JOURNAL OF BACTERIOLOGY, Mar. 1990, p. 1457–1463
0021-9193/90/031457-07$02.00/0

Vol. 172, No. 3

Characterization of Superoxide Dismutases Purified from Either Anaerobically Maintained or Aerated *Bacteroides gingivalis*

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Received 9 August 1989/Accepted 11 December 1989

Superoxide dismutases (SODs) were purified from extracts of either anaerobically maintained or aerated *Bacteroides gingivalis*. Each purified enzyme (molecular weight, 46,000) was a dimer composed of two subunits of equal sizes. SOD from anaerobically maintained cells (anaero-SOD) contained 1.79 g-atom of Fe and 0.28 g-atom of Mn, and SOD from aerated cells (aero-SOD) contained 1.08 g-atom of Mn and 0.36 g-atom of Fe. Spectral analysis showed that anaero-SOD had the characteristic of Fe-SOD and that aero-SOD had that of Mn-SOD. Both enzyme preparations contained three isozymes with identical isoelectric points. On the basis of inactivation of SOD by H_2O_2, it was found that aero-SOD consisted of one Mn-SOD and a small quantity of two Fe-SODs, whereas anaero-SOD contained only Fe-SOD. However, each apoprotein from anaero-SOD and aero-SOD, prepared by dialysis in guanidinium chloride plus 8-hydroxyquinoline, showed only one protein band each with the same isoelectric point on an isoelectric focusing gel. Subsequent dialysis of both apoenzymes with either $MnCl_2$ or $Fe(NH_4)_2(SO_4)_2$ restored the activity. These reconstituted SODs showed only one protein band with SOD activity on native polyacrylamide gel electrophoresis. Furthermore, the two enzymes had similar amino acid compositions, and their amino-terminal sequences were identical through the first 12 amino acids. These results suggest that the three isozymes of anaero-SOD and aero-SOD in *B. gingivalis* are formed from a single apoprotein.

Superoxide dismutases (SODs; EC 1.15.1.1) catalyze dismutation of superoxide (O_2^-) into O_2 and H_2O_2. These enzymes are a family of metalloproteins which have iron (Fe-SOD), manganese (Mn-SOD), or copper (CuZn-SOD) in the active site (4, 10). In general, procaryotes contain Fe-SOD, Mn-SOD, or a combination of these enzymes. Earlier metal replacement studies with Fe-SOD and Mn-SOD enzymes produced by several bacterial species indicated strict metal cofactor specificity for these enzymes (18). *Escherichia coli* cells contained only Fe-SOD when grown anaerobically, but when grown aerobically they contained Fe-SOD, Mn-SOD, and a hybrid of these (16). In an experiment on metal binding for these apo-SODs, only Fe restored SOD activity to apo-Fe-SOD, and only Mn restored SOD activity to apo-Mn-SOD (23). However, bacteria such as *Propionibacterium sherimanii* (22) and *Streptococcus mutans* (19) were found to use the same protein moiety to form either Fe-SOD or Mn-SOD, depending on the metal supplied in the growth medium. Although anaerobically grown *Bacteroides fragilis* contained only Fe-SOD (13), the SOD in *B. fragilis* exposed to oxygen contained only Mn-SOD, which had the same physiological properties and amino acid composition as Fe-SOD (12). A similar result has been obtained with *B. thetaiotaomicron* (24). Thus, it has been hypothesized that these bacteria synthesize a single SOD apoprotein capable of binding either Mn or Fe.

There are many studies suggesting that *B. gingivalis* plays a central role in the etiology of certain forms of periodontal disease (20, 29). Since *B. gingivalis* can be established and maintained in a periodontal pocket, bacterial enzymes that destroy and neutralize the toxic products of oxygen metabolism may be an important virulence factor in the pathogenesis of periodontal infections. Our previous study (1) has demonstrated that *B. gingivalis* shows the highest activities of SOD and that *B. gingivalis* is also the most tolerant in the presence of oxygen among the black-pigmented *Bacteroides* species tested. Furthermore, the SOD activity increased during aeration of *B. gingivalis*. In this study, we purified SODs from anaerobically maintained or aerated *B. gingivalis* and characterized both of the SODs, which contained three isozymes that were separated electrophoretically.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *B. gingivalis* 381 was obtained from stock strains at Research Laboratories of Oral Biology, Sunstar Inc., Osaka, Japan. The strain was maintained anaerobically by weekly transfer on plates containing Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood, 1 mg of yeast extract (BBL) per ml, 5 μg of hemin per ml, and 1 μg of menadione per ml. The bacterial cells were cultured anaerobically in prereduced Trypticase soy broth (BBL) supplemented with 1 mg of yeast extract per ml, 5 μg of hemin per ml, and 1 μg of menadione per ml and were incubated to the mid-logarithmic phase (A_{660}, 1.0) at 37°C in an anaerobic system 1024 (Forma, Morietta, Ohio) in an atmosphere of 80% N_2–10% CO_2–10% H_2. For aeration studies, cells grown anaerobically in 1 liter of broth to the mid-logarithmic phase were harvested aseptically by centrifugation at 3,500 × *g* for 30 min at 4°C, suspended in 3 liters of nonreduced fresh broth, transferred to sterile 5-liter flasks stoppered with cotton plugs, and incubated at 37°C in air with vigorous shaking for 6 h. Cells were harvested by centrifugaton at 8,000 × *g* for 30 min at 4°C and washed three times with 50 mM potassium phosphate buffer (pH 7.8).

