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Production and Some Properties of Catalase and Superoxide Dismutase from the Anaerobe *Bacteroides distasonis*

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The catalase level of *Bacteroides distasonis* (ATCC 8503, type strain) varied with the amount of hemin supplied to the medium when the cells were grown in either a prereduced medium containing 0.5% peptone, 0.5% yeast extract, and 1% glucose or in a prereduced, defined heme-deficient medium. The effect of hemin on catalase production could not be duplicated by ferrous sulfate or ferrous ammonium citrate. Catalase activity reached peak values in late log phase, whereas superoxide dismutase specific activity remained constant throughout the culture growth cycle. The catalase was a nondialyzable, cyanide- and azide-sensitive, heat-labile protein that coeluted with bovine erythrocyte catalase from Sepharose 6 B. Analysis of polyacrylamide gels stained for catalase activity and for heme showed a correspondence between the single catalytic activity band and one of three heme-protein bands. These data suggest a heme-protein of approximately 250,000 molecular weight. The superoxide dismutase was a cyanide-insensitive protein of approximately 40,000 molecular weight that migrated electrophoretically on acrylamide gels as a single band of activity.

Organisms that utilize oxygen must also possess some molecular mechanism to maintain reduced oxygen metabolites at levels compatible with normal biochemical activity. One reduced oxygen metabolite, the superoxide radical, is disproportionated by superoxide dismutase (5, 16). This reaction gives rise to molecular oxygen and hydrogen peroxide. Hydrogen peroxide may also be generated by oxidation of various other reduced cellular components. Several mechanisms can be utilized to control hydrogen peroxide levels. Thus, catalase (17) may be present to reduce H_2O_2 concentrations, or H_2O_2 may be excreted into the surrounding milieu where it can be reduced by other components. Whereas superoxide dismutase is a common protective mechanism among the aerobes, its presence in anaerobic bacteria has only recently been suggested (11). The presence of catalase in aerobes has been well established, but its presence in anaerobes has not been thoroughly investigated. In this communication, we describe the levels of superoxide dismutase and catalase in the anaerobe *Bacteroides distasonis*, some factors affecting production of those enzymes, and some molecular properties that argue for each enzyme as a specific protein species.

MATERIALS AND METHODS

A 2% inoculum of *B. distasonis* ATCC 8503 was transferred under O_2-free CO_2 from chopped-meat carbohydrate medium (12) into a prereduced medium containing 0.5% peptone, 0.5% yeast extract, and 1% glucose (PYG) (12), supplemented with 7.7 μM hemin and 2.2 μM vitamin K_1, and was incubated at 37°C for 15 h. This procedure was used for all the *B. distasonis* strains listed in Table 1.

Alternatively, ATCC 8503 was grown in Varel-Bryant basal medium (19) plus vitamin B_{12}, modified by the deletion of $CoCl_2 \cdot 6H_2O$ and NaCl and by the substitution of 0.1 M glycerol phosphate buffer and 0.5% $NaHCO_3$ for the sodium carbonate (J. B. Kowalski and J. L. Johnson, unpublished data). The medium was prepared, dispensed, and autoclaved as previously described for prereduced media (12), except that a nitrogen atmosphere was used for both media preparation and inoculation. The final pH after autoclaving was 6.8. Histidine, lysine, leucine, isoleucine, and valine, at a final concentration of 20 μg/ml each, were added aseptically after autoclaving. Heme concentration in this medium was 1.5 μM unless otherwise specified (4). The inoculum for the heme-deficient medium had been cultured from the chopped-meat carbohydrate medium through one passage in the heme-limiting medium. Hemin or iron concentrations in excess of endogenous content were obtained by aseptically adding appropriate amounts of sterile stock hemin (12), ferrous sulfate, or ferrous ammonium citrate to the medium prior to inoculation. Culture growth was monitored by the turbidimetric method of Koch (15), and 50- or 100-ml portions of the cells were removed at various time intervals for catalase and superoxide dismutase assays. Cells were harvested by centrifugation, washed with 25-ml portions of 50 mM potassium phosphate plus 1 mM ethylenediaminetetraacetic

TABLE 1. *Levels of catalase and superoxide dismutase in strains of Bacteroides distasonis*[a]

