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Characterization of the superoxide dismutase of the denitrifying bacterium, *Bacillus halodenitrificans*

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Summary. *Bacillus halodenitrificans* produced a dimeric, manganese-containing superoxide dismutase constitutively when grown either aerobically or as a denitrifier. The molecular mass of the enzyme was determined by sedimentation equilibrium to be 41.4±3 kDa with each subunit estimated at 26 kDa. Plasma emission spectroscopy indicated the presence of 1.22 mol manganese atoms/mol holoenzyme. The electronic absorption spectrum displayed a broad band centered at approximately 474 nm ($\varepsilon = 560\ mM^{-1} \cdot cm^{-1}$) and a shoulder at 595 nm. In the ultraviolet range, the spectrum exhibited split maxima at 278 nm and 283 nm and a shoulder at 291 nm, thus resembling the spectra of superoxide dismutase from *Bacillus subtilis* and *Escherichia coli*. The amino acid composition of the *B. halodenitrificans* enzyme differed slightly quantitatively but little qualitatively from counterpart enzymes from other sources. Like the superoxide dismutases of *Mycobacterium lepraemurium* and human mitochondria, the *B. halodenitrificans* enzyme exhibited several cysteine residues. As expected from the capacity superoxide dismutase exhibits for protecting NO as neutrophil cytotoxicity factor, the *B. halodenitrificans* superoxide dismutase did not interfere with accumulation of NO produced by the organism's nitrite reductase.

Key words: Denitrifier – Mn-superoxide dismutase – Dimeric protein – Cysteine-containing protein – NO protection

Introduction

Superoxide dismutase (EC 1.15.1.1) catalyzes dismutation of superoxide anion. Present in all aerotolerant organisms, it appears to provide a defense against oxygen toxicity (Bannister and Rotilio 1984; Hassan and Moody 1986). However, it occurs in some anaerobes, including *Desulfovibrio gigas* (Hatchikian et al. 1977), *Methanobacterium bryantii* (Kirby et al. 1981), *Chlorobium thiosulfatophilum* (Kanematsu and Asada 1978), *Bacteroides fragilis* (Gregory and Dapper 1983) and *Actinomyces naeslundii* (Gregory and Dapper 1980). The three types of superoxide dismutase isolated to date contain iron (FeSOD), manganese (MnSOD), or copper-zinc (CuZnSOD) as catalytic metal ion. Procaryotes typically contain FeSOD, MnSOD, or a combination of these enzymes. CuZnSOD was thought to be restricted to eucaryotes, but *Photobacterium leiognathi* (Puget and Michelson 1974), *Caulobacter crescentus* (Steinman 1982) and *Paracoccus denitrificans* (Vignais et al. 1980) also produce it. An MnSOD is produced in the mitochondrial matrix of eucaryotes (Ravindranath and Fridovich 1975; Barra et al. 1984).

Why three types of superoxide dismutase are distributed among living organisms, and even two types simultaneously in some (e.g., FeSOD and MnSOD in *Escherichia coli* and MnSOD and CuZnSOD in eucaryotes) is not yet obvious (Parker et al. 1986). *B. fragilis* and *Bacteroides thetaiotaomicron* appear to synthesize only one apoprotein that take up either Fe or Mn (Pennington and Gregory 1986).

In our current studies a purple protein found in *Bacillus halodenitrificans* cells grown under denitrifying conditions was subsequently identified as MnSOD. The present paper describes purification and characterization of this enzyme and presents discussion of its possible role in this denitrifying bacterium.

Materials and methods

Preparation of cell extracts. B. halodenitrificans was grown aerobically or anaerobically to early stationary phase at 37° C. Cells were harvested by centrifugation, suspended at 4° C in 20 mM Tris/HCl pH 7.6 supplemented with 10 mM phenylmethylsulfonyl fluoride (protease inhibitor), and ruptured by two passages through a Manton-Gaulin homogenizer at 633 $kg \cdot cm^2$. A few milligrams of DNase I and II (Sigma) were added, the mixture was treated with neutralized streptomycin sulfate (0.5 $mg \cdot ml^{-1}$), stir-

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red for 20 min at 4° C and centrifuged at 13200 × *g* for 30 min. The supernatant was centrifuged at 144000 × *g* for 90 min at 4° C. The resulting pellet was extracted with 50 mM Tris/HCl pH 7.6 containing 600 mM NaCl at 25° C for 60 min and centrifuged again at 144000 × *g* at 4° C for 60 min. The dark-red pellet was retained for other studies. The supernatant fluids from both centrifugations were dialyzed against 10 mM Tris/HCl pH 7.6 for 24 h with two changes of dialysis buffer and centrifuged at 13200 × *g* for 30 min to remove precipitates formed during dialysis of this soluble fraction of the extracts.