Enzyme assay. SOD activity was assayed by a modification of the method of McCord and Fridovich (21). The reaction mixture (3 ml) contained samples of enzyme solution and 50

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mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.05 mM NaCN, 0.01 mM cytochrome *c* (grade III; Sigma Chemical Co., St. Louis, Mo.), 0.1 mM xanthine (Sigma), and a volume of xanthine oxidase (Sigma) sufficient for constant reduction of cytochrome *c*, the rate of which was an A_{550} increase of 0.025 ± 0.001 (standard deviation [SD])/min. Inhibitors, when present, were added to the mixture to the appropriate concentration. The assay was performed at 25°C. One unit of SOD activity was defined as the amount which gave a 50% decrease in the rate of reduction of cytochrome *c*. Protein content was determined by the Lowry method as modified by Hartree (15), with recrystallized bovine serum albumin as the standard.

Purification of anaero-SOD and aero-SOD. All operations were performed at 4°C unless otherwise noted. For purification of SOD from anaerobically grown cells (anaero-SOD), 10 g (wet weight) of anaerobically maintained cells was suspended in a sample of 50 mM potassium phosphate buffer (pH 7.8) and lysed by five 1-min sonication treatments (Ultrasonic Disruptor UR 200P; 170 W; Tomy Seiko Co. Ltd., Tokyo, Japan). The disrupted products were centrifuged at 25,000 × *g* for 60 min. The supernatant was brought to 50% saturation by addition, with stirring, of solid ammonium sulfate. The solution was stirred for 3 h and then centrifuged at 25,000 × *g* for 60 min. The pellet was discarded, the supernatant was applied to a Phenyl-Sepharose gel column (1.8 by 10 cm; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer (pH 7.8) containing 50% ammonium sulfate, and the column was washed with ca. 80 ml of the same buffer. A linear gradient of ammonium sulfate (50 to 0%) in this buffer was then applied. The flow rate was 15 ml/h, and the eluate was collected into 4-ml fractions. The fractions displaying SOD activity were pooled and dialyzed against three 5-liter volumes of 5 mM potassium phosphate buffer (pH 8.5). The dialysate was applied to a Q-Sepharose column (1.8 by 10 cm; Pharmacia LKB) equilibrated in the same buffer with a Fast-Protein Liquid Chromatography (FPLC) system (Pharmacia LKB). The column was washed with ca. 80 ml of 5 mM potassium phosphate buffer (pH 8.5) and eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer. The flow rate was 1 ml/min, and the eluate was collected into 2-ml fractions. Fractions having SOD activity were collected and concentrated by ultrafiltration over a UP-20 membrane (Toyo Roshi Ltd., Tokyo, Japan). The resultant solution was applied to a Superose-12 column (1 by 30 cm; Pharmacia LKB) equilibrated with 50 mM potassium phosphate buffer (pH 7.8) with the FPLC system. The flow rate was 0.4 ml/min, and the eluate was collected into 0.5-ml fractions. The SOD activity was eluted as a single symmetrical peak which was congruent with an A_{280} peak. The active fractions were collected as the preparation of the purified enzyme.

For purification of SOD from aerobically grown cells (aero-SOD), sonicated extracts of aerated cells were treated with 50% saturated ammoium sulfate, and then Phenyl-Sepharose gel column chromatography was performed in the same way as for purification of anaero-SOD. The pooled fractions were dialyzed against 20 mM potassium phosphate buffer (pH 8.5). The dialysate was applied to a Mono-Q column (0.5 by 5 cm; Pharmacia LKB) equilibrated with a 20 mM potassium phosphate buffer (pH 8.5) with an FPLC system at a flow rate of 1 ml/min. SOD activity passed unhindered through this column. The unbound active fractions were collected and dialyzed against 5 mM potassium phosphate buffer (pH 8.5). The dialysate was adsorbed onto a Q-Sepharose column equilibrated with 5 mM potassium phosphate buffer (pH 7.8) with an FPLC system. The column was washed with ca. 80 ml of the same buffer and eluted with a linear gradient of NaCl (0 to 1 M). The active fractions were collected as the preparation of purified aero-SOD.

