) as routes to reduce oxygen [3].

The oxygen metabolites superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH\cdot$) [4] are produced inside the cell in normal aerobic metabolism. They pose a potential threat to the chemical integrity of the living cell [5, 6] because these products can lead to deleterious effects, including lipid peroxidation, enzyme oxidation and further free radical formation.

Superoxide radical has both direct and indirect toxicity to biological systems. Superoxide inhibits the enzymes catalase and glutathione peroxidase [9, 10], causes the release of Fe from the storage form ferritin [11], and breaks single strands in DNA. Indirectly, dismutation of superoxide leads to production of H_2O_2. Hydrogen peroxide is the most stable of the intermediates of the reduction of oxygen, but it can penetrate cell membranes rapidly. H_2O_2 may cause peroxidation of membrane lipids [12] or react with Fe (II) or Cu (I) ions to form the hydroxyl radical with attendant cell damage. It has been suggested that superoxide radical and hydrogen peroxide can generate the highly reactive

hydroxyl radical via the iron-catalyzed Haber-Weiss reaction in the presence of certain iron chelates [7, 8]. The processes are depicted in the following reactions:

$$O_2^- + O_2^- + 2H^+ = = = \blacktriangleright H_2O_2 \quad (1)$$

$$H_2O_2 + Fe^{+2} = = = \blacktriangleright OH^- + OH\cdot + Fe^{+3} \quad (2)$$

$$Fe^{+3} + O_2^- = = = \blacktriangleright Fe^{+2} + O_2 \quad (3)$$

Hydroxyl radical is very reactive and likely generates a secondary radical within a single diffusion pathlength. Superoxide dismutes in biological systems much faster than the formation of $OH\cdot$ through the Haber-Weiss reaction, which is extremely slow [1]. It has been suggested that metal ions, such as copper (II) and iron (III) and some of their complexes, catalyze the reactions shown above. This assumption was verified through the study of the effects of metal ions and chelators on the yield of $OH\cdot$ in such system.

Cells that metabolize oxygen have evolved several classes antioxidant enzymes. Superoxide dismutases, a family of metalloenzymes, are widely distributed in obligate aerobes, aerotolerant anaerobes, and some obligate anaerobic organisms. The enzymes catalytically disproportionates O_2^- into H_2O_2 and O_2. The catalases disproportionate hydrogen peroxide into water and oxygen. The peroxidases utilize a variety of electron donors to reduce hydrogen peroxide into water. These reactions catalyzed by these enzymes are:

$$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \quad \text{(Superoxide dismutase)}$$

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \quad \text{(Catalase)}$$

$$H_2O_2 + AH_2 \longrightarrow 2H_2O + A \quad \text{(Peroxidase)}$$

Superoxide dismutase is essential in defending against toxicity of the partially reduced oxygen intermediates generated during biological reduction of dioxygen. Superoxide dismutases, the topic of this study, are metalloproteins divided into three classes, based on the type of metal bound at the active site.

Copper-Zinc Superoxide Dismutases

McCord and Fridovich [13] were the first to describe the superoxide dismuting activity of a green copper-containing protein which been isolated some 30 years earlier. This enzyme, previously called erythrocuprein, is now called CuZn SOD. According to the gross structural properties [14, 15], the copper-zinc dismutases are a conserved family. The Cu-Zn SODs are dimers (native molecular weight usually 31,000 to 33,000) containing about 2.0 g-atoms of copper and 2.0 g-atoms of zinc per mole. The two subunits are connected by noncovalent bonding and are apparently identical. The subunit molecular weights are 15,600-16,000 in bovine blood [16, 17, 18], human eythrocytes [19], yeast [20, 21] and horse liver

[22]. These data and the crystal structure of the bovine erythrocyte enzyme [23, 24, 25] also demonstrate that each subunit contains one intrachain disulfide bond and one active site, containing an atom of copper and an atom of zinc. It has been demonstrated that the copper is the catalytically active metal and the zinc plays a structural role.

Iron superoxide dismutase

Superoxide dismutases containing iron at the active site have been isolated from a variate of procaryotes and from several plant families. Anaerobically grown *E. coli* produces FeSOD [26] whereas oxygenation of the cells induces MnSOD [27]. The Fe-enzymes have a molecular weight of 40,000, are dimeric and usually contain 1 or 2 g-atoms of iron per mole of enzyme [28]. The iron in the resting state is Fe (III) and likely oscillates between the Fe (III) and Fe (II) states during the catalytic cycle. The FeSODs are acidic proteins, although there are a few exceptions.

Manganese superoxide dismutase

The first manganese-containing superoxide dismutase (MnSOD) isolated was from *E. coli* [27]. The molecular weight of the dimer is 40,000. However, tetrameric, 80-100,000 M.W. MnSODs have been isolated from a variety of

sources. These large molecular weight MnSODs are found in some species of bacteria and in extracts of animal and plant tissues [29]. In *E. coli* and *B. fragilis* , MnSOD is produced under special conditions. The synthesis of the Mn-containing protein in *E. coli* is repressed under anaerobic conditions [30], but when the cells were grown aerobically, a large increase in the level of MnSOD was observed [31]. *B. fragilis* produces only FeSOD. However, aeration of the cells at mid-logarithmic growth phase induces a MnSOD. MnSOD has a pI of 4 to 5 and the metal content of the purified enzymer ranges from 0.5-1.0 gram atom Mn/subunit.

The three forms of SODs are distinctly different and are distinguished by selective inhibition or inactivation. The FeSOD is reversibly inhibited by 1mM NaN_3 and irreversibly inactivated by H_2O_2. The MnSOD is resistant to H_2O_2 and is inhibited only 50% by 20mM NaN_3. The CuZn SOD is inhibited by CN^- and inactivated by H_2O_2. Structure and amino acid sequence data show distinct differences between the Cu/Zn SOD and the FeSOD or MnSOD. However, FeSODs are extremely similar to MnSODs from all sources and both show decreased catalytic activity at high pH values (compared to pH 7.8) and are not inhibited by CN^- [32]. This observation strongly suggests that there are at least two families of SODs that evolved along separate lines. It has also been proposed that an iron-containing SOD was the common ancestor of both the present-day Mn-and Fe-containing superoxide dismutases. This proposal is based on the premise that anaerobes contain exclusively the Fe form of superoxide dismutase

and that anaerobic organisms are believed to have preceded aerobic species in the evolution of life [33]. It is clear that these two families of SODs have evolved independently in response to a common selective pressure, that is, the evolution of oxygen and the threat of its toxicity.

Metal Substitution

SOD is induced in response to oxidative stress [34] in both prokaryotes and eukaryotes: *Streptococcus faecalis* [31], *E. coli* [35, 36, 37], *S. cerevisiae* [38], rat lung [39], guinea pig leukocytes [40], *Bacteroides fragilis* [41], *Bacteroides thetaiotaomicron* [42], blue-green algae [43], and green algae. The SOD level generally is responsive to the degree of oxygenation. Aerobically grown *E. coli* contains three isozymes of superoxide dismutase, a FeSOD, a MnSOD and a hybrid consisting of one subunit each from the FeSOD and MnSOD [44]. Anaerobically grown *E. coli* , in contrast, contains only FeSOD. It is proposed that MnSOD and hybrid-forms of SOD are rapidly synthesized upon exposure to air and that FeSOD is a important defense mechanism against sudden exposure to oxygen [45]. Superoxide dismutases in the anaerobes *B. fragilis* [41] and *B . thetaiotaomicron* [42] are O_2 -inducible, and each organism apparently synthesizes a single apoprotein capable of binding either Fe or Mn in a catalytically competent manner. Both *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* contain FeSOD when grown anaerobically. But when exposed 20% oxygen, superoxide dismutase levels increased about 10-20 fold compared

with anaerobic controls. The induced SODs are stable to H_2O_2 (5mM) for 2 hours and only 50% inhibited by 20mM NaN_3 , indicating manganese superoxide dismutase production.