Enzymatic assay. Superoxide dismutase activity was determined using the xanthine oxidase/cytochrome *c* system as described by McCord and Fridovich (1969).

Activity staining. Disc gel electrophoresis was performed according to Jovin et al. (1964) with photopolymerization with riboflavin of both the resolving and concentrating gels. Superoxide dismutase was located by the method of Beauchamp and Fridovich (1971). The gels were first soaked in 2.45 mM (0.2%) nitroblue tetrazolium for 20 min, then in a solution containing 28 mM tetramethylethylenediamine, 2.8 mM riboflavin, and 36 mM potassium phosphate pH 7.8 for 15 min. The gels were then placed in small dry test tubes and illuminated for 5–15 min. Photoreduced riboflavin reduced nitroblue tetrazolium, thus coloring the gels blue except at positions containing superoxide dismutase, which remained clear.

Analytical methods. Protein content was measured by a modification of the method of Bradford (Read and Northcote 1981), using bovine serum albumin as a standard. Metal analysis was carried out by plasma emission spectroscopy using the Jarrel-Ash model 750 atom-comp. Molecular masses were determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (Laemmli 1970) and sedimentation equilibrium with a Beckman model E analytical centrifuge (Schachman 1959). Protein standards for SDS/PAGE were lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase *b* (92.5 kDa). Electronic absorption spectra were recorded with a Shimadzu UV-265 spectrophotometer at room temperature (slit width = 1 nm).

Amino acid composition. Protein hydrolysis was performed at 110° C in 6 M HCl *in vacuo* for 24, 48 and 74 h. Samples for cysteine determination were first treated in performic acid (formic acid/hydrogen peroxide, 1:9) for 3 h at 4° C. Samples to be analyzed for tryptophan were hydrolyzed in the presence of 2% thioglycollic acid. Amino acid residues were determined with a Beckman 119 CL amino acid analyzer.

Materials. DE-52, Sephadex G-75 and DEAE-Bio-Gel A were products of Whatman, Pharmacia and Bio-Rad Laboratories, respectively. Xanthine oxidase and horse heart cytochrome *c* (type III) were purchased from Sigma.

Results

Purification of superoxide dismutase

The soluble fractions obtained from extracts of *B. halodenitrificans* cells were loaded onto a DE-52 column (35 × 6 cm) equilibrated with 10 mM Tris/HCl pH 7.6. All purification procedures were carried out at 4° C. The column was washed with 1 l identical buffer and proteins eluted with a 2-l gradient of 0–300 mM NaCl in 10 mM Tris/HCl pH 7.6. A purple protein band eluted near 100 mM NaCl, ahead (with some overlapping) of a pale yellow fraction. The purple fractions from several runs, also discernable spectroscopically by the presence of a broad shoulder at 470 nm, were pooled and concentrated by ultrafiltration on an Amicon YM 10 membrane. After several exchanges with 50 mM Tris/HCl pH 7.6, the protein was loaded onto a Sephadex G-75 column (1 m × 2 cm) equilibrated with 50 mM Tris/HCl pH 7.6. The chromatography was carried out at a flow rate of 10 ml · h^{-1}. This step removed the yellow protein, which eluted before the purple protein. All the fractions with a broad light-absorption shoulder at 470 nm were pooled, dialyzed against 10 mM Tris/HCl pH 7.6 for 20 h and loaded onto a DEAE-Bio-Gel A column (50 × 3 cm) equilibrated with identical buffer. The column was washed with 300 ml of the buffer, and the proteins were eluted with 1-l gradient of 0–100 mM NaCl. The purple band eluted near 30 mM NaCl. The fractions displaying a distinctive broad band at 470 nm were pooled and found to be pure by native and SDS/PAGE electrophoresis.