Electrophoresis and molecular weight determination. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed with a Phast System (Pharmacia LKB) on PhastGel Gradient 10-15 (Pharmacia LKB) with a molecular weight separation range of 10,000 to 250,000 as recommended by the manufacturer. Samples were dissolved in 10 mM Tris hydrochloride buffer (pH 8.0) with 2.5% sodium dodecyl sulfate–1 mM EDTA–0.01% bromphenol blue and immersed in a boiling water bath for 5 min. An Electrophoresis Calibration Kit (Pharmacia LKB) was used for molecular weight markers. The gels were stained with 0.1% Coomassie brilliant blue R-350. The molecular weights of the native purified enzymes were estimated with a Superose-12 gel filtration column (Pharmacia LKB) on the basis of the procedure of Andrews (2). The molecular weight markers for this analysis were aldolase (molecular weight, 158,000), bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 43,000), and cytochrome *c* (molecular weight, 12,000). Disc electrophoresis of the native protein (native PAGE) was performed on gels containing 7.5% acrylamide by the Davis method (7). The gels were stained for protein with 0.2% Coomassie brilliant blue R-250, and SOD activity was detected in gels by the photochemical Nitro Blue Tetrazolium stain of Beauchamp and Fridovich (5). To distinguish between Mn-SOD and Fe-SOD, the gel was soaked for 1 h in 1 mM NaCN–2 mM H_2O_2–50 mM potassium phosphate buffer (pH 7.8). The position of the activity band was recorded by linear densitometric scanning at 560 nm with a CS 910 gel scanner (Shimadzu Ltd., Kyoto, Japan). Isoelectric focusing (IEF) PAGE was performed with a Phast System on PhastGel IEF 3-9 (Pharmacia LKB) with a pH range of 3 to 9. An IEF Calibration Kit (Pharmacia LKB) was used for standard pH markers. The gels were stained with 0.02% Coomassie brilliant blue R-350.

Analytical methods. UV and visible spectra of the purified enzymes were prepared with a recording spectrophotometer (UV-265FW; Shimadzu Ltd.). The metal contents of the enzymes were determined by an atomic absorption spectroscope (AA-8500; Nippon Jarrell-Ash, Kyoto, Japan). For amino acid analysis, protein was hydrolyzed in 5.7 M HCl at 110°C in evacuated, sealed tubes for 22 to 24 h. The hydrolysates were analyzed with Hitachi 835 S amino acid analyzer (Hitachi Ltd., Tokyo, Japan). Cysteine was determined as cysteic acid by hydrolysis in 5.7 M HCl for 24 h at 110°C after performic acid oxidation of the intact protein (17). Tryptophan was determined after hydrolysis of the intact protein for 24 h at 110°C in 4 M methanesulfonic acid with 0.2% tryptamine (28). The amino-terminal sequence of the protein was determined with a 470A gas phase automatic sequencer (Applied Biosystems, Inc., Foster City, Calif.). Phenylthiohydantoin amino acids were identified by reverse-phase high-performance liquid chromatography as described by Tsunasawa et al. (32).

Metal removal and replacement study. Metal removal and replacement studies were performed by the methods of Gregory and Dapper (13). Apo-SOD was prepared from purified SOD by dialysis for 18 h in 5 M guanidinium chloride containing 20 mM 8-hydroxyquinoline (pH 3.2) and then dialyzed for 8 h in 5 M guanidinium chloride to remove the organic chelator. The holoenzyme was reconstituted by

TABLE 1. Purification of anaero-SOD from *B. gingivalis* 381

Step	Vol (ml)	Protein concn (μg/ml)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Purification (fold)
Crude extract	180	3,190	1,950	3.4		1
Ammonium sulfate (50% saturation)	197	2,300	3,270	7.2	100	2
Phenyl-Sepharose CL-4B	303	46.0	3,210	229	98	68
Q-Sepharose	12.8	188	2,450	1,020	75	300
Superose-12	71.0	24.5	1,610	931	49	272

dialysis for 12 h in 20 mM Tris buffer (pH 7.0) containing either 1 mM $MnCl_2$ or 1 mM $Fe(NH_4)_2(SO_4)_2$. Excess metal was removed by dialysis in 20 mM Tris–1 mM EDTA (pH 7.0).

