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IN VIVO METAL SUBSTITUTION IN *BACTEROIDES FRAGILIS* SUPEROXIDE DISMUTASE

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Bacteroides fragilis, an obligate anaerobe, synthesizes an azide-inhibitable iron-containing superoxide dismutase when grown in complex medium. Cells grown anaerobically in complex media containing desferrioxamine (Desferal™, Ciba-Geigy) and graded concentrations of Mn synthesize the azide-resistant manganese-containing SOD. The fraction of MnSOD activity in dialyzed cell extracts increased progressively as the Mn concentration in the medium increased. The fraction of MnSOD activity also increased in extracts of cells grown in the medium with 1 mM Mn but with graded concentrations of desferrioxamine (0-10 micromolar). The SOD activity in the cells grown under the various conditions varied but not in a causal relationship with either Mn or desferrioxamine concentration. Electrophoresis revealed that the SOD activity in cells grown in the absence or presence of 1 mM Mn migrated with the same relative mobility and exhibited identical activity patterns when examined separately or as a mixture. These data are consistent with substitution of Mn for Fe in the *B. fragilis* apoprotein under anaerobic conditions and support the model of a single protein binding either Fe or Mn.

KEY WORDS: Mn-superoxide dismutase, Fe-superoxide dismutase, *Bacteroides fragilis*, desferrioxamine.

INTRODUCTION

The anaerobes *Bacteroides fragilis, B. thetaiotaomicron* and *B. distasonis* synthesize metallosuperoxide dismutases. Anaerobically grown and maintained *B. fragilis* and *B. thetaiotaomicron* produce iron-containing superoxide dismutase (FeSOD), but aeration of the cells, grown anaerobically to mid log phase, induced Mn-containing SOD (MnSOD). The FeSODs from *B. fragilis* and *B. thetaiotaomicron* are dimers whose molecular weights are 42,000 and 46,000, respectively.[1,2] Superoxide dismuting activity and metal cofactor are lost upon treatment of either enzyme with 5 M guanidinium chloride plus 8-hydroxyquinoline. Renaturation of the denatured apoprotein in buffer containing either ferrous or manganous salt restored superoxide scavenging activity. Renaturation in the presence of ferrous ammonium sulfate yielded enzymic activity that was 50% inhibited by 0.2 mM NaN_3 and was rapidly inactivated by H_2O_2, as was the native, iron-containing enzyme. Renaturation in $MnCl_2$ restores an azide- and H_2O_2-resistant SOD activity. With either enzyme, the Mn-reconstituted protein bound approximately 1 gm-atom Mn per mol dimer.

The oxygen-induced MnSOD isolated from each species was subjected to metal removal under denaturing conditions and renaturation in either Fe or Mn salts.[2,3] Superoxide scavenging activity was again restored by either metal. Physical properties and amino acid compositions of the constitutive FeSOD and O_2-induced MnSOD

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from the same species were in each case indistinguishable. The data are consistent with the synthesis of an apoprotein capable of inserting either Fe or Mn depending upon intracellular conditions. These enzymes display "cambialistic" properties as defined by Martin, *et al.*[4] to describe *Streptococcus mutans* SOD, an enzyme functional with either Fe or Mn. Were this the case, depletion of Fe from the medium and an increase in Mn content should cause the *Bacteroides* to form MnSOD anaerobically. This hypothesis was explored using *B. fragilis*.