Iron chelators also effect SOD biosynthesis. Moody and Hassan [46] demonstrated that iron chelators greatly increase the biosynthesis of MnSOD in *E. coli.* under anaerobic conditions where MnSOD is normally not expressed [47]. A model for the regulation of MnSOD biosynthesis in *E. coli* proposes that MnSOD biosynthesis is negatively regulated at the transcriptional level by an iron-containing protein. The repressive effect of the regulatory protein is dependent upon the presence of iron in the proper valence (Fe^{2+}). Ferrous iron chelators and redox active compounds inactivate the repressor protein and thus result in the induction of MnSOD [47]. The iron chelator, 2,2′-dipyridyl (2,2′-DP) induced SOD in *Proteus vulgaris* , *Enterbacter cloaeae* and *Staphylococococcus aureus* in a manner similar to that found in *E. coli*

Metal replacement studies have been performed with dimeric manganese-containing dismutases from *B. stearothermophilus* [48, 49, 50] and *E. coli* [51, 52], dimeric iron-containing dismutases from *Pseudomonas* ovalis [53, 54] and *Photobacterium leiognathi* [55], the tetrameric manganese-containing enzyme from *Thermus aquaticus* [56], and the dimeric manganese-containing enzyme from the eukaryote alga, *Porphyridium cruentum.* In all cases, inactive apoprotein has been reconstituted with the native metal ion, producing an active

enzyme of approximately the same metal stoichiometry as native holoenzyme. The specific activity regained was about 100% that of native enzyme, except *P. leiognathi* Fe dismutase in which 50% of the specific activity was restored [55]. *B. stearothermophilus, E. coli, P. leiognathi, P. ovalis, and B. fragilis* apoproteins were also reconstituted with a non-native divalent metal ion. In these four cases can replace the native manganese can be replaced by iron, or the native iron by manganese [41, 42, 49, 50, 52, 53, 55]. However, only *P. ovalis* and *B. fragilis* SODs exhibit activity with the alternate metal.

There are some common properties for the metal replacement derivatives of Mn/Fe dismutases. Native enzyme contains 1 metal ion per dimer, or about 0.5 metal per subunit. The *E. coli* studies suggest that the replacement metal occupies the native metal-binding site, and only the divalent forms of the metal binds to the apoproteins.

Bacteroides are anaerobic, gram-negative organisms. *B. fragilis, B. distasonis,* and *B. thetaiotaomicron* are part of the normal flora of the intestine, oral cavity, nasopharynx, oropharynx, vagina, and urethra and in these locations, they are relatively harmless. However, they may establish severe infections and tissue destruction under certain conditions and are the major pathogen in anaerobic soft tissue infections. The organisms have great significance for clinical bacteriology. Thus, studies of the antioxidant differents of those aerobes may lead to an understanding of their survival during brief O_2 exposure.

MATERIALS

Fisher Scientific was the source of following products: hydrogen peroxide, atomic absorption standards (Mn, Fe, Zn), ammonium sulfate, ammonium persulfate, dialysis tubing and glycerol. Biorad was the source of Chelex 100 and protein A-horseradish peroxidase conjugate. Sigma Chemical Company was the source of following products: cytochrome c (type III), xanthine, riboflavin, EDTA, hemin, vitamin K_1 , cysteine, phenol red, bromphenol blue, protamine sulfate, phenyl-sepharose, Wide Range Isoelectric Focusing Marker Kit, sodium azide, sodium cyanide, bovine serum albumin, 4-chloro-1-naphthol, tris-hydroxymethyl-aminomethane (Tris), riboflavin, and 8-hydroxyquinoline. Acrylamide and bis-acrylamide were purchased from Research Organic Inc.. Heico was the source of ultrapure guanidine hydrochloride. Resazurin, trypticase soy broth, yeast extract, tryptic peptone and gelatin were from Difco. LKB was the souce of ampholines (pH 3-10). ICN was the source of Freund's Complete

and Incomplete Adjuvants. Xanthine oxidase was purified from unpasteurized cream by the method of Waud *et al.* [57].

Bacteroides fragilis (VPI 2393), *Bacteroides distasonis* (ATCC 8503), and *Bacteroides thetaiotaomicron* (VPI 3388) were obtained from the Virginia Polytechnic Institute and State University (VPI) Anaerobe Laboratory.

METHODS

Bacterial Culture

Bacteroides fragilis (VPI 2393), *Bacteroides distasonis* (ATCC 8503) and *Bacteroides thetaiotaomicron* (VPI 3388) were cultured in anaerobically prereduced media which contained 5.5g Brain Heart Infusion Broth (dehydrated) and 0.75g Yeast extract per 150ml supplemented with Vitamin K_1[58]. The media were prepared separately in 250 flasks and were supplemented with different amounts of manganese chloride, desferrioxamine mesylate (desferal), magnesium chloride or hemin after sterilization.

In some experiments, the media were chromatographed through Chelex 100 resin. The media were then supplemented with Vitamin K_1 and hemin prereduced, and sterilized. Stock cultures were maintained in chopped meat medium

and were transferred under oxygen free CO_2. About 1.5ml inoculum was transferred to 150ml media and was incubated at 37 C. Cells were harvested by centrifugation (11,000 X g for 15 minutes) and washed once in 50mM potassium phosphate, 1mM EDTA, pH 7.8 (SOD buffer), and were disrupted by sonication. Approximately 70 watts was applied through the microtip of a Heat system W 385 sonifier for a total of 6 minutes. The sonicate was centrifugated 15 minutes in 17,000 X g, and the pellet was discarded.

Measure of Enzyme Activity

Superoxide dismutase activity was measured using the cytochrome c reduction assay described by McCord and Fridovich except that 0.5mM NaCN was present in each assay [13]. The amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% (i.e. 0.0125 absorbance unit/per min.) was defined as one unit of activity.

Xanthine oxidase was added to the assay solution to obtain a standard slope of 0.025 A/minute. When sodium azide was used in the assay solution as inhibitor for FeSOD, the amount of added xanthine oxidase was adjusted to obtain the same standard slope as in the absence of sodium azide.

Superoxide dismutase activity was also determined histochemically on native polyacrylamide gels as described by Beauchamp and Fridovich [59].

SOD inhibition and inactivation reaction

FeSOD is inhibited by 1mM NaN_3 and inactivated by H_2O_2, whereas MnSOD is not. MnSOD and FeSOD can be distingished in the sample. Different amount of NaN_3 were added to the SOD assay and the volume of xanthine oxidase was adjusted to give a standard slope (absorbance 0.025 + or - 0.001/min at 550nm). The sample was also incubated with 2.5mM H_2O_2 and then activity was measured as a function of incubation time. The following equations were used to determine the relative content of FeSOD and MnSOD in extracts:

$X + Y$ = Total SOD Activity

$0.9X + 0.1Y$ = Activity remaining in 2mM NaN_3

Where Y is the fraction of FeSOD and X is the fraction of MnSOD. The algorithm is based on the inhibition of MnSOD and FeSOD isolated from *B. fragilis* and *B. thetaiotaomicron* .

Metal Content

The enzyme samples were dialyzed against 50mM potassium phosphate, 1mM EDTA, (pH 7.8) overnight. Metal content of a buffer blank and the samples were determined by flame aspiration on a Perkin - Elmer Model 560 Atomic Absorption Spectrophotometer. The instrument response was calibrated with metal solutions whose concentration were known.

Reconstitution Experiments

The cell extracts or the purified enzymes were dialyzed in a solution of 5M guanidinium chloride and 20mM 8-hydroxyquinoline (pH 3.2) for 12 hour with gentle stirring at 4 C. The extracts or purified enzyme in the tubes were dialyzed for an additional 12 hours in 5M guanidinium chloride. When the two dialysis steps were finished, there was no measurable SOD activity. The denatured SOD was reconstituted by dialysis in 20mM Tris buffer (pH 7.0) containing either 1mM $FeSO_4$ or 1mM $MnCl_2$ for 12 hours at 4C. The excess metal was removed by dialysis of the samples in 2 change of SOD buffer for 12 hours.