Enzymatic properties

Its distinctive color and absorption spectrum suggested that the purple protein was a manganese-containing superoxide dismutase, an assumption confirmed by demonstration that the protein inhibited cytochrome *c* reduction by the superoxide-generating system, xanthine/xanthine oxidase. The specific activity, expressed as the amount of enzyme inhibiting by 50% the reduction of cytochrome *c*, was 5150 units · mg^{-1} protein. This activity is greater than that reported for other MnSODs (around 3500 units · mg^{-1}) (Keele et al. 1970; Britton et al. 1978; Ravindranath and Fridovich 1975), except for the enzyme of *Bacillus subtilis* with a reported specific activity of 4600 units · mg^{-1} (Tsukuda et al. 1983). The activity found in crude extracts was also great, i.e. 97 units · mg^{-1}, compared to 1.9 units · mg^{-1} for *Halobacterium halobium* (Salin and Oesterhelt 1988), 9.8 units · mg^{-1} for *B. fragilis* (Gregory 1985), 25 units · mg^{-1} for *E. coli*, 24.5 units · mg^{-1} for *Alcaligenes faecalis*, 72.6 units · mg^{-1} for *Streptococcus sanguis*, 42 units · mg^{-1} for *Bacillus megaterium*, 50 units · mg^{-1} for *Acholeplasma laidlawii* (Kirby et al. 1980), and 40–140 units · mg^{-1} for various strains of *B. subtilis* (Tsukuda et al. 1983).

Crude extracts prepared from aerobically grown cells of *B. halodenitrificans* displayed activity similar to that of anaerobically grown cells (97.5 units · mg^{-1}). This activity was not inhibited by incubation in 10 mM H_2O_2 for 80 min and was 40% inhibited after addition of 20 mM NaN_3 to the assay mixture, a behavior typical of MnSOD (Misra and Fridovich 1978). It appears that superoxide dismutase occurs constitutively in *B. halodenitrificans*, an observation further confirmed by the presence of a single band of activity on non-denaturing electrophoresis gels prepared with extracts from cells grown either aerobically or anaerobically. The activity bands corresponded identically with the position

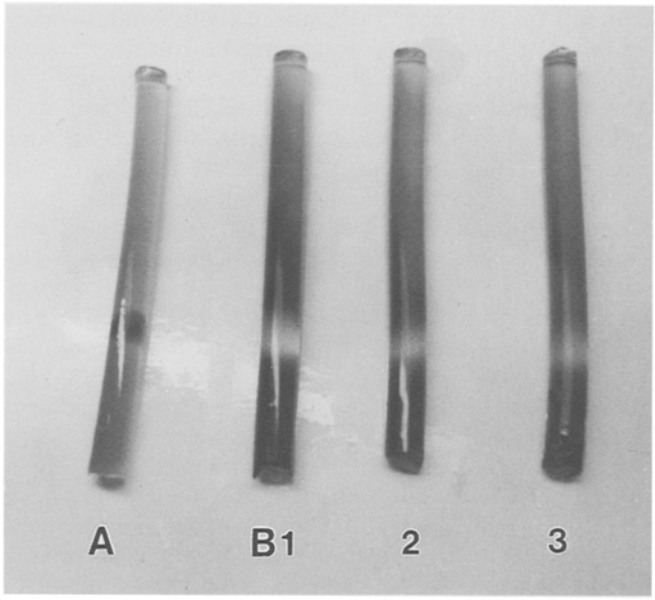

Fig. 1 A, B. Disc gel electrophoresis of superoxide dismutase. **(A)** Protein staining of the pure enzyme. **(B)** Activity staining. Lane 1, pure enzyme; lane 2, crude extract from aerobically grown cells; lane 3, crude extract from anaerobically grown cells

of the purified enzyme subjected to electrophoresis (Fig. 1).

The enzyme was stable for at least one year at −80° C.