VPI strain no.	Source	Sp act (U/mg)	
		Catalase	Superoxide dismutase
4243	Type strain, ATCC 8503	96.0	3.2–3.9
J15-49A	Human feces	1.2	0.2
C18-7	Human feces	2.0	0.2
C14-2	Human feces	4.7	0.4
S1-35	Human feces	0.8	0.4
T3-25	Human feces	5.1	1.2
C50-2	Human feces	1.6	0.4
C33-3	Human feces	0.5	0.3
S6A-50	Human feces	2.5	0.1
C19-17	Human feces	5.2	0.2
0052	Feces, conventional mice	1.5	0.5
6815	Lung tissue, Medical College of Virginia, no. 4015443	2.6	0.2

[a] The strains of *B. distasonis* listed were grown to late logarithmic phase in PYG plus 7.7 μM hemin and 2.2 μM vitamin K_1. The cells were harvested, extracts were prepared, and the catalase and superoxide dismutase specific activities were assayed as described in the text. Catalase was assayed on extracts which had never been frozen.

acid (pH 7.8), and sonically disrupted with a Bronson W185D Sonifier at a power setting of 65 W in the microtip. The cell suspension was cooled in an ice-salt bath during sonic treatment. The extract was clarified by centrifugation at 37,000 × *g* for 15 min. Protein content of the crude extract was measured by absorbance at 280 nm, assuming that a 1-mg/ml solution of protein in a 1-cm cuvette would give an absorbance of 1.0. Catalase was assayed spectrophotometrically by the method of Beers and Sizer (2), and the specific activity is reported in international units. Superoxide dismutase was measured by the McCord and Fridovich method (16).

Crude cell extracts containing 500 to 600 U of catalase were chromatographed in 10 mM sodium phosphate, pH 7.0, on a column (1.5 by 98 cm) of Sepharose 6 B at 4°C. Fractions of 1.1 ml were collected for catalase assay. Bovine erythrocyte catalase was chromatographed as a 250,000 molecular weight marker enzyme (18). The molecular weight of superoxide dismutase was determined by chromatography of the extract in 100 mM potassium phosphate plus 1 mM ethylenediaminetetraacetate (pH 7.0) on a column (1.5 by 95 cm) of Sephadex G-150 that had been calibrated with appropriate molecular weight standards. Fractions of 1.3 ml were assayed for superoxide dismutase activity to determine the elution volume.

The effects of sodium cyanide or sodium azide were tested by addition of those reagents to a final concentration of 1 or 5 mM in the assay mixture prior to the addition of the enzyme. Extracts were either frozen and thawed or heated in a boiling-water bath to test the effects of those treatments on the enzymatic activity.

Disc gel electrophoresis was performed in 5 or 7.5% acrylamide gels (3), and catalase activity (9) or heme (10) was visualized by staining the gels. Superoxide dismutase activity was localized on gels by the method of Beauchamp and Fridovich (1).

Hydrogen peroxide (30%), ferrous sulfate, ferrous ammonium citrate, disodium salt of ethylenediaminetetraacetic acid, sodium cyanide, and sodium azide were purchased from J. T. Baker. Cytochrome *c* (type III), xanthine, xanthine oxidase, diaminobenzidine, benzidine, and bovine erythrocyte catalase were products of Sigma Chemical Co. Sodium nitroprusside was obtained from Fisher Chemical Co. Acrylamide and bisacrylamide were Aldrich Chemical's Gold Label products.

All strains of bacteria used were from the VPI Anaerobe Laboratory Culture Collection and were verified as *B. distasonis* by phenotypic and deoxyribonucleic acid homology studies.

RESULTS

Catalase and superoxide dismutase activity in selected strains of *B. distasonis*. The specific activities of catalase and superoxide dismutase for 12 strains of *B. distasonis* grown to late log phase in PYG medium supplemented with hemin (7.7 μM) and vitamin K_1 (2.2 μM) are shown in Table 1. Except for the type strain, ATCC 8503, most strains tested produced relatively small amounts of catalase (0.5 to 5.2 U/mg). Strain ATCC 8503 produced catalase at a specific activity of 96 U/mg.

Superoxide dismutase activity was detected in all of the strains tested. The highest levels of catalase and superoxide dismutase were detected in ATCC 8503. Since this strain produced higher levels of those enzymes than any other anaerobe we have thus far tested, factors affecting the levels of those enzymes were tested.