Protein Determination

The protein concentrations of crude extracts were based on absorbance at 280nm, corrected for nucleic acid contamination by the absorbance at 260nm [13].

The protein content of the isolated enzymes was determined by the Lowry method [60] using bovine serum albumin as the standard.

Antibody Production

A rabbit was injected with filter sterilized purified enzyme (100ug) in 1ml 0.9% NaCl and 1ml of Freund's Complete Adjuvant. After three weeks, the rabbit was injected with sterile solution of the pure enzyme (100ug) in 1ml of 0.9% NaCl and 1ml of Freund's incomplete Adjuvant. This step was repeated every three weeks. The serum (20ml to 30ml) was collected three weeks after each booster injection.

Serum immunoglobulin G was precipitated by addition of solid ammonium sulfate (243g/l) followed by centrifugation 35,000 X g for 15 minutes. The precipitate was resuspended in 50mM potassium phosphate (pH 7.4). This proce-

dure was repeated until the ammonium sulfate precipitate was white. The white precipitate was resuspended in 20mM potassium phosphate (pH 8.0) and was dialyzed in that buffer overnight. The sample was loaded on DE-52 (13 x 1.2cm) and washed with 20mM potassium phosphate buffer (pH 8.0). The fractions with A_{280} greater than 0.9 were pooled and concentrated under N_2 on a YM 10 ultrafilter. The concentration of protein was determined by the Lowry method [60].

Ouchterlony Immunodiffusion

Nine ml of 1% agarose in PBS buffer (10mM sodium phosphate, 0.15M NaCl, 0.1% NaN_3) (pH 7.4) were poured into a 9.5 X 4.5 cm plate. When the agarose was hardened, it was cut into wells (4mm in diameter). The antigen and antibody were separately put in wells and incubated for 2 days in 4 C. The plate was washed with 3 changes of PBS buffer then with distilled H_2O , on a glass plate at 37 C overnight, and stained with 0.5% coomassie blue [61].

RESULTS

I. In situ substitution of Mn into Bacteroides SOD

Bacteroides fragilis (2393)

Effect of Mn concentration on B. fragilis SOD activity

B. fragilis was grown anaerobically in prereduced BHI media which was passed twice on chelex 100 and supplemented with different concentrations of Mn or Fe (Table 1). The SOD activity of crude extracts were determined and the relative amount of FeSOD and Mn SOD content was measured by azide inhibition [13].

The specific activity of SOD in the extracts varied from 4.6 - 5.9 U/mg (Table 1). The SOD activity of cells grown with or without 2mM Mn was similar. However, the fraction of MnSOD increased with increasing $MnCl_2$ concentration in the media, whereas FeSOD content decreased. MnSOD activity represented 71% and FeSOD 29% of the total activity of cells grown in media containing 2 mM $MnCl_2$. (Table 1).

Effect of Desferal concentration on B. fragilis SOD activity

B. fragilis was grown anaerobically in chelex-treated BHI media supplemented with different concentrations of desferal. The specific activity of SOD in the cell extracts were 6.7 - 7.6 U/mg, independent of the desferal concentration. The total activity of MnSOD increased with increasing desferal concentration in the media while the FeSOD activity decreased. Extract of cells grown in medium with 5 uM desferal contained 51% MnSOD and 49% FeSOD (Table 2).

Effect of Desferal plus 1mM Mn on B. fragilis SOD content

B. fragilis was grown anaerobically in chelex-treated BHI supplemented with 1mM $MnCl_2$ and different concentrations of desferal.

The specific activities of the samples varied for 2 - 4.9 U/mg. Increasing desferal concentration in the presence of 1mM $MnCl_2$ did not change the specific

Table 1. Effect of Mn concentration on B. fragilis SOD

[Mn] (mM)	Density A_{660}	SOD Activity (Units/mg)	% Inh. (2mM N_3)	% Ina. (H_2O_2)	% FeSOD	% MnSOD
0.0	1.67	5.4	71	90	78	22
*0.0(Fe)	1.90	5.0	83	89	91	9
1	1.50	4.6	52	58	45	55
2	1.00	5.9	30	34	29	71

B. fragilis was grown anaerobically in BHI media which were passed twice through Chelex 100.

* B. fragilis was grown anaerobically in BHI media plus 9.6 X 10^{-3}mM $FeSO_4$.

Table 2. Effect of Desferal concentration on B. fragilis SOD

[Desferal] (uM)	Density A660	SOD Activity (Units/mg)	% Inh. (2mM N_3)	% Ina. (H_2O_2)	% FeSOD	% MnSOD
0.0	1.52	6.7	86	89	89	11
1	1.34	7.0	76	77	84	16
2	1.48	7.1	55	67	60	40
5	1.00	7.6	46	58	49	51

B. fragilis was grown anaerobically in BHI media which were passed twice through Chelex 100.

activity of SOD in cell extracts. However, addition of desferal to the media affected the growth of cells. High concentrations of desferal (5uM and 10uM) slowed the growth of cells and decreased cell density. The MnSOD content increased with increasing desferal concentration, whereas FeSOD decreased. At 5 or 10uM desferal in the media with 1mM $MnCl_2$, only MnSOD could be detected in the cell extract (Table 3).

Effect of Mn plus 1uM Desferal on B. fragilis SOD activity

B. fragilis was grown anaerobically in chelex-treated BHI media with 1uM desferal and different concentrations of Mn. The specific activity of the samples varied from 2.6-5.7 U/mg. The total SOD activity of the extracts did not vary directly with increasing $MnCl_2$ concentration indicating that $MnCl_2$ did not induce SOD in the bacteria. However, the proportion of MnSOD increased with increasing $MnCl_2$ concentration in the media. At 2mM Mn and 1uM Desferal, MnSOD represented 100% of the total SOD (Table 4).

Bacteroides distasonis (8503)

Effect of Mn plus 1uM Desferal in B. distasonis SOD activity

Table 3. Effect of Desferal concentration with 1mM Mn on *B. fragilis* SOD

[Desferal] (uM)	Density A660	SOD Activity (Units/mg)	% Inh. (2mM N_3)	% Ina. (H_2O_2)	% FeSOD	% MnSOD
0.0	1.67	2.0	65	64	70	30
1	1.38	3.0	48	47	47	53
2	1.19	3.8	21	25	19	81
5	0.85	4.9	0	0	0	100
10	0.67	3.5	0	0	0	100

B. fragilis was grown anaerobically in BHI media which were passed twice through Chelex 100 and were supplemented with 1mM $MnCl_2$ prior to sterilization.

Table 4. Effect of Mn concentration with 1uM Desferal on B. fragilis SOD

[Mn] (mM)	SOD Activity (Units/mg)	% Inh. (2mM N_3)	% Ina. (H_2O_2)	% FeSOD	% MnSOD
0.0	5.7	76	77	84	16
*0.0(Fe)	5.0	83	89	91	9
0.1	3.4	75	76	82	18
0.25	4.4	56	57	60	40
0.5	3.7	47	50	50	50
1.0	3.7	42	47	43	57
1.5	2.6	16	29	13	87
2.0	4.9	0	0	0	100

B. fragilis was grown anaerobically in BHI media which were passed twice through Chelex 100 and were supplemented with 1uM prior to sterilization.

* B. fragilis was grown anaerobically in BHI media plus 9.6×10^{-3}mM $FeSO_4$.

B. distasonis was grown anaerobically in chelex-treated BHI media supplemented with 1uM desferal and different concentrations of Mn. The specific activity of the samples varied from 3.0 to 5.2 U/mg. Increasing the concentration of $MnCl_2$ in the media did not change the SOD specific activity in the cell extracts. 2mM $MnCl_2$ and 1uM desferal significantly affected the growth of *B. distasonis* . The absorbance of the cells grown in media containing 2mM Mn and 1uM Desferal after 28 hours incubation was 0.82 compared to the absorbance of 1.36 of the cells grown in media containing no added Mn and 1uM Desferal after 16 hours incubation. At 2mM Mn and 1uM desferal, MnSOD constituted 100% of the total SOD activity in the cell extracts (Table 5).