MATERIALS AND METHODS

B. fragilis (VPI 2393) was maintained anaerobically in chopped meat medium[5] and was transferred aseptically to prereduced, anaerobically sterilized Brain-Heart Infusion, (BHI, 37 gm/L) plus yeast extract (5 gm/L). Where indicated, the medium was passed twice through Chelex 100 resin (Biorad, Richmond, CA) to diminish the trace metal concentration. Desferrioxamine (Desferal, Ciba-Geigy) was added to the media after sterilization, but Mn was added as the filter sterilized solution of $MnCl_2$ in water just before inoculation. Cells, grown at 37° overnight, were harvested by centrifugation, washed once in 50 mM potassium phosphate, 1 mM EDTA (pH 7.8) and resuspended in 10–15 mL of the buffer at 4°. Cells were disrupted by 70 watts of sonic oscillation applied through the microtip of a Heat Systems W-385 sonicator in 30 sec. bursts for a total of 6 min. Cell debris was removed by centrifugation and the supernatant solution was dialyzed overnight at 4° in 4 L of the phosphate buffer. Superoxide dismutase activity was measured by the cyt. *c* competition method of McCord and Fridovich.[6] Inhibitors, when present, were added to the assay mixture prior to initiation of the assay with xanthine oxidase. Crude cell extracts were separated electrophoretically in gels containing 7.5% acrylamide and the SOD activity visualized as described by Beauchamp and Fridovich.[7] Proteins were focussed to their isoelectric pH in acrylamide gels containing the appropriate ampholytes. The algorithm used to calculate amounts of the Mn- and FeSODs was based on the assumption that 2 mM NaN_3 inhibited the activity of FeSOD 90% and of MnSOD 10%. The values are based on experimentally determined inhibition values for the *B. fragilis* Fe- and MnSODs.[1,3]

RESULTS

Effect of Mn concentration on SOD content

Superoxide dismutase activity of *B. fragilis* grown anaerobically in Chelex-treated BHI, supplemented with 1 micromolar desferrioxamine (Table I), is shown for cells cultured with graded concentrations of Mn in the medium. The specific activities varied from 2.2 to 5 U/mg but not in a causal relationship with Mn concentration. Thus the SOD activity in extracts of cells grown with no added Mn or with 2 mM Mn were similar. The fraction of azide-inhibitable SOD activity in cell extracts decreased as the Mn concentration of the media increased. SOD activity in extracts of cells grown without Mn supplementation was inhibited 89% by 2 mM azide but SOD in extracts of cells grown in 2 mM Mn was not inhibited. The data are consistent with increased proportion of MnSOD in the extracts in response to graded increases in Mn in the medium (Figure 1).

TABLE I
Effect of Mn concentration on *B. fragilis* SOD activity

[Mn]* (mM)	SOD Activity (U/mg)	% Inhibition (2 mM Azide)
0.0	5.0	83
0.1	3.4	75
0.25	4.4	56
0.5	3.7	47
1.0	3.7	42
1.5	2.6	16
2.0	4.9	0

*BHI media were passed twice through Chelex-100 and were supplemented with 1 micromolar desferrioxamine after sterilization. Mn, as the filterilized solution of $MnCl_2$ in water, was added to the appropriate concentration just prior to inoculation.

Effect of desferrioxamine concentration on B. fragilis *SOD*

SOD activity was determined in dialyzed extracts of *B. fragilis* grown in Chelex-treated BHI with 1 mM $MnCl_2$ but different concentrations of desferrioxamine (Table II). The specific activity of SOD in the extracts varied within the same range as shown in Table I but was independent of the desferrioxamine concentration. The fraction of MnSOD activity in the extracts increased with increasing desferrioxamine concentration in the medium. MnSOD comprised 37% of the SOD activity in cells grown in chelex-treated medium lacking desferrioxamine but was the sole detectable form of SOD in cells grown in medium with 5 micromolar desferrioxamine.