RESULTS

Purification of anaero-SOD and aero-SOD. The purification procedures for anaero-SOD and aero-SOD are summarized in Tables 1 and 2, respectively. The purification procedure for anaero-SOD was almost identical to that for aero-SOD. However, a final Superose-12 gel filtration step was required for purification of anaero-SOD, and Mono-Q chromatography was used to isolate aero-SOD. The two enzyme activities migrated as single peaks in a variety of chromatography assays. On chromatography with Phenyl-Sepharose CL-4B and Q-Sepharose Fast Flow, both enzymes were eluted at the same position by the eluting buffer with a gradient. One enzyme was purified from each extract of anaerobically maintained or aerated cells. Anaero-SOD was purified 272-fold from the initial crude extract to a specific activity of 931 U/mg of protein, and aero-SOD was purified 240-fold to a specific activity of 1,438 U/mg of protein.

Characterization of purified anaero-SOD and aero-SOD. The native molecular weights of anaero-SOD and aero-SOD were both estimated to be 46,000 by gel filtration. On sodium dodecyl sulfate-PAGE in the absence of β-mercaptoethanol, each of the purified enzymes displayed only a single protein band with an apparent molecular weight of about 23,000 (Fig. 1). This observation was not affected by β-mercaptoethanol treatment. Thus, each of the native SODs was probably a dimer of equal-size subunits joined noncovalently. Purified anaero-SOD contained 1.79 g-atom of Fe, 0.28 g-atom of Mn, 0.08 g-atom of Cu, and 0.17 g-atom of Zn per mol of dimer, whereas aero-SOD contained 1.08 g-atom of Mn, 0.36 g-atom of Fe, 0.09 g-atom of Cu, and 0.23 g-atom Zn per mol of dimer. The UV and visible spectra of each purified enzyme showed a peak at 280 nm with a shoulder at

TABLE 2. Purification of aero-SOD from *B. gingivalis* 381

Step	Vol (ml)	Protein concn (μg/ml)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Purification (fold)
Crude extract	178	8,360	8,970	6.0		1
Ammonium sulfate (50% saturation)	183	3,290	9,600	15.9	100	2
Phenyl-Sepharose CL-4B	340	65.0	7,100	321	74	54
Mono-Q	355	14.0	5,850	1,177	61	196
Q-Sepharose	15.0	160	3,450	1,438	36	240

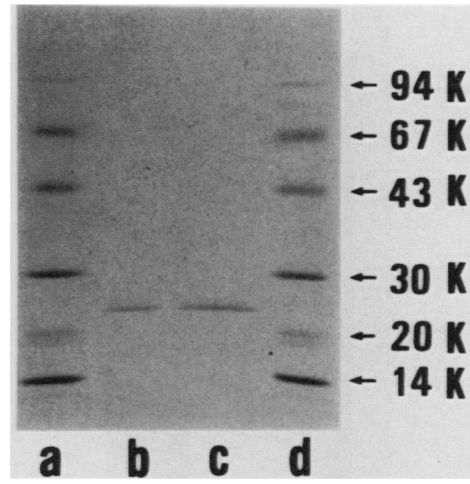

FIG. 1. Sodium dodecyl sulfate-PAGE profile with Coomassie blue stain of purified SODs from *B. gingivalis* 381. Lanes: a and d, standard molecular weight markers (phosphorylase *b* [molecular weight, 94,000], bovine serum albumin [molecular weight, 67,000], ovalbumin [molecular weight, 43,000], carbonic anhydrase [molecular weight, 30,000], soybean trypsin inhibitor [molecular weight, 20,100], and α-lactalbumin [molecular weight, 14,400]); b, anaero-SOD (1.1 μg of protein); c, aero-SOD (1.3 μg of protein).

292 nm (Fig. 2). The molecular extinction coefficients at 280 nm of both of the enzymes were identical at 8.1×10^4 M^{-1} cm^{-1}. However, anaero-SOD displayed a peak of visible absorption intensity at 350 nm and aero-SOD displayed a peak at 475 nm (Fig. 2).