Bacteroides thetaiotaomicron (3388)

Effect of Mn plus 1uM Desferal in B. thetaiotaomicron SOD activity

B. thetaiotaomicron was grown anaerobically in chelex-treated media as described for *B. distasonis* . The SOD specific activity of the cell extracts varied from 4.9-9.2 u/mg. Again there was no increase in SOD activity with increasing $MnCl_2$ concentration in the media. The pattern of azide-inhibitable and H_2O_2-inactivated SOD decreased with increasing $MnCl_2$ concentration in the BHI media. The MnSOD content increased with $MnCl_2$ concentration in the media. At 2mM Mn and 1uM Desferal, the MnSOD represented 100% of the SOD activity (Table 6).

Table 5. Effect of Mn concentration with 1uM Desferal on B. distasonis SOD

[Mn] (mM)	Density A660	SOD Activity (Units/mg)	% Inh. (2mM N_3)	% Ina. (H_2O_2)	% FeSOD	%MnSOD
0.0	1.36	4.02	77	80	85	15
*0.0(Fe)	1.43	5.05	83	90	91	9
0.1	1.23	3.76	68	64	74	26
0.25	1.06	3.0	66	67	71	29
0.5	1.34	5.20	49	63	52	48
1.0	1.03	4.23	45	54	47	53
2.0	0.82	4.36	0	0	0	100

B. distasonis was grown anaerobically in BHI media which were passed twice through Chelex 100 and were supplemented with 1uM Desferal prior to sterilization.

* B. distasonis as grown anaerobically in BHI media plus 9.6×10^{-3}mM $FeSO_4$.

Table 6. Effect of Mn concentration with 1uM Desferal on *B. thetaiotaomicron*

[Mn] (mM)	Density A660	SOD Activity (Units/mg)	% Inh. (2mM N_3)	% Ina. (H_2O_2)	% FeSOD	% MnSOD
0.0	1.60	4.87	77	75	84	16
*0.0(Fe)	1.83	7.89	92	90	96	4
0.1	1.36	8.76	77	69	84	16
0.5	1.68	9.17	44	55	46	54
1.0	1.29	7.71	40	46	42	59
1.5	0.86	7.7	23	25	18	82
2.0	0.56	5.77	0	0	0	100

B. thetaiotaomicron was grown anaerobically in BHI media which were passed twice through Chelex 100 and were supplemented with 1uM Desferal prior to sterilization.

* *B. thetaiotaomicron* was grown anaerobically in BHI media plus 9.6 X 10^{-3}mM $FeSO_4$.

II. Electrophoretic characterization of Bacteroides SODs

B. fragilis SOD

Extracts of cells grown with or without 1mM $MnCl_2$ were electrophoresed on 7.5% polyacrylamide gels and were stained for SOD activity (Fig. 1).

The SOD activity in the extract of cells grown with or without Mn migrated with the same relative mobility individually (Lane 1,2,4,5), or as mixtures of two samples (Lane 3,6).

Similarly, SOD from extracts grown with or without Mn or a mixture of the two samples focussed to same isoelectric point.

B. fragilis, B. distasonis and B. thetaiotaomicron SOD

Extract of cells grown with or without 1mM $MnCl_2$ media were separated on a 7.5% polyacrylamide gel. The SOD activity in extracts of each organism migrated with different relative mobilities (compare lane 1, 4 and 7 in Fig.2). However, within the same species, the SOD activity in extract of cells grown with or without 1mM Mn migrated identically (Lane 1, 5; 4, 5; and 7, 8) or in combination (lane 3, 6, 9) (Fig.2).

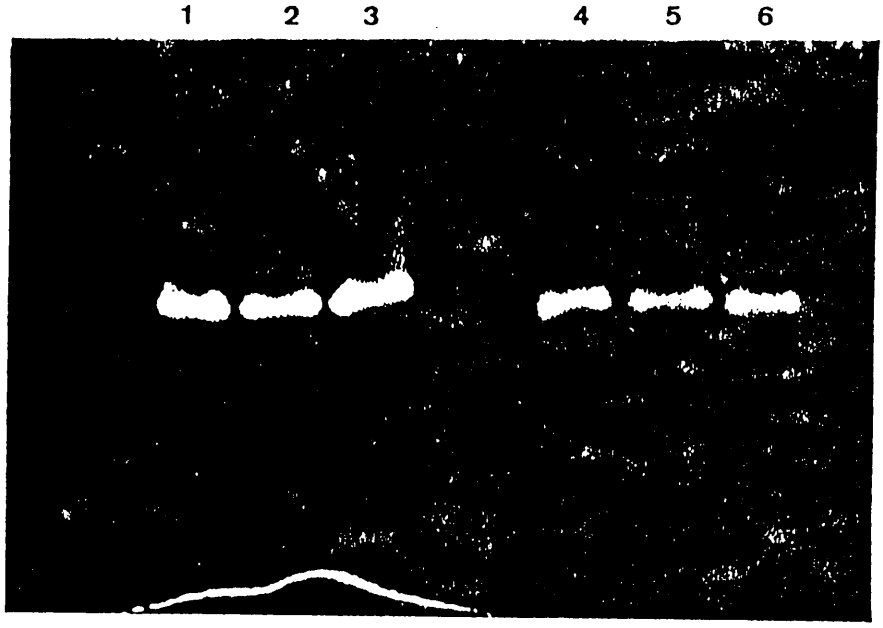

Fig. 1. Electropherogram of SOD in B. fragilis cell extracts: activity stain.

Lane 1 & 4 - Extracts of cells grown in chelex-treated media with 1uM desferal.
Lane 2 & 5 - Extracts of cells grown in chelex-treated media with 1uM desferal and 1mM Mn.
Lane 3 & 6 - Equal volume mixtures of the extracts described.

(Lane , 0.9 U; Lane 2, 0.4 U; Lane 3, 0.7 U; Lane 4, 0.5 U; Lane 5, 0.5 U; Lane 6, 0.4 U)

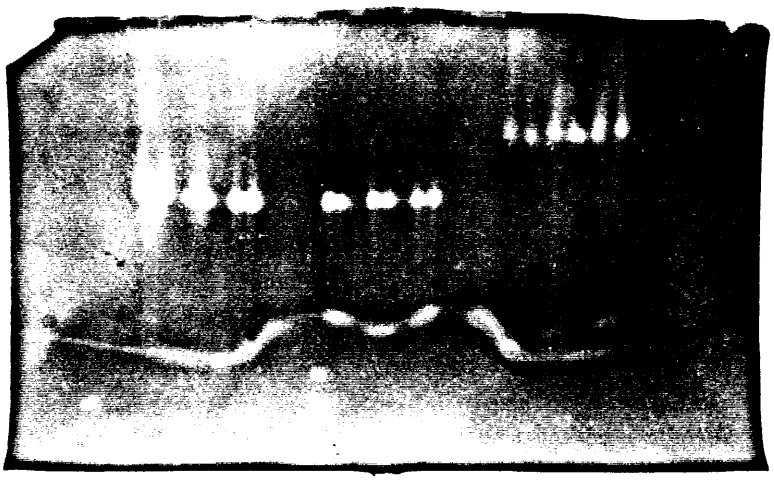

Fig. 2. Electropherogram of SOD in Bacteroides cell extracts: activity stain.

B. fragilis:
Lane 1, OmM Mn, 0.38 U; Lane 2, 1mM Mn, 0.4 U; Lane 3, Equal volume mixture of 0 and 1mM Mn, 0.25 U.

B. distasonis:
Lane 4, OmM Mn, 0.22 U; Lane 5, 1mM Mn, 0.44 U; Lane 6, Equal volume mixtures of 0 and 1mM Mn, 0.25 U.

B. thetaiotaomicron:
Lane 7, OmM Mn, 0.6 U; Lane 8, 1mM Mn, 0.5 U; 0.5 U; Lane 9, Equal volume mixture 0 and 1mM Mn, 0.55 U.