Effect of growth medium components on enzyme levels in ATCC 8503. The highest level of catalase was measured when PYG (12) medium was supplemented with hemin and vitamin K_1. Table 2 outlines the effects of elevation or diminution of hemin levels in PYG medium. When that medium was supplemented with hemin at 7.7 μM, but without vitamin K_1, the catalase specific activity was 60% of the level observed when both components were supplied to the growth medium. However, if hemin was omitted, the cells produced no more catalase with vitamin K_1 present than in its absence. Vast growth rate differentials could not explain this phenomenon, since the cell densities at harvest were approximately the same. It appears that vitamin K_1 did not directly affect catalase synthesis but did function synergistically with hemin in catalase production. When

TABLE 2. *Effect of vitamin K_1 and hemin on enzyme levels in ATCC 8503*[a]

Growth medium	Sp act (U/mg)	
	Catalase	Superoxide dismutase
PYG	19.7	2.8
Plus 2.2 μM vitamin K_1	20.7	2.8
Plus 7.7 μM hemin	66.7	3.2
PYG + 2.2 μM vitamin K_1		
Plus 7.7 μM hemin	96.0	3.9
Plus 37.5 μM hemin	187	2.8
Plus 77 μM hemin	214	1.0[b]

[a] *B. distasonis* ATCC 8503 was grown in PYG medium alone or supplemented as shown. Cell harvest, crude extract preparation, and enzyme assays were performed as described in the text. Sterile stock hemin at the desired concentration was added aseptically to the sterilized medium.

[b] The number of cells per milliliter of culture grown with 77 μM hemin was only 40% the number of cells per milliliter of culture in the presence of 7.7 μM hemin.

the cells were grown in PYG plus 2.2 μM vitamin K_1 supplemented with 37.5 μM hemin rather than 7.7 μM, the catalase specific activity doubled (Table 2). Upon increasing the hemin concentration to 77 μM, there was only a slight increase in catalase level over the test level observed in the presence of 37.5 μM hemin. This indicated that either saturation of the medium with hemin or saturation of the cells' ability to utilize hemin had been reached. The substitution of ferrous ions, either as ferrous sulfate or ferrous ammonium citrate, at concentrations 10- to 100-fold greater than hemin would not elicit the catalase response that hemin did.

The superoxide dismutase levels were independent of hemin or vitamin K_1 additions to PYG medium, except that levels were markedly lower in cells grown in PYG plus 77 μM hemin. Total extractable protein and cell density were also lower in those cultures than in cultures grown under the other conditions tested, so that some general inhibition of growth could account for that observation.

Since the peptone and yeast extract might contain some endogenous heme or heme-containing proteins, *B. distasonis* ATCC 8503 was grown to late log phase in defined heme-deficient medium with and without added hemin. The catalase specific activity again responded to the hemin concentration as shown in Table 3, although the absolute level of catalase (44 U/mg) was lower in cells grown in this medium than in cells grown in PYG. The heme saturation phenomenon was again observed, and cell growth was partially inhibited when 15 μM hemin was added to the defined medium as compared with growth in the defined medium plus 0.15 μM hemin. In the defined medium with no hemin supplementation, the cell growth was diminished by 60% when compared with growth in defined medium plus 0.15 μM hemin, and there was no measurable catalase activity. The inoculum for this culture was a 1% inoculum directly from the chopped-meat carbohydrate, and some hemin may have been transferred to the defined medium in this case.

Superoxide dismutase levels were independent of hemin concentrations in the defined medium, but the specific activity was only 20% of that measured in cells grown in the PYG medium.

Catalase specific activity increased during the logarithmic growth phase and reached maximal values during the late logarithmic growth phase. This was true whether the cells were grown in PYG or defined medium. Catalase activity levels remained constant, even after 36 h in the stationary growth phase. Superoxide dismutase specific activity remained constant throughout the growth cycle into stationary phase in either of the two media tested.

Enzyme characteristics. The crude extract from *B. distasonis* ATCC 8503 was subjected to electrophoresis in 5% acrylamide gels; then companion gels were stained for catalase activity and for the presence of heme. One of three

TABLE 3. *Effect of hemin concentration on catalase and superoxide dismutase levels in a defined medium*[a]

Growth conditions	Sp act (U/mg)	
	Catalase	Superoxide dismutase
Defined medium	0	—[b]
Plus 1.5×10^{-2} μM hemin	1.0	0.6
Plus 1.5×10^{-1} μM hemin	25	0.6
Plus 1.5 μM hemin	44	0.8
Plus 15 μM hemin	Inhibition of cell growth	

[a] *B. distasonis* ATCC 8503 was grown to late log phase in the defined medium described in the text. Hemin was added prior to sterilization of defined medium. Culture inocula, harvesting, and enzyme assays were as described in the text.