PAGE. On native PAGE of both purified SODs, each gel stained for enzyme activity revealed three zones of SOD activity that migrated coincidentaly with three bands stained for protein (Fig. 3). This suggests that each of the SODs has three isozymes. To distinguish three isozymes between Mn-SOD and Fe-SOD, the gels were stained for SOD activity in the presence of 2 mM H_2O_2. Figure 4 shows densitometric scans of the gels. Three isozymes of anaero-SOD were entirely inactivated by H_2O_2. In aero-SOD, two isozymes showing rapid migration were inactivated by H_2O_2, but the most active isozyme with the slowest migration was little affected. Thus, it is considered that anaero-

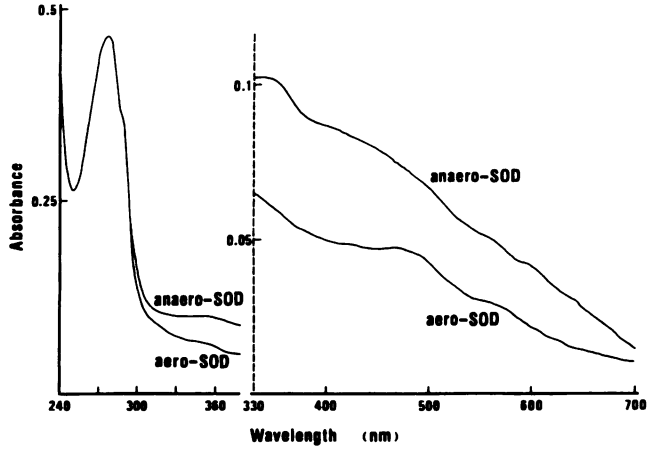

FIG. 2. UV and visible absorption spectra of purified SODs from *B. gingivalis* 381. The protein concentration of each enzyme was 0.21 mg/ml in 50 mM potassium phosphate (pH 7.8).

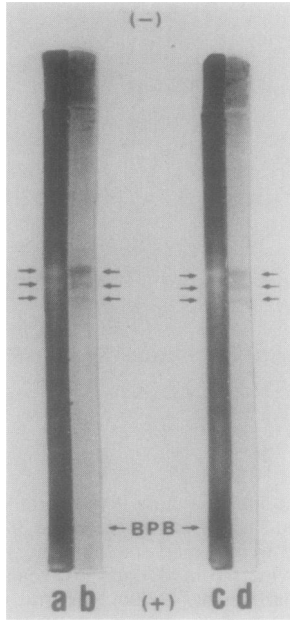

FIG. 3. Activity- and protein-stained native PAGE of purified anaero-SOD and aero-SOD from *B. gingivalis* 381. Lanes: a, anaero-SOD stained for SOD activity; b, anaero-SOD stained for protein; c, aero-SOD stained for SOD activity; d, aero-SOD stained for protein. Approximately 6 μg of protein (total activity, 6.5 U) was applied to each gel for activity or protein staining. BPB, Bromphenol blue.

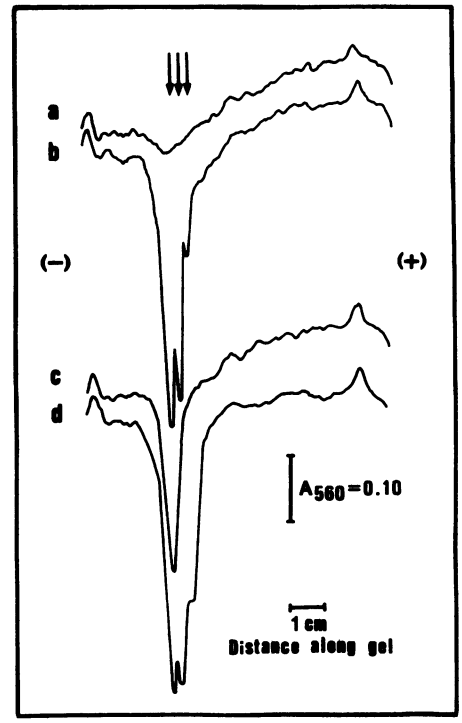

FIG. 4. Effects of hydrogen peroxide on isozymes of anaero-SOD and aero-SOD from *B. gingivalis*. After native PAGE of each sample, gels were stained for SOD activity with Nitro Blue Tetrazolium and then scanned spectrophotometrically. Samples (5 μg) of protein were applied to each gel. Gel a, Anaero-SOD incubated in 50 mM potassium phosphate buffer containing 1 mM NaCN and 2 mM H_2O_2 for 1 h at 25°C before staining for activity; gel b, anaero-SOD; gel c, aero-SOD incubated under the same conditions as gel a; gel d, aero-SOD.

SOD may consist of three isozymes of Fe-SOD and aero-SOD may contain one Mn-SOD and small quantities of two Fe-SODs. The purified enzymes were subjected to IEF PAGE in a pH 3 to 9 gradient (Fig. 5). Both of the native SODs also showed three protein bands with identical pIs (5.25, 5.10, and 5.00). However, each of the denatured enzymes exposed to 5 M guanidinium chloride and 20 mM 8-hydroxyquinoline exhibited a single band with the same isoelectric point (pI, 5.30). Furthermore, each reconstituted SOD derived from each apoprotein by dialysis in either $Fe(NH_4)_2(SO_4)_2$ or $MnCl_2$ was subjected to native PAGE and stained for both the protein and enzyme activity (Fig. 6). Each reconstituted SOD revealed only one band corresponding to the most slowly migrating band of native SOD.