Similarly, SODs from the three strains of *Bacteroides* which grown with or without Mn or a mixture of the two samples focussed to same isoelectric point in acrylamide gel.

III. Characterization of B. distasonis SOD

Purification of B. distasonis SOD

(1) Five grams of lyophilized *B. distasonis* cells grown anaerobically in BHI media were hydrated in 150ml of 25mM potassium phosphate, 0.5mM EDTA (pH 7.8). This and all subsequent steps were at 4 C. Cells (75ml) were sonicated at 70 watts into the microtip for a total of 6 min. and cell debris was removed by centrifugation (17,000 X g, for 30 min.). Protamine sulfate was added to the supernatant (0.1% final concentration) and the mixture was stirred for 30 minutes. The precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 60% saturation and the solution was stirred one hour. After centrifugation (17,000 X g) for 15 min. to remove the precipitate, the solution was concentrated under N_2 over a YM 10 Ultrafilter to about 20ml and the supernatant was pumped on phenyl sepharose (18cm X 1.5cm) equilibrated in 50% ammonium sulfate in SOD buffer. Fractions were eluted with a gradient of 50% ammonium sulfate 50% to SOD buffer (650ml/each side). The fractions with SOD activity were pooled, concentrated to about 20ml, and dialyzed in 10mM Tris, 1mM EDTA (pH 8) (Tris buffer). The supernatant was pumped on DE-53 (15cm X 1.5cm) equilibrated with the Tris buffer. Fractions were eluted with a gradient of KCl (0 to 100mM) in the Tris buffer (280ml/each side).

Fractions with SOD activity were pooled, concentrated to 30ml, and stored in the freezer.

The superoxide dismutase from *B. distasonis* grown anaerobically in BHI media had a specific activity of 2300 U/mg, and was purified 460-fold in 73% yield (Table 7).

(2). Lyophilized *B. distasonis* cells (6.28 grams) grown in 1mM Mn, 1uM Desferal, 0.08mM Mg and Chelex- treated BHI media were hydrated in 188ml of 25mM potassium phosphate, 0.5mM EDTA (pH 7.8). The cells were sonicated in two butches for 6 min. at 70 watts to break the cells. The cell debris was removed by centrifugation (17,000 X g for 30 min.). Protamine sulfate was added to the supernatant to 0.1% final concentration and stirred 0.5 hour room (4 C). After centrifugation, ammonium sulfate was added to the supernatant to 60% saturation and the solution stirred one hour. The precipitate was removed by centrifugation (17,000 X g for 15 min.). The supernatant concentrated under N_2 over a YM 10 Ultrafilter to about 20ml, and was pumped on phenyl sepharose (18cm X 1.5cm) column equilibrated in 50% ammonium sulfate in SOD buffer. Fractions were eluted with a gradient of 50% ammonium sulfate to SOD buffer (650ml/each side). The fractions with SOD activity were pooled and concentrated to about 20ml. The supernatant was pumped on DE-53 (15cm X 1.5cm) equilibrated with 10 mM Tris, 1mM EDTA (pH 8). Fractions were eluted with a gradient (0 to 100mM KCl, 280ml/each side) in 10mM Tris, 1mM EDTA, pH

Table 7. Isolation of FeSOD from B. distasonis

Step	Volume ml	Total units	Total protein mg	Sepecific activity u/mg	% Yield	Fold puri.
Crude	278	8340	1600	5	100	---
Protamine sulfate	292	10700	970	11	100	2
60% Ammonium sulfate	270	11500	600	19	100	4
Phenyl-sepharose	315	10100	44	230	100	44
DE-53	40	6200	2.7	2300	74	460

Based on 280nm absorbance
$E = 1.2$ ml $mg^{-1}cm^{-1}$.
(B. distasonis was grown in BHI media)

8. The fractions with SOD activity were pooled, concentrated to 30ml, and stored frozen.

The superoxide dismutase from *B. distasonis* anaerobically grown in chelex treated BHI media supplemented with 1mM Mn, and 1uM Desferal had a specific activity of 2400 U/mg, and was purified 350 fold in 54% yield (Table 8).

Characterization of B. distasonis SODs

Purified *B. distasonis* SOD isolated from cells grown anaerobically in untreated BHI media was electrophoresed on 7.5% polyacrylamide gels. The gels were stained for protein (Fig. 3) and SOD activity (Fig. 4).

Purified *B. distasonis* of SOD which isolated from cells grown anaerobically in treated BHI media also was separated on 7.5% polyacrylamide gels. The gels were stained for protein (Fig. 5) and SOD activity (Fig. 6). The purified SOD in each sample migrated as a single protein and activity band on gels.

Purified *B. distasonis* SOD isolated from cells grown anaerobically in untreated or treated BHI media was focused to its isoelectric point in

Table 8. Isolation of SOD from B. distasonis

Step	Volume ml	Total units	Total protein mg	Specific activity u/mg	% Yield	Fold puri.
Crude	178	10300	1500	6.8	100	---
Protamine sulfate	182	11300	870	13	100	2
60% Ammonium sulfate	206	12500	530	24	100	4
Phenyl-sepharose	64	10400	10	1040	100	153
DE-53	30	5600	2.3	2400	54	353

Based on 280nm absorbance
E = 1.2 ml $mg^{-1}cm^{-1}$.
(E. distasonis was grown in chelex-treated BHI media which contained 1mM Mn and 1uM Desferal)

Fig. 3. Electropherogram of purified B. distasonis SOD grown anaerobically in untreated BHI media.

Lane 1, 0.8ug; Lane 2, 1.0ug; Lane 3, 1.2ug; Lane 4, 1.5ug; Lane 5, 1.8ug; Lane 6, 2.4ug; Lane 7, 3.2ug; Lane 8, 4.0ug. The values are amounts of protein loaded onto the gel.

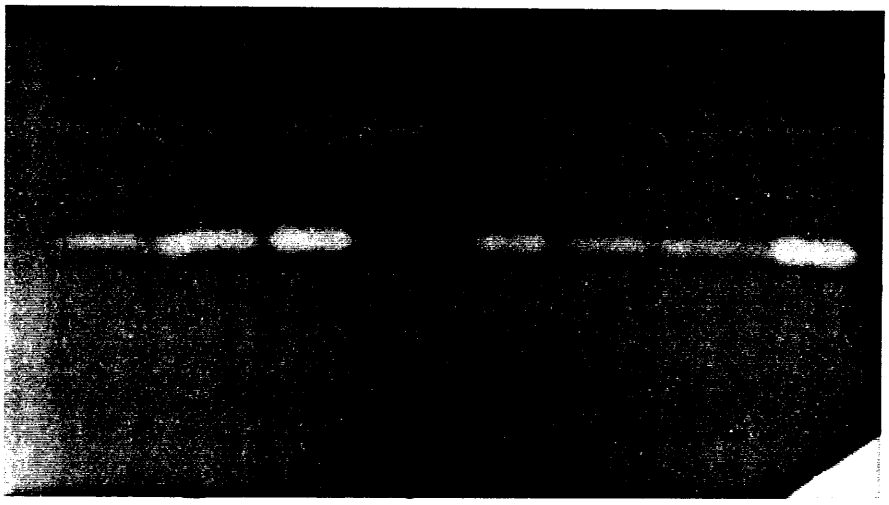

Fig. 4. Electropherogram of purified B. distasonis FeSOD: activity stain.