Spectroscopic properties

The electronic absorption spectrum of superoxide dismutase (Fig. 2) displayed a broad band centered at approx 474 nm ($\varepsilon = 560$ $mM^{-1} \cdot cm^{-1}$) and a shoulder around 595 nm. The ultraviolet region of the spectrum was complex, with a split maximum at 278 nm and 283 nm and a shoulder at 291 nm, indicating the contribution of tryptophan residues to the spectrum. The spectrum closely resembled that of MnSOD from *B. subtilis* (Tsukuda et al. 1983) and *E. coli* (Keele et al. 1970).

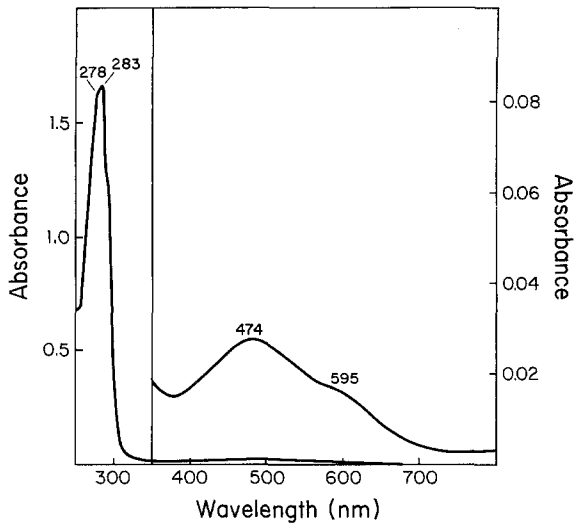

Fig. 2. Electronic absorption spectrum of Mn-containing superoxide dismutase. Protein concentration, 1.1 $mg \cdot ml^{-1}$ in 10 mM Tris/HCl pH 7.6

Physical properties

The molecular mass of the enzyme determined by sedimentation equilibrium was 41.4±3 kDa (mean value computed from several runs), assuming a partial specific volume of 0.7373 $ml \cdot g^{-1}$, SDS/PAGE revealed a single protein band at 26 kDa, consistent with a dimeric structure. The molecular mass of the subunits was unexpectedly high, which may signal an erratic electrophoretic migration of the polypeptide. Other organisms also yielded dimeric MnSODs, with molecular masses of 40–45 kDa (Keele et al. 1970; Britton et al. 1978; Ichihara et al. 1977; Brock and Walker 1980; Barra et al. 1984; Tsukuda et al. 1983).

Analysis of the enzyme by plasma emission spectroscopy indicated the presence of 1.22 mol manganese atom/41.4-kDa molecule. No other metal was detected.

Amino acid composition

Some differences can be seen among superoxide dismutases from various organisms (Table 1), but the patterns appear similar for all of them. The *B. halodenitrificans* enzyme was rich in lysine and aspartic acid/asparagine residues and, like the enzymes from *Mycobacterium lepraemurium* and from human mitochondria, it contained several cysteines. The tryptophan content was lower than that in other superoxide dismutases, an apparent contradiction with the high absorbance at 291 nm of the protein. Poor recovery of tryptophan after hydrolysis may be responsible for this discrepancy.

Discussion

Like most Gram-positive bacteria (Britton et al. 1978), *B. halodenitrificans* synthesized an MnSOD similar to others in its electronic absorption spectrum, molecular mass and amino acid composition; but it proved dissimilar in exhibiting a specific activity greater than those of many other superoxide dismutases. As the enzyme from *B. subtilis* also displays a high activity (Tsukuda et al. 1983), this property may be an attribute common to *Bacillus* species. It was a major protein in *B. halodenitrificans* grown under either anaerobic or denitrifying conditions.