Amino acid composition and amino-terminal sequences. The amino acid compositions of anaero-SOD and aero-SOD were virtually identical (Table 3). For amino acid sequencing, 1 nmol of each enzyme was subjected to an automatic sequencer and the phenylthiohydantoin derivatives from each cycle were analyzed by high-performance liquid chromatography. The amino-terminal sequences of the two SODs were identical and were identified as Met-Thr-His-Glu-Leu-Ile-Ser-Leu-Pro-Tyr-Ala-Val.

Inactivation and inhibition characteristics of reconstituted SOD. The results of the resolution-reconstitution experiment are summarized in Table 4. Native anaero-SOD activity was inhibited by 80% by inclusion of 5 mM NaN_3 in the assay mixture and inactivated by 68% upon incubation at 25°C with 1 mM H_2O_2 for 20 min. Native aero-SOD activity was inhibited by 26% on addition of 5 mM NaN_3 and inactivated by only 30% by incubation in 1 mM H_2O_2 for 20 min. Treatment of both purified enzymes with 5 M guanidinium chloride and 20 mM 8-hydroxyquinoline resulted in complete loss of enzyme activity. For determination of metal specificity, each denatured apoprotein was dialyzed in Tris (pH

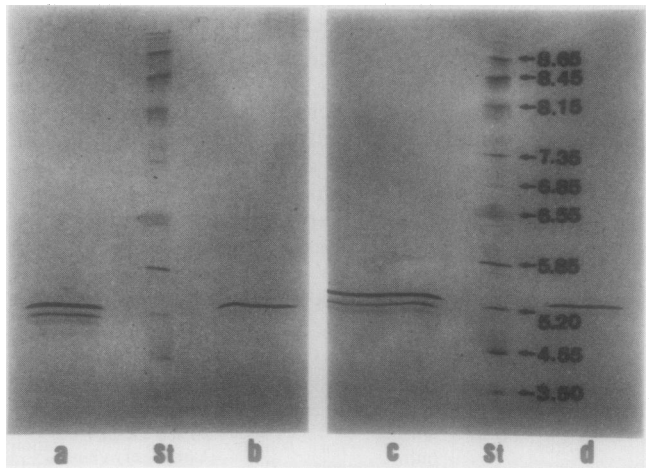

FIG. 5. IEF PAGE of native SOD and apo-SOD from *B. gingivalis* 381. IEF gels with an ampholyte pH gradient of 3 to 9 were stained for protein. Treatment with 20 mM 8-hydroxyquinoline in 5 M guanidinium chloride was used to remove the metal from native anaero-SOD and aero-SOD. Lanes: a, native enzyme of anaero-SOD (2 μg of protein); b, apoenzyme of anaero-SOD (1.7 μg of protein); c, native enzyme of aero-SOD (2 μg of protein); d, apoenzyme of aero-SOD (1.7 μg of protein); St, standard pI marker.

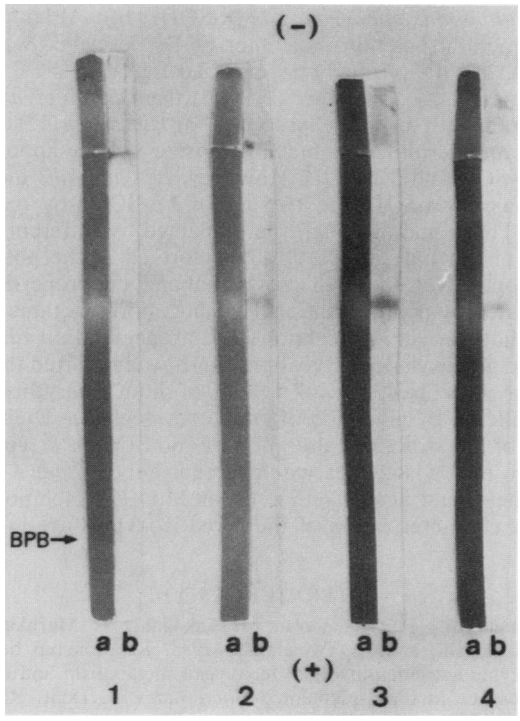

FIG. 6. Native PAGE of reconstituted SODs from *B. gingivalis*. Each apoenzyme of anaero-SOD and aero-SOD was dialyzed in 20 mM Tris buffer (pH 7.0) containing either 1 mM $MnCl_2$ or 1 mM $Fe(NH_4)_2(SO_4)_2$. Lanes: 1, Mn-reconstituted SOD from anaero-SOD (12 μg of protein); 2, Mn-reconstituted SOD from aero-SOD (12 μg of protein); 3, Fe-reconstituted SOD from anaero-SOD (10 μg of protein); 4, Fe-reconstituted SOD from aero-SOD (10 μg of protein); a, staining for SOD activity; b, staining for protein. BPB, Bromphenol blue.