Lane 1, 0.1 U; Lane 2, 0.15 U; Lane 3, 0.2 U; Lane 4, 0.25 U; Lane 5,0.3 U; Lane 6, 0.35 U; Lane 7, 0.4 U; Lane 8, 0.45 U;

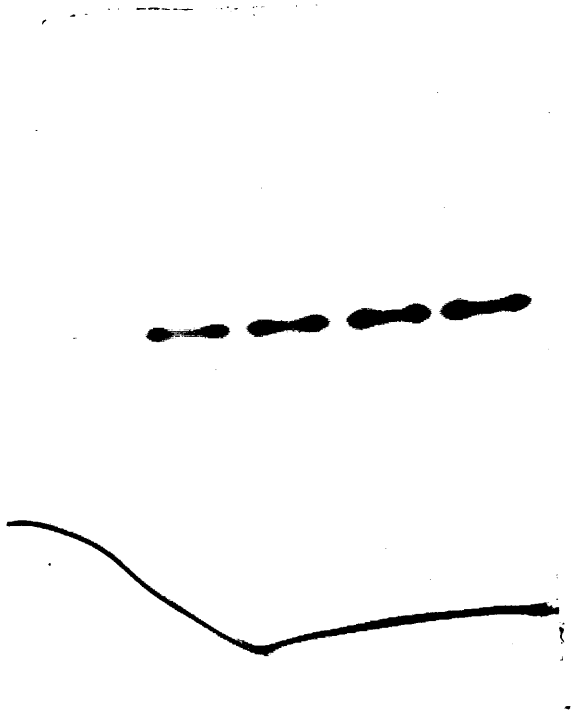

Fig. 5. Electropherogram of purified B. distasonis Mn/Fe SOD.

Lane 1, 0.9 ug; Lane 2, 1.8 ug; Lane 3, 2.7 ug; Lane 4, 3.6 ug;

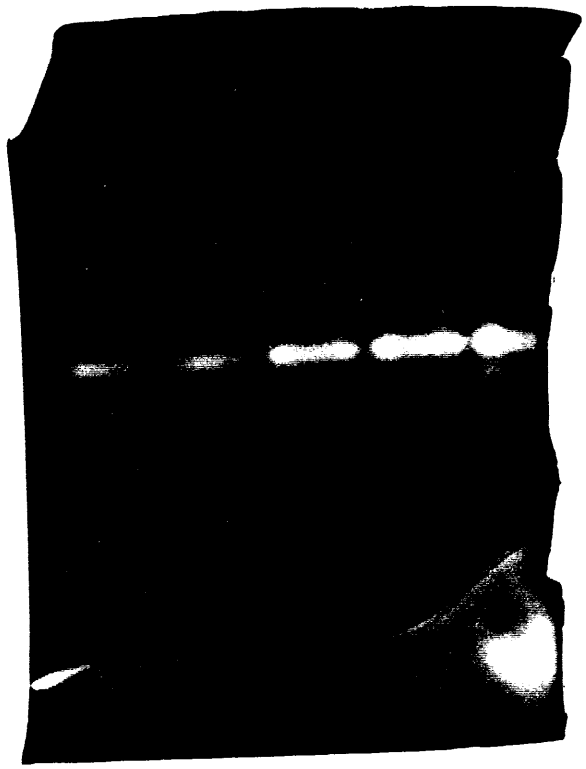

Fig. 6. Electropherogram of purified B. distasonis Mn/Fe SOD: activity stain.

Lane 1, 0.25 U; Lane 2, 0.5 U; Lane 3, 0.75 U; Lane 4, 1.0 U; Lane 5, 1.25 U;

polyacrylamide gels. Both purified SODs of *B. distasonis* have the similar isoelectric point in the gel (Fig. 7).

Metal content

Atomic absorption spectrophotometry of the purified native *B. distasonis* SOD (control) (Table 9) and *B. distasonis* SOD from Mn-supplemented cells revealed that the native SOD contained about 2 gram-atoms/mol Fe, <0.05 gram-atoms/mol Mn and 0.16 gram-atoms/mol Zn. SOD from Mn-supplemented cells contained 1.1 gram-atoms/mol Fe, 1.2 gram-atoms/mol Mn and 0.2 gram-atoms/mol Zn (Table 9).

Effect of sodium azide and hydrogen peroxide on B. distasonis SOD

Purified *B. distasonis* SOD which was anaerobically grown in BHI media was sensitive to NaN_3 and H_2O_2. As previously reported for FeSOD, NaN_3 (2mM) inhibited SOD activity by 90% and incubation with 2.5mM H_2O_2 for 10 min. inactivated SOD activity by 92%.

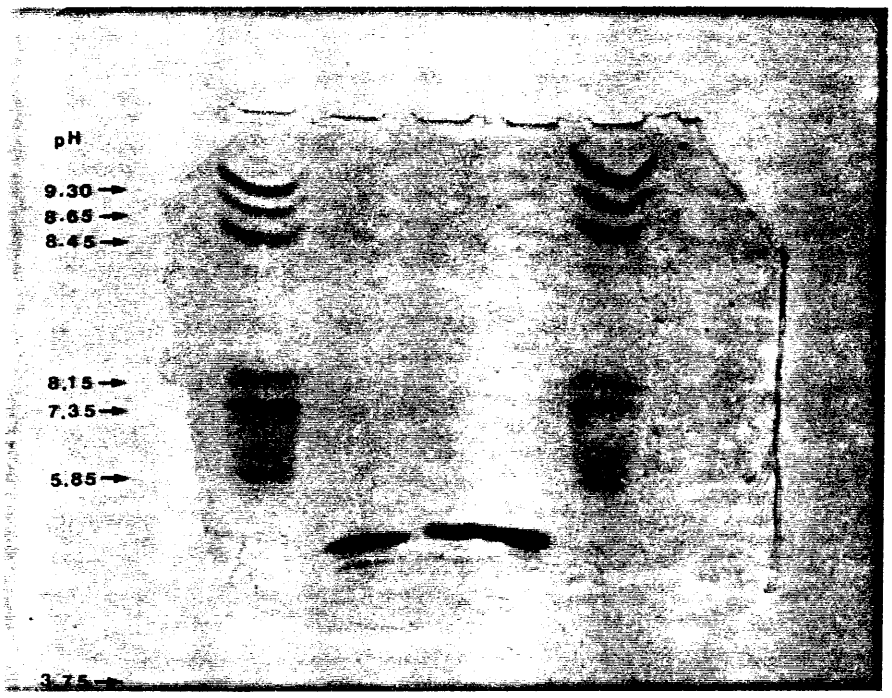

Fig. 7. Isoelectric focusing of purified B. distasonis SOD

Lane 1, 5 - pH marker proteins (3.75 - 9.3)

Lane 2 - B. distasonis grown anaerobically in BHI media (5 ug).

Lane 3 - B. distasonis grown anaerobically in chelex-treated plus 1mM Mn, 1uM Desferal BHI media (3 ug).

Lane 4 - Mixture of equal amount of Lane 2 and Lane 3 (6 ug).

Table 9. Metal Analysis of B. distasonis SOD

Sample	Metal Content (gram-atoms/mol enzyme)		
	Fe	Mn	Zn
B. distasonis (8503) (Control)	1.5	0.04	0.16
B. distasonis (8503) (Sample)	1.05	1.16	0.20

[Control] ----- B. distasonis was grown anaerobically in BHI media.

[Samples] ----- B. distasonis was grown anaerobically in Chelex -treated, Mn-supplemented BHI media.

IV. Metal reconstitution of Bacteroides SODs

Metal reconstitution of extracts of B. distasonis

The six crude samples of *Bacteroides* (*B. fragilis, B. distasonis* , and *B. thetaiotaomicron* grown anaerobically in BHI and in BHI media treated with Chelex 100 and supplemented with $MnCl_2$, 1uM Desferal, 0.08mM $MgCl_2$) were assayed for SOD activity. The extracts obtained from cultures grown anaerobically in BHI contained about 90% FeSOD and 10% MnSOD according to the equation:

X + Y = Total SOD Activity

0.9X + 0.1Y = Activity remaining in 2mM NaN_3. The extracts from cultures grown anaerobically in chelex-treated BHI contained about 50% FeSOD and 50% MnSOD. The apoproteins of these extracts were prepared by denaturation, and the apoproteins were reconstituted with either $FeSO_4$ or $MnCl_2$. About 50-70% of the SOD activity and 60-80% specific activity were recovered upon reconstituting with Fe. The SOD activity upon reconstitution with $MnCl_2$ yielded 80-90% recovery and SOD specific activity was 90-100% recovery. The crude extracts reconstituted with Fe was inhibited 90% on addition of 2mM NaN_3 to the assay. No inhibition of activity by 2mM NaN_3 was observed with Mn-reconstituted SOD (Table 10, Table 11).