[b] The inoculum for this experiment was chopped-meat carbohydrate (11), and some hemin was carried over with the inoculum. Hemin was growth limiting in this instance. Superoxide dismutase activity was not assessed.

visible heme bands (R_f = 0.53 to 0.54) corresponded to the enzymatic activity band (R_f = 0.53). There was only a single activity band visible after staining for catalase activity. The catalase was sensitive to sodium azide or sodium cyanide when these reagents were added to the assay mixture during spectrophotometric assay of this enzyme. A final concentration of 1 mM sodium azide or sodium cyanide completely inhibited the enzymatic activity in crude extracts. That the catalase activity was labile to freezing and thawing was demonstrated by a 50% loss of the catalytic activity after freezing for 8 h and subsequent thawing at room temperature. However, if the whole cells were frozen prior to sonic treatment, there was negligible loss of catalase activity, even after 4 days of frozen storage. Crude extracts could be frozen quickly at dry-ice temperatures and lyophilized without loss of activity upon subsequent rehydration with water.

When the crude *B. distasonis* catalase was chromatographed on a Sepharose 6 B column, approximately 90% of the total activity was recovered in a single band of catalase activity and protein. This activity was eluted by a volume of buffer identical to the elution volume for bovine erythrocyte catalase, indicating a molecular weight of approximately 250,000.

The superoxide dismutase also exhibited a single band of activity after electrophoretic separation of the crude extract on 7.5% acrylamide gels and subsequent staining for superoxide dismutase activity. This enzymatic activity was insensitive to 5 mM cyanide and was not labile to repeated freezing and thawing of the cell extract. All of the superoxide dismutase activity eluted from a Sephadex G-150 column in a single peak, and the estimated molecular weight was 40,000.

DISCUSSION

B. distasonis ATCC 8503 produced intracellular levels of catalase and superoxide dismutase that surpassed the levels of those enzymes measured in any other anaerobe thus far tested and rivaled the enzyme specific activities of crude extracts of the aerotolerant species *Escherichia coli* (7) and *Streptococcus faecalis* (8). The catalase specific activity responded to the heme levels in the growth medium under the growth conditions tested. This response might be expected since, with very few exceptions (13), catalases contain heme as the active center component (17). Our data show correspondence between the catalase activity and a heme-containing protein when those entities were specifically stained on companion acrylamide gels. These data suggest, but do not prove, that the catalase of *B. distasonis* is a heme protein. With respect to cyanide and azide sensitivity, molecular weight, and lability to freezing, the *B. distasonis* catalase had properties quite similar to bovine erythrocyte catalase. The facts that only a single catalase activity band was observed after electrophoretic separation on acrylamide gels and that virtually all of the catalase activity eluted as a single band from Sepharose 6 B suggested that a single, high-molecular-weight protein was responsible for the catalase activity. Thus, the catalase did not appear to be an artifact generated by spurious heme binding to polydisperse proteins in the cell.

We observed that although vitamin K_1 had no effect on growth or catalase specific activity, K_1 was synergistic with hemin in elevating the catalase specific activity. The physiological mode of action of vitamin K_1 is not known, even though it enhances the growth rate of certain anaerobic organisms and is required by others (6). *B. distasonis* ATCC 8503 did not appear to require vitamin K_1 in excess of the quantities that may be present in PYG medium, and it grew quite well in the defined medium without vitamin K_1. The culture in defined minimal medium reached late log phase after approximately 40 h of incubation compared with growth in PYG (12), where late log phase occurred after 17 h of incubation. Trace concentrations of hemin were required for growth of the organism, since no growth was observed in heme-deficient defined minimal medium when the inoculum was also heme deficient.

Ferrous ion was not able to substitute for hemin in ability to induce catalase synthesis. Even at ferrous ion levels 10- to 100-fold that of hemin levels, no elevation of the catalase specific activity could be measured. The lack of ability to utilize ferrous ion may be due to defects in iron transport or defects in protoporphyrin synthesis. It is not known which specific defect is true for the *B. distasonis* strain, but several *Bacteroides* do require preformed hemin as a growth factor (20).