7.0) containing either 1 mM $Fe(NH_4)_2(SO_4)_2$ or 1 mM $MnCl_2$. By reconstitution of anaero-SOD, 69% of the initial activity was recovered on dialysis with Mn^{2+} and 25% was recovered on dialysis with Fe^{2+}. With reconstituted anaero-SOD, Mn^{2+} treatment caused 58% recovery and Fe^{2+} treatment caused 36% recovery. In both apoproteins, the Fe-reconstituted enzyme activities were inhibited by 70 to 80% by 5 mM NaN_3 and inactivated by 60 to 80% by incubation in 1 mM H_2O_2 for 20 min, showing characteristics of Fe-SOD. Both Mn-reconstituted enzyme activities were inhibited by about 15% by 5 mM NaN_3 and inactivated by about 25% by incubation in 1 mM H_2O_2 for 20 min, showing characteristics of Mn-SOD.

TABLE 3. Amino acid compositions of anaero-SOD and aero-SOD from *B. gingivalis* 381

Amino acid	No. of residues/mol of dimer	
	Anaero-SOD	Aero-SOD
Lys	22.0	24.4
His	11.8	11.6
Trp[a]	6.0	6.1
Arg	9.5	10.3
Asp	45.2	43.4
Thr	19.7	20.0
Ser	15.5	15.9
Glu	40.7	40.6
Pro	15.5	15.0
Gly	31.3	32.8
Ala	23.1	25.5
Cys[b]	<0.5	<0.5
Val	17.5	16.0
Met	1.9	2.0
Ile	16.0	15.7
Leu	35.5	34.7
Tyr	14.2	12.7
Phe	21.2	20.4

[a] Obtained by hydrolysis for 24 h in 4 M methanesulfonic acid (29).
[b] Half-cystine was determined as cysteic acid after performic acid oxidation.

DISCUSSION

SOD plays an important role in diminishing antioxidant damage imposed upon cells (16, 25–27). SOD activities in a number of bacteria have been demonstrated to be inducible on exposure to oxygen (12, 16, 19, 24). In this study, exposure of *B. gingivalis* to air resulted in a twofold increase in SOD specific activity. Such inductions increased SOD activity twofold in *B. fragilis* (12, 13) and fourfold in *B. thetaiotaomicron* (24). The amount of protein extracted from aerated cells was also approximately three times that obtained from anaerobically maintained cells. Aeration may cause some damage to organisms, such as cell structural abnormalities, occasional cell breakage, and general damage to the cytoplasm and cell wall (16). Thus, the purification

TABLE 4. Inactivation and inhibition of reconstituted anaero-SOD and aero-SOD from *B. gingivalis* 381

Enzyme or metal salt added (concn)	Initial activity (total U)[a]	Activity recovered (total U)[b]	% Recovery	% Inactivation incubation in H_2O_2 (1 mM) for (min):				% Inhibition by NaN_3 at (mM):			
				1	5	10	20	0.1	0.2	1.0	5.0
Anaero-SOD											
Native SOD				10	24	60	68	35	60	71	80
Apo-SOD	300	<10	<3								
$MnCl_2$ (2 mM)	300	207	69	4	10	20	22	3	4	8	18
$Fe(NH_4)_2(SO_4)_2$ (1 mM)	300	75	25	5	30	55	60	32	52	66	70
Aero-SOD											
Native SOD				5	19	27	30	5	7	18	26
Apo-SOD	400	<10	<3								
$MnCl_2$ (2 mM)	400	230	58	9	15	24	27	3	3	10	12
$Fe(NH_4)_2(SO_4)_2$ (1 mM)	400	144	36	15	33	55	75	30	57	70	82

[a] Total SOD units before dialysis to denaturation.
[b] Total SOD units after reconstitution of activity and dialysis to remove excess metal ions.

procedure of aero-SOD was required to modify that of anaero-SOD.