Table 10. Azide inhibition of native and Fe-reconstituted SODs from Bacteroides

	% Inhi. of SOD Act. by 2mM NaN_3 conc.	Sp. activity U/mg	Recovered U/Mg %
B. fragilis [control]			
Native Enzyme	86	7.8	
Fe Recon.	81	5.4	69
B. fragilis [1mM Mn]			
Native Enzyme	45	10.2	
Fe Recon.	92	8.9	87
B. distasonis [Control]			
Native Enzyme	84	4.9	
Fe Recon.	85	3.9	80
B. distasonis [1mM Mn]			
Native Enzyme	52	6.5	
Fe Recon.	84	5.3	81
B. thetaiotaomicron [Control]			
Native Enzyme	88	9	
Fe Recon.	89	6.2	69
B. thetaiotaomicron [1mM Mn]			
Native Enzyme	49	10	
Fe Recon.	91	8.2	82

Table 11. Azide inhibition of native and Mn-reconstituted SODs from Bacteroides.

	% Inhi. of SOD Act. by 2mM NaN_3 conc.	Sp. activity U/mg	Recovered U/Mg %
B. fragilis [control]			
Native Enzyme	86	7.8	
Mn Recon.	0	10.4	100
B. fragilis [1mM Mn]			
Native Enzyme	45	10.2	
Mn Recon.	0	13.8	100
B. distasonis [Control]			
Native Enzyme	84	4.9	
Mn Recon.	0	6.1	100
B. distasonis [1mM Mn]			
Native Enzyme	52	6.5	
Mn Recon.	0	8.5	100
B. thetaiotaomicron [Control]			
Native Enzyme	88	9	
Mn Recon.	0	10	86.4
B. thetaiotaomicron [1mM Mn]			
Native Enzyme	49	10	
Mn Recon.	0	9.8	98

Metal reconstitution of purified B. distasonis SOD

The purified *B. distasonis* SOD which was anaerobically grown in BHI media was virtually all FeSOD and the purified *B. distasonis* SOD which was anaerobically grown in Chelex 100 treated BHI media containing 1mM $MnCl_2$ and 1uM Desferal had about 50 % FeSOD and 50% MnSOD. Both of these samples were denatured and reconstituted with 1mM $FeSO_4$. About 60 - 80% of specific activity was recovered. The total activity of the reconstituted enzyme was about 50-70% that of native SOD. Fe - reconstituted SOD was inhibited 90% by 2mM NaN_3 (Table 12). 2mM NaN_3 inhibited 90% SOD activity (Table 12)

Reconstitution with 1mM $MnCl_2$ resulted in recovery of about 80 - 90% SOD activity and over 90% specific activity. Mn - reconstituted was not inhibited by 2mM NaN_3 . (Table 13).

Table 12. Azide inhibition of native and Fe-reconstituted SODs from purified Bacteroides

	% Inhi. of SOD Act. by 2mM NaN_3 conc.	Sp. activity U/mg	Recovered U/Mg %
B. distasonis [Control]			
Native Enzyme	90	2300	
Fe Recon.	92	1679	73
B. distasonis [1mM Mn]			
Native Enzyme	44	2400	
Fe Recon.	85	1512	62

[Control] ------- *B. distasonis* was grown anaerobically on BHI media.

[sample] ------- *B. distasonis* was grown anaerobically on chelex-treated plus 1mM Mn, 1uM Desferal BHI media.

Table 13. Azide inhibition of native and Mn-reconstituted SODs from purified Bacteroidem

	% Inhi. of SOD Act. by 2mM NaN_3 conc.	Sp. activity U/mg	Recovered U/Mg %
B. distasonis [Control]			
Native Enzyme	90	2300	
Mn Recon.	0	2093	91
B. distasonis [1mM Mn]			
Native Enzyme	44	2400	
Mn Recon.	0	2184	91

[Control] -------- B. distasonis was grown anaerobically on BHI media.

[sample] -------- B. distasonis was grown anaerobically on chelex-treated plus 1mM Mn, 1uM Desferal BHI media.

V. Effect of Hemin on B.distasonis SOD

B. distasonis was grown anaerobically in BHI and in chelex-treated BHI supplemented with different $MnCl_2$ and hemin concentrations. Hemin had no effect on the fraction of FeSOD in the extracts. For example, the bacterium grown in BHI with hemin and 1mM $MnCl_2$ had 65% FeSOD and the bacterium grown in BHI without hemin and 1mM $MnCl_2$ had 66% FeSOD.

Similarly, *B. distasonis* was grown anaerobically in BHI which was stirred in Chelex 100 for two hours to remove transition metals. By this chelex way, it will get better transition metal-restricted in the media than passing twice column of chelex 100. Under same condition (0mM $MnCl_2$, 1mM $MnCl_2$ or 2mM $MnCl_2$ sopplemented in the media), MnSOD content did not change With or without hemin solution in the media. The MnSOD content and FeSOD content were independent in Hemin solution in BHI Media (Table 14).

Table 14. Effect of Hemin on B. distasonis SOD

Treatment	Hemin	[Mn] (mM)	Density A_{660}	Sp. Act. (u/mg)	% Inh. (2mM N_3^-)	%MnSOD
Untreated	+	0	1.23	5.5	83	8
	+	1	1.22	9.2	65	30
	+	2	1.26	6.6	47	51
Untreated	-	0	1.30	3.6	83	8
	-	1	1.52	6.0	66	28
	-	2	1.45	7.9	51	46
Chelex	+	0	1.49	3.4	78	18
	+	1	0.9	5.0	50	47
	+	2	1.2	8.8	45	45
Chelex	-	0	1.28	4.2	77	15
	-	1	1.2	5.8	59	37
	-	2	1.3	4.4	49	48
Chelex	-	0	1.31	4.4	77	22
(1uM DF)	-	1	1.6	8.5	43	56
	-	2	0.7	7.4	0	100

B. distasonis anaerobically grew in BHI media which were stired in Chelex 100 for two hours.

VI. Immunological studies of FeSOD of B. distasonis

The antibodies to *B. distasonis* FeSOD reacted with the homologous antigen in Ouchterlony double diffusion gels. The antibody molecules can combine with antigen which elicited their formation to form an antigen-antibody complex. In the Ouchterlony plate, antibody and antigen diffuse toward each other and an opaque line is formed (Fig. 8). The antibodies raised from FeSOD of *B. distasonis* had light reaction with FeSOD of *B. fragilis* but no obvious reaction with FeSOD of *B. thetaiotaomicron*

Fig. 8. Ouchterlony double diffusion plates for cross reaction between antigen and antibody of B. distasonis SOD

Center - antibody.
Outside - antigen.

DISCUSSION

Superoxide dismutases in procaryotes contain either iron or manganese at the catalytic site. Iron and manganese superoxide dismutases evolved from a common ancestor unrelated to that of copper/zinc superoxide dismutase. The crystal structure studies demonstrate that FeSOD and MnSOD share a common polypeptide fold and have very similar amino acid sequence alignments which are completely different from those of CuZnSOD. *Escherichia coli,* like many gram-negative bacteria, contains two SODs: FeSOD and MnSOD. The two SODs are expressed differently in the cell. Usually, FeSOD is synthesized anaerobically whereas MnSOD is not, but MnSOD is induced by exposure to oxygen. *Bacteroides fragilis* , *Bacteroides distasonis* and *Bacteroides thetaiotaomicron* are anaerobic gram-negative bacteria that contain SOD. These anaerobic bacteria synthesize an iron-containing SOD (FeSOD) when grown anaerobically. MnSOD, if any, was not detectable. It seems likely that the cells tightly control either availability or concentration of the metals involved in SOD synthesis and

that Fe, rather than Mn, is preferentially utilized in the cells. Exposure of mid-logarithmic growth phase *B. fragilis* and *B. thetaiotaomicron* to 20% O_2 induced MnSOD formation in the cells, increasing specific activity 10-20-fold. Since SOD is an antioxidant enzyme, it is assumed that SOD in organisms is under some sort of regulatory control and the level of SOD reflects the oxidative stress imposed. However, in each case, the induced SOD had physical and chemical characteristics that except for metal content were identical. Moreover, the denatured FeSOD was reconstituted upon addition of Mn and the denatured MnSOD was reconstituted by Fe. These data are consistent with the synthesis of a single apoprotein that expresses SOD activity upon binding either transition metal. If it is the case, anaerobic synthesis of MnSOD might be accomplished by restricting Fe and reconstituting Mn of the media. Studies reported here support that contention.