Whereas *B. halodenitrificans* appeared to synthesize the enzyme constitutively, superoxide dismutase is generally induced by aerobic stress in other bacteria. *E. coli* produces an FeSOD at all times, and an additional

Table 1. Amino acid composition of MnSOD from various organisms

Amino acid	*B. halodenitrificans*[a]	*B. stearothermophilus*[a,f]	*E. coli*[b]	*S. faecalis*[c]	*M. lepraemurium*[d]	Human[e,f]
Ala	34	40	47	43	50	34
Gly	30	30	26	31	31	36
Val	22	16	20	23	20	18
Leu	29	38	38	29	42	40
Ile	16	18	14	20	14	22
Ser	13	22	22	15	17	14
Thr	14	22	19	28	21	14
Pro	21	26	15	20	14	20
His	14	18	12	17	19	20
Asx	57	50	42	54	50	44
Glx	42	42	37	42	40	44
Cys	4	0	ND	0	6	4
Met	6	4	3	9	0	4
Arg	7	12	10	8	6	8
Lys	31	24	29	22	23	30
Tyr	16	16	12	17	21	18
Phe	13	16	18	16	18	12
Trp	5	12	ND	12	13	10

ND, no data
[a] Brock and Walker (1980)
[b] Keele et al. (1970)
[c] Britton et al. (1978)
[d] Ichihara et al. (1977)
[e] Barra et al. (1984) for the human mitochondrial enzyme
[f] The amino acid composition derived from the sequence of these proteins. In accord with the dimeric structure, these values are doubles

MnSOD under aerobic conditions (Gregory and Fridovich 1973). *S. sanguis* synthesizes only MnSOD, which increases 50–100-fold under high oxygen concentrations (Diguiseppi and Fridovich 1982). The apoprotein in *B. fragilis,* an anaerobe, can be reconstituted with either Fe or Mn, whereas uptake of Zn yields an inactive protein (Gregory 1985). The Fe form is found in anaerobically grown cells, the manganese form in cells exposed to oxygen, with an increase of 30-fold of the total dismutase activity. This exchange in metal center is attributed to a decreased availability of soluble Fe under aerobic conditions (Gregory 1985). The enzyme from *Propionibacterium shermanii* can also incorporate Mn or Fe, according to availability in the medium (Meier et al. 1982). FeSOD and MnSOD in other organisms cannot be reconstituted with the counterpart metal and often display different electrophoretic migrations, although some sequence similarities exist (Bannister and Rotilio 1984), suggesting that the two forms of the enzymes may be evolutionarily related. *B. halodenitrificans* produced only MnSOD during anaerobiosis where iron was supplied in excess, thus reinforcing the notion of a constitutive occurrence of superoxide dismutase in this bacterium.

Physiological roles for this enzyme remain unsettled. Although the enzyme dismutates superoxide anion, so do Mn chelates; Fee (1981) questions the necessity for a specific enzyme to do the job. *Lactobacillus plantarum* apparently lives aerobically without superoxide dismutase but utilizes high concentrations of Mn to dispose of superoxide radicals (Epp et al. 1986). Mutants of *E. coli* devoid of both FeSOD and MnSOD reportedly survived under aerobic conditions, although growth was weak and proceeded only with provision of all 20 amino acids, especially leucine and valine, in minimal medium (Carlioz and Touati 1986). The presence of a constitutive enzyme in *B. halodenitrificans* raises the question of whether it is synthesized at all times as a means of preventing damage produced by rapid changes in oxygen concentrations, as proposed for strict anaerobes (Bannister and Rotilio 1984), or whether it has other physiological functions, besides elimination of superoxide radicals. The need for a 'backup' amount of superoxide dismutase is understandable for a facultative anaerobe, but the elevated activity found in anaerobically grown cells may be consistent with other roles for this enzyme. Miyake (1986) reported an increase in its activity to a level almost identical to that of aerobically grown cells in anaerobically grown *E. coli* cells placed in the presence of nitrate. This induction was apparently linked to nitrate respiration, as mutants unable to produce nitrate reductase did not show a response to nitrate. Henry and Bessieres (1984) pointed out that the dismutation of NO to NO_2^- and N_2O is thermodynamically favorable, but does not occur for kinetic reasons in the absence of a catalyst. However, we found no reduction of NO to N_2O by superoxide dismutase in a system containing nitrite/nitrite reductase/superoxide dismutase and supplied with ascorbate/phenozine methosulfate. In view of the use of superoxide dismutase to protect NO produced from L-arginine as neutrophil cytotoxicity factor

(Wright et al. 1989), such a finding was expected. Studies with mutants of *B. halodenitrificans* devoid of superoxide dismutase activity might provide some indication of the relevance of this enzyme to denitrification.

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