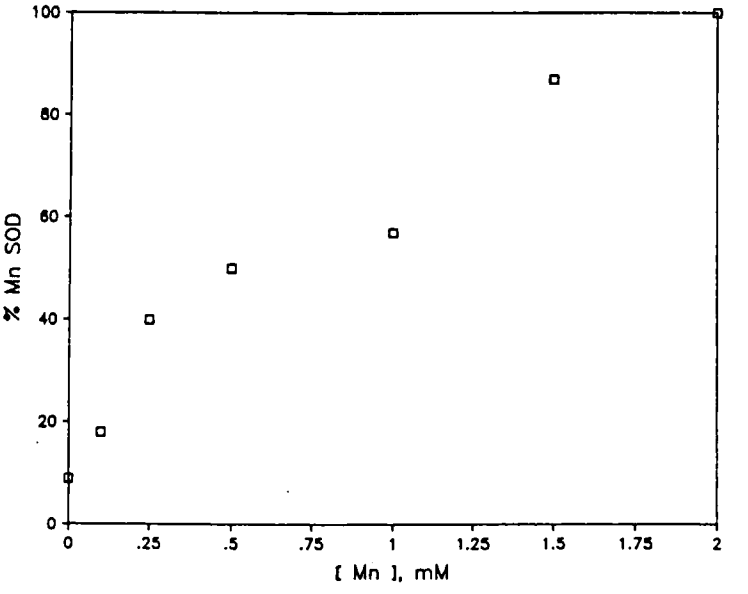

FIGURE 1 Effect of medium Mn concentration on MnSOD activity in *B. fragilis*. The fraction of MnSOD activity, based on azide-resistant activity, is shown as a function of Mn concentration. The percentage of MnSOD activity was calculated based on the algorithm described (Methods).

TABLE II
Effect of desferrioxamine on *B. fragilis* MnSOD content[a]

Desferrioxamine (Micromolar)	Sp.Act. (U/mg)	% Inhibition (2 mM azide)	% MnSOD
0	5.8	59	37
1	3.0	48	53
2	3.8	21	81
5	4.9	0	100
10	2.4	0	100

[a]Cells were grown in BHI medium that was treated with Chelex-100 resin and supplemented with 1 mM $MnCl_2$ prior to inoculation.

Electrophoretic characterization of B. fragilis *SOD*

Extracts of cells grown in the absence or presence of 1 mM Mn were separated on 7.5% acrylamide gels and stained for superoxide dismutase activity (Figure 2). In each case, the SOD in extracts grown with or without 1 mM Mn migrated with the same relative mobility. Identical activity patterns, a single SOD major activity band and faintly active electromorphs, were observed when the extracts were electrophoresed individually (Lanes 1, 2, 4 and 5) or as a mixture (Lanes 3 and 6). The extracts were