The superoxide dismutase specific activity levels were independent of vitamin K_1 and hemin concentrations, but they were influenced by the growth medium. This difference is readily seen by comparing Table 2 with Table 3. The superoxide dismutase activity was not inhibited by sodium cyanide or by repeated freeze-thaw treatment. All of the procaryotes thus far examined possess either a manganisuperoxide dismutase (14), a ferrisuperoxide dismutase (21), or both enzyme forms. These cya-

nide-insensitive forms are distinguished from the cupro zinc eucaryotic form of the enzyme that is cyanide sensitive (5). The superoxide dismutase from *B. distasonis* does not appear to be an exception. The single activity band of superoxide dismutase exhibited after electrophoresis on acrylamide gels, and the elution of all the enzymatic activity from Sephadex G-150, again argue for a single specific enzyme.

The presence of superoxide dismutase and catalase in some anaerobes may provide a modicum of aerotolerance and may explain the diverse range of sensitivities of the anaerobes to oxygen. This hypothesis is now being tested in our laboratory.

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LITERATURE CITED

1. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44:276–287.
2. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195:133–140.
3. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404–427.
4. **Falk, J. E.** 1964. Porphyrins and metalloporphyrins, p. 181. Elsevier Publishing Co., New York.
5. **Fridovich, I.** 1975. Superoxide dismutase. Annu. Rev. Biochem. 44:147–159.
6. **Gibbons, R. J., and J. B. McDonald.** 1960. Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J. Bacteriol. 80:164–170.
7. **Gregory, E. M., and I. Fridovich.** 1973. Oxygen toxicity and the superoxide dismutase. J. Bacteriol. 114:1193–1197.
8. **Gregory, E. M., and I. Fridovich.** 1973. Induction of superoxide dismutase by molecular oxygen. J. Bacteriol. 114:543–548.
9. **Gregory, E. M., and I. Fridovich.** 1974. Visualization of catalase on acrylamide gels. Anal. Biochem. 58:57–62.
10. **Haut, A., G. R. Tudhope, E. G. Cartwright, and M. M. Wintrobe.** 1962. The nonhemoglobin erythrocyte proteins studied by electrophoresis on starch gel. J. Clin. Invest. 41:579–587.
11. **Hewett, J., and J. G. Morrison.** 1975. Superoxide dismutase in some obligately anaerobic bacteria. FEBS Lett. 50:315–317.
12. **Holdeman, L. V., and W. E. C. Moore (ed.).** 1975. Anaerobe laboratory manual, 3rd ed. Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va.
13. **Johnston, M. A., and E. A. Delwiche.** 1965. Isolation and characterization of the cyanide-resistant and azide-resistant catalase of *Lactobacillus plantarum*. J. Bacteriol. 90:352–356.
14. **Keele, B. B., Jr., J. M. McCord, and I. Fridovich.** 1970. Superoxide dismutase from *Escherichia coli B*. A new manganese-containing enzyme. J. Biol. Chem. 245:6176–6181.
15. **Koch, A. L.** 1970. Turbidity measurements of bacterial cultures in some available commercial instruments. Anal. Biochem. 38:252–259.
16. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase: an enzymatic function for erythrocuprein. J. Biol. Chem. 244:6049–6055.
17. **Schonbaum, G. R., and B. Chance.** 1976. Catalase, p. 363–408. *In* P. Boyer (ed.), The enzymes, vol. 13. Academic Press, Inc., New York.
18. **Tanford, C., and R. E. Lovrein.** 1962. Dissociation of catalase into subunits. J. Am. Chem. Soc. 84:1892–1895.
19. **Varel, V. H., and M. P. Bryant.** 1974. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. Appl. Microbiol. 28:251–257.
20. **Wilkins, T. D., S. L. Chalgren, F. Jimenez-Ulate, C. R. Drake, Jr., and J. L. Johnson.** 1976. Inhibition of *Bacteroides fragilis* on blood agar plates and reversal of inhibition by added hemin. J. Clin. Microbiol. 3:359–363.
21. **Yost, F. J., Jr., and I. Fridovich.** 1973. An iron-containing superoxide dismutase from *Escherichia coli*. J. Biol. Chem. 248:4905–4908.