Moody and Hassan presented data on the increased biosynthesis of MnSOD by ferrous iron chelators and increased manganese content in *E. coli* . They found that adding Mn^{+2} caused 2-fold increase in MnSOD while it had no effect on FeSOD or the hybrid SOD composed of one subunit each of MnSOD and FeSOD [46]. They proposed a model that the synthesis of MnSOD in *E. coli* is under the control of ferrous ion. It is possible that the regulatory protein of MnSOD is an iron-containing protein and that removal of the iron signals the biosynthesis of the enzyme. But so far there is no report about *in vivo* metal substitution into *Bacteroides* SOD. The *Bacteroides* synthesize FeSOD under

anaerobic culture, but they synthesize MnSOD in anaerobic culture under specific conditions. The content of MnSOD is dependent on Mn concentration and on the presence of iron chelator in the medium. Although the formation of MnSOD was increased and that of FeSOD was decreased when the *Bacteroides* were grown in Chelex-treated media and in media supplemented with $MnCl_2$, pure manganese-containing SOD was not formed. The inhibition by 2mM NaN_3 and inactivation with 2.5mM H_2O_2 were consistent with a mixture of Fe-containing and Mn-containing superoxide dismutase in each cell extract. When the cells were grown anaerobically in Chelex 100 treated media and supplemented with 1uM desferal and 2mM $MnCl_2$, only manganese-containing SOD in *Bacteroides* was obtained. It was shown that MnSOD content is proportional to the concentration of manganese in the medium. However, the specific activity of superoxide dismutase in the cell did not vary proportionally with the Mn concentration. The result also strongly support this viewpoint. SOD from *Bacteroides* grown anaerobically in BHI and in chelex-treated BHI supplemented with 1mM Mn, 1uM Desferal migrated in 7.5% polyacrylamide gel with the same relative mobility in the native gel and yielded identical activity patterns. Similarly, in IEF gel, the extracts focussed to identical activity patterns, whether electrophoresed separately or in combination. It is proposed that the conversion of inactive apoprotein to catalytically holoenzymes depends on the availability of the appropriate metal cofactor.

It is known that nutrient depletion affects the envelope structure and related properties. Much evidence indicated that iron is an important nutrient required by most bacteria. Bacteria used in our laboratory have been traditionally cultured without regard to iron content of the media. Various microorganisms have also shown to adapt to iron-limited conditions and exhibited a range of phenotypic changes. For example, they will produce new proteins, iron-binding compounds and t-RNA molecules with incorrect nucleotides. Fontaine, et al. demonstrated that the anaerobe, *Bacteroides ureolyticus* produced proteins under conditions of reduced iron availability, that were not detected under conditions of iron availability. Hemin is a iron-porphyrin prosthetic compound usually supplemented in BHI media. It has been considered that the Hemin is a potential resource of iron for FeSOD biosynthesis. Based on our experimental results, however, we find that *Bacteroides distasonis* grown anaerobically in BHI media supplemented with hemin did not have increased FeSOD content. Similarly, FeSOD content of *Bacteroides* grown anaerobically in chelex-treated BHI supplemented with hemin were similar to those of *Bacteroide* grown anaerobically in BHI media without hemin solution. Hemin did not increase the fraction of FeSOD in cells grown in iron restricted medium.

Metal is important for catalytic activity of superoxide dismutases, and removing metal from *Bacteroides* SOD abolished catalytic SOD activity. Apoenzymes of *Bacteroides* prepared in the presence of a denaturant and a chelator are capable of binding either Fe or Mn at their active site and consc-

quently regain significant O_2^- scavenging activity. SOD apoenzymes prepared from crude extracts of *Bacteroides* (*B. fragilis, B. distasonis* and *B. thetaiotaomicron*) grown anaerobically in BHI media and apoenzymes of the same bacteria grown anaerobically in BHI media treated with Chelex 100 and supplemented with 1mM $MnCl_2$ and 1uM Desferal were reconstituted with either Mn or Fe metal. Purified apoenzyme of *B. distasonis* grown anaerobically in BHI and in BHI treated with Chelex 100 and supplemented with 1mM $MnCl_2$ and 1uM Desferal were also reconstituted with Mn or Fe, with regain of the enzymatic activity. The activity recovered upon reconstitution with Fe salt was less than that reconstituted with Mn salt. The reason is that the $MnCl_2$ is more soluble in 20mM Tris buffer, (pH 7.0) than is $FeSO_4$. $FeSO_4$ is also oxidized easily. In each reconstitution study, there was some loss of protein by precipitation, so the recovery of specific activity is higher. Each metal confers upon the apoprotein characteristics specific for the metal. The Fe-reconstituted SOD was inhibited by azide and inactivated by H_2O_2 . But the Mn-reconstituted SOD was resistant to azide and H_2O_2. The reconstituted SOD also had electrophoretic properties which is identical to those of holoproteins. These data are also consistent with the presence of a single binding site of per subunit whose ligands accept either iron or manganese.

The fraction of FeSOD and MnSOD in crude extracts was estimated by using experimental equation[62]: X + Y = total activity, 0.9X + 0.1Y = activity uninhibited by 2mM NaN_3 . Y represent fraction of FeSOD, X represent fraction

of MnSOD. The algorithm was based on inhibition of the pure FeSOD and MnSOD from *B. fragilis* . Azide inhibition studies and metal content of SOD isolated from *B. distasonis* grown in untreated or chelex-treated Mn supplemented BHI validate the assumptions used to calculate MnSOD content of crude extracts. Superoxide dismutase isolated from *B. distasonis* grown anaerobically in BHI media produced FeSOD. The specific activity was around 2300 u/mg which is higher than those of *B. distasonis* which was grown anaerobically in PYG (Peptone-Yeast Extract Glucose) media [58]. This suggests that BHI is more suitable for SOD synthesis in *B. distasonis* than is PYG. The isolated superoxide dismutase from *B. distasonis* which was grown anaerobically in chelex-treated BHI media containing 1mM Mn, 1uM Desferal, and Mg was about half FeSOD and half MnSOD. Chelex treatment plus desferal depleted iron and manganese supplementation increased manganese availability. Specific activity of the MnSOD-FeSOD mixture is around 2400 u/mg. SOD from cells grown in BHI had higher FeSOD and Fe-content (Fe 1.5 gram-atoms/mol enzyme, Mn 0.04 gram-atoms/mol enzyme) than did the enzyme from Mn supplemented cells (Fe 1.05 gram-atoms/mol enzyme, Mn 1.16 gram-atoms/mol enzyme). Azide inhibition and hydrogen peroxide inactivation reaction demonstrated that the SOD from the cells grown in BHI media lost 90% activity of superoxide dismutase in 2mM NaN_3 and in 2.5mM H_2O_2 in ten minutes whereas the SOD from the cells grown in treated BHI media plus 1mM Mn lost only 50% activity in 2mM N_3 or on incubation in 2.5mM H_2O_2 . The conclusion is that

anaerobic conditions, *Bacteroides* can form FeSOD on MnSOD, depending on the availability of transition metals in the media.

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