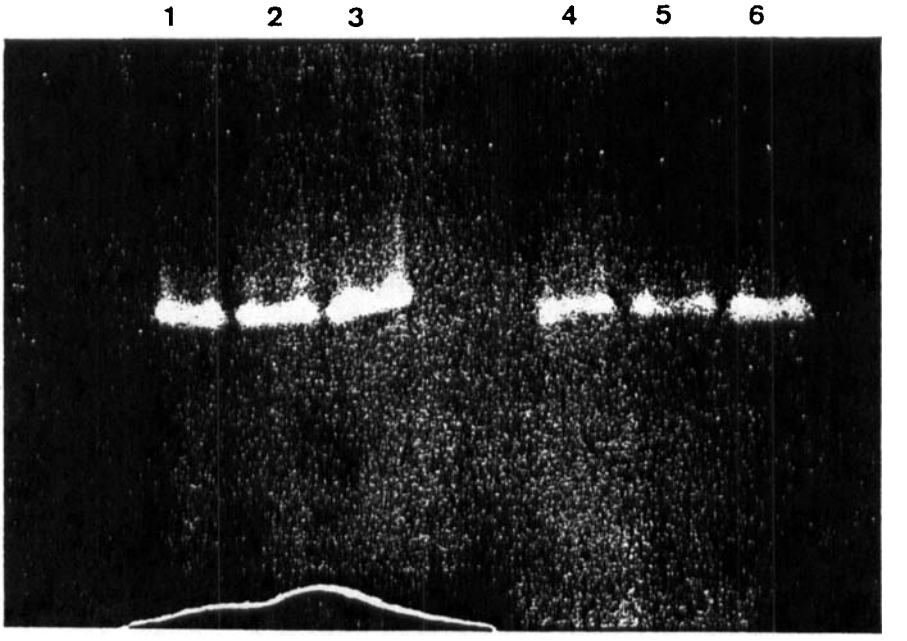

FIGURE 2 Electropherogram of SOD activity in *B. fragilis*. Dialyzed cell extracts of *B. fragilis* grown in the medium indicated were separated on 7.5% acrylamide slab gels. After electrophoresis, the gels were stained for SOD activity as described by Beauchamp and Fridovich.[7] Extracts of cells grown in complex medium with 7 micromolar hemin, contain 90% FeSOD (Lanes 1 and 4). Extracts of cells grown in chelex-treated medium with 1 mM Mn and 1 micromolar desferrioxamine contain 57% MnSOD, 43% FeSOD (Lanes 2 and 5). Lanes 3 and 6 are equal volume mixtures of the extracts described. SOD activity units applied: Lane 1, 0.9 units; Lane 2, 0.4 units; Lane 3, 0.7 units; Lane 4, 0.5 units; Lane 5, 0.5 units, Lane 6, 0.4 units.

focussed to their isoelectric point in gels containing ampholytes. The Mn-deficient, Mn-supplemented and the mixture of the extracts revealed identical isoform patterns (data not shown).

DISCUSSION

Almost all the SODs examined display a strict specificity for the active site metal. Although the apoproteins may bind other transition metals, those metals are usually catalytically incompetent. The *Bacteroides* species used in these studies is among those with relaxed metal specificity. We have shown that the holoenzymes from *B. fragilis* and *B. thetaiotaomicron* are FeSODs but the denatured apoproteins derived from those enzymes are reconstituted with either iron or manganese. The enzymes used in those studies were isolated from cells grown in complex media with no attention to metal supplementation or restriction.

Increased concentration of Mn in chelex-treated anaerobic BHI medium resulted in an increased proportion of MnSOD in the crude extract. This increase was not accompanied by significant increases in the SOD specific activity in *B. fragilis*. Thus, the supplementation of the cells with Mn failed to induce SOD. The fraction of MnSOD in extracts of cells approximates a hyperbolic response to increased Mn concentration in the medium (Figure 1). The concentration of Mn (1.5–2.0 mM) yielding only the Mn-form of SOD is large compared with Mn concentration in the medium. However, a significant fraction of the MnSOD (35–50%) was formed when Mn concentrations were 250–500 micromolar in the chelex-treated medium. The formation of MnSOD was also favored by increased concentrations of desferrioxamine in the chelex-treated medium supplemented with 1 mM Mn (Table II).

Synthesis of MnSOD in oxygenated *B. fragilis* was accomplished with the amount of Mn available in the unsupplemented complex medium and was dependent on dioxygen and *de novo* protein synthesis. Under anaerobiosis, formation of FeSOD was favored even in medium passed through chelex resin. These observations suggest that the apoSOD competes strongly for Fe and that under anaerobic conditions, this competition is overcome only by limitation of Fe in the face of large concentrations of Mn. It is not yet known, for this enzyme, the relative affinity of the apoprotein for the Fe^{++}/Fe^{+++} couple compared with the Mn^{++}/Mn^{+++} couple. Pugh and Fridovich[8] have suggested that Mn^{+++} binds to apoMnSOD in *E. coli* more avidly than does Mn^{++}. In that model, Fe^{++} effectively competes with either Fe^{+++} or Mn^{++} but not Mn^{+++} for the apoprotein. The requirement for increased concentrations of Mn^{++} in the anaerobic formation of *B. fragilis* MnSOD may reflect the poor competition of the apoSOD for Mn^{++}. If *B. fragilis* apoSOD competes equally well for Fe^{++} and Mn^{++}, the cellular response to increased Mn concentration may reflect differential transport of iron and manganese. These possibilities remain to be explored.

B. distasonis and *B. thetaiotaomicron* also formed MnSOD in chelex-treated medium supplemented with Mn (data not shown). The fraction of MnSOD in cells grown in each Mn concentration was similar to those shown for *B. fragilis*.

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