Both purified samples were determined to be homogeneous by FPLC using Superose-12 gel and sodium dodecyl sulfate-PAGE. The two proteins were apparently dimers of two equal-size subunits. The molecular weight of each of these enzymes was 46,000, which was identical to those of SODs from numerous other microbial sources (12, 13, 19, 24). On spectral analysis, anaero-SOD showed spectra typical of Fe-SOD (30) and aero-SOD had optical spectra generally characteristic of Mn-SOD (9). Metal analysis revealed that 77% of the metal content of anaero-SOD was Fe and that about 61% of that of aero-SOD was Mn. The total metal contents bound to anaero-SOD (2.32 g atom/mol of SOD) and aero-SOD (1.76 g atom/mol of SOD) are consistent with the existence of one metal-binding site per monomer (13, 24). Anaero-SOD was inhibited by 80% by 5 mM NaN_3 and inactivated by 68% by incubation in 1 mM H_2O_2 for 20 min, whereas aero-SOD was inhibited by 26% by 5 mM NaN_3 and inactivated by 30% by incubation in 1 mM H_2O_2 for 20 min. It thus appears that anaero-SOD contains mainly Fe-SOD and aero-SOD contains mainly Mn-SOD.

Electrophoretic and isofocusing analyses performed on the two purified SODs revealed the presence of a major isozymic form and two minor forms in each enzyme. The isozymes migrated identically on gels and focused on the same three isoelectric points. Our preliminary study showed that crude homogenates from anaerobically maintained or aerated cells were also separated into three bands on native PAGE stained for activity. H_2O_2 inactivation proved that anaero-SOD contained three Fe-SODs and that aero-SOD contained one major Mn-SOD and two minor Fe-SODs. The ratios of the activities of the three isozymes were similar in anaero-SOD and aero-SOD. Furthermore, preincubation of cells with puromycin (20 μg/ml) prevented induction of the three isozymes from aerated cells (data not shown). These results suggest that the SOD of *B. gingivalis* induced by oxygen may not be only Mn-SOD but also Fe-SOD. Gregory (12) has reported that SOD isolated from O_2-induced *B. fragilis* contains only Mn-SOD with the same isoelectric point as Fe-SOD isolated from anaerobically grown cells. *E. coli* grown in the presence of oxygen contained three isozymes with different electrophoretic mobilities, including Mn-SOD, Fe-SOD, and a hybrid SOD, and Fe-SOD did not respond to oxygenation (8). Since each isozyme from anaerobically grown or aerated *B. gingivalis* exhibited the same electrophoretic mobility, it is not possible to determine whether Fe-SOD induced in aerated cells is a hybrid SOD.

When metal was removed from each purified SOD by chelators, the preparation of each apo-SOD exhibited only a single protein band on an IEF gel. Both denatured apoproteins accepted Fe or Mn with restoration of SOD activity, and the reconstituted holoenzymes migrated as a single band on native PAGE and IEF PAGE. Thus, it is probable that the three isozymes are formed from a single apoprotein. Moreover, the identical compositions and partial amino acid sequences of the two enzymes agree with the above observation. The first 12 amino-terminal amino acids of anaero-SOD and aero-SOD were identical. The amino acid sequences of anaero-SOD and aero-SOD were homologous to those of other Fe-SODs or Mn-SODs (14, 19, 31). A 5-amino-acid sequence of the first 12 amino-terminal acids was identical at positions 6 (Leu) and 9 to 12 (Leu-Pro-Tyr-Ala) to those of *S. mutans* (cambialistic: Fe/Mn-SOD) (19), *E. coli* (Fe-SOD and Mn-SOD) (31), *Pseudomonas ovalis* (Fe-SOD) (14), *Chromatium vinosum* (Fe-SOD) (14), and *Rhodopseudomonas sphaeroides* (Mn-SOD) (14). Although the amino acid at position 7 of other SODs described was Pro (14, 19, 31), the purified enzymes had Ile.

The results of this study of SODs from *B. gingivalis* are consistent with the model proposed for *B. fragilis* (12) and *B. thetaiotaomicron* (24), which synthesize a single apoprotein for Fe-SOD and Mn-SOD. However, *B. gingivalis* induced one major Mn-SOD and two minor Fe-SODs by oxygenation. These findings might be explained by different regulation of the type of metallo-SOD formed at the posttranscriptional level. It is well known that enzyme properties are modulated by posttranslational modification reactions, such as a phosphorylation reaction (11), an acetylation reaction (6), and glycosylation (glycation) (3). It was reported that the protein structures of CuZn-SOD of human erythrocytes were altered by glycation of amino residues (3). Thus, as a result of the structural alteration of apo-SOD in *B. gingivalis*, two minor isozymes would accept only Fe but a major isozyme would accept either Fe or Mn. The isolation and further characterization of the three isozymes are in progress.

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