J. Gen. Appl. Microbiol., **25**, 367–374 (1979)

SUPEROXIDE DISMUTASE ACTIVITY IN *VIBRIO EL TOR* IN RELATION TO OXYGEN TOXICITY AND BACTERICIDAL ACTION OF NITROFURANTOIN

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(Received July 17, 1979)

Superoxide dismutase has been characterized in normal and also in nitrofurantoin-resistant mutant strain of *Vibrio el tor*. An induction of this enzyme, which is noted in *Vibrio el tor* when grown under hyperbaric oxygen, is inhibited by nitrofurantoin. This induction of superoxide dismutase becomes insensitive to nitrofurantoin in *Vibrio el tor* after it acquires resistance towards the antibacterial agent. The lethal effect of superoxide radicals on *Vibrio el tor* is shown by both photochemically and enzymically generated superoxide radicals. Nitrofurantoin aggravated the lethal effect of superoxide radicals in normal as well as in nitrofurantoin-resistant mutant of the microorganism. Bovine superoxide dismutase preparation exhibited a protective effect on the enhanced lethality of superoxide radicals towards *Vibrio el tor* strains in the presence of nitrofurantoin. Superoxide dismutase could also inhibit the photochemical and enzymic activation of nitrofurantoin.

Superoxide radicals are common intermediates in the biological reduction of oxygen, and the enzyme superoxide dismutase constitutes the primary defence mechanism against the toxicity of superoxide radicals by catalytically dismutating the free radicals (*1–3*). Hyperbaric oxygen causes an induction of the level of superoxide dismutase in microorganisms like *Streptococcus faecalis* (*4*), *E. coli* (*5*), and *Saccharomyces cerevisiae* (*6*), and these cells are much more resistant to hyperbaric oxygen compared to the uninduced cells. In the case of *B. subtilis,* however, exposure to hyperbaric oxygen does not result in any change in the superoxide dismutase level and the organism remains equally sensitive to hyperbaric oxygen (*5*). Hassan and Fridovich (*7*) reported that bovine superoxide dismutase can counteract the lethal effect of streptonigrin which has the ability to produce superoxide radicals by its alternative reduction and reoxidation. Xanthine oxidase/xanthine system, a potent source of superoxide radicals (*3*), can reduce

nitrofurantoin for its subsequent lethal effect on bacteria (*8*). Thus it will be interesting to study the involvement of superoxide radicals in nitrofurantoin reduction which is the prerequisite for its lethal action and also in nitrofurantoin resistance in *Vibrio el tor*. The extent of induction of superoxide dismutase by hyperbaric O_2 and the inhibition pattern of this induction by nitrofurantoin and also by chloramphenicol, a protein synthesis inhibitor, along with the effect of superoxide dismutase on the enhanced bactericidal effect of superoxide radicals in the presence of nitrofurantoin in normal as well as in nitrofurantoin-resistant mutants of *Vibrio el tor* and also on the reduction of nitrofurantoin have been studied.

MATERIALS AND METHODS

Organism. *Vibrio el tor* Visak 20/69, a non-hemolytic *el tor* strain, obtained from Cholera Research Centre, Indian Council of Medical Research, Calcutta, was used. The nitrofurantoin resistance in *Vibrio el tor* was developed by serial passage of the normal strain through increasing concentrations of the antibacterial agent and the strain resistant to 50 μg/ml of nitrofurantoin was selected for the experiments. The nitrofurantoin-resistant *Vibrio el tor* is referred as *Vibrio* NFT.

Chemicals and enzymes. Xanthine, xanthine oxidase, ferricytochrome *c*, nitrofurantoin and nitrobluetetrazolium were all purchased from Sigma Chemical Co., U.S.A. Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine were obtained from E. Merck Co., West Germany. Bovine superoxide dismutase was kindly donated by Dr. S. P. MUKHERJEE of the Department of Biochemistry, Duke University Medical Centre, Durham, N.C., U.S.A. All the other reagents used were of analytical grade.

Medium and growth conditions. In order to study the lethality of *Vibrio el tor* towards superoxide radicals, a medium containing Na_2HPO_4, 10 g; KCl, 5 g; NH_4Cl, 2 g; $MgSO_4$, 0.01 g; amino acid mixture (containing amino acids in equal proportions) 1 g; and glucose, 2 g per liter, pH 8.0 was used for the growth of the bacteria. The salts used in this medium were of ultrapure grade. In all the other experiments, peptone-water containing 1.0% peptone and 0.5% NaCl was used for the growth of the organism. The pH of the media was adjusted to 8.0 with 1 M NaOH and the organisms were grown at 37°. Deep still cultures were prepared by inoculating the peptone media filled up to 10 cm of 3×15 cm tubes with 2% inoculum. These were then incubated at 37° without shaking.

Cells were harvested in the late log phase by centrifugation and cell-free extracts were prepared by sonic disruption of the cells suspended in 50 mM potassium phosphate and 1 mM EDTA, pH 7.8, at 4° in a Raytheon Sonic Oscillator. The cell debris was removed by centrifugation at $37,000 \times g$ for 15 min. The supernatants were used as the enzyme source. The cell-free extract was centrifuged at $105,000 \times g$ for 90 min in an ultracentrifuge and the supernatant was used for

application on polyacrylamide gel.

Assay of superoxide dismutase. Superoxide dismutase activity was assayed according to the method of McCord and Fridovich (*9*). The reaction mixture contained 1×10^{-5} M ferricytochrome *c*, 5×10^{-5} M xanthine, 6×10^{-9} M xanthine oxidase, and 0.05 M potassium phosphate (pH 7.8) in 3 ml. The reaction mixture was equilibrated with air and the reduction of ferricytochrome *c* was determined by following the decrease in OD at 550 nm. Under these conditions the amount of superoxide dismutase required to inhibit the rate of reduction of ferricytochrome *c* by 50% is defined as 1 unit of activity. This reaction mixture was also employed in the study for the determination of the inhibition of ferricytochrome *c* reduction with different amounts of cell-free extracts. Proteins were measured by the biuret method (*12*).

Localization of superoxide dismutase on gel. 7.5% polyacrylamide gels were prepared according to the method of Davis (*10*) and for staining the gel the method of Beauchamp and Fridovich (*11*) was followed. After electrophoresis, gels were first soaked in 2.45×10^{-3} M nitrobluetetrazolium for 20 min and then in a solution containing 0.028 M tetramethylethylenediamine, 2.8×10^{-5} M riboflavin, and 0.036 M potassium phosphate, pH 7.8, for 15 min, after which the gels were illuminated till maximum contrast of the bands were obtained.

Determination of the lethality of photochemically generated superoxide radicals. The cells (100 Klett reading with green filter) (10 ml) grown in the chemically defined medium were incubated in the presence of 2% glucose, 5×10^{-4} M riboflavin, 0.015 M methionine, and 0.05 M potassium phosphate, pH 7.8, in the light at 37°, in an aluminium foil-lined box fitted with a 20-W fluorescent lamp. At definite time intervals, samples were withdrawn, diluted, and plated on nutrient agar and incubated overnight at 37° in the dark. The number of colonies was counted to determine the number of viable cells.

Determination of the lethality of enzymically generated superoxide radicals. Ten milliliters of the cells (100 Klett reading with green filter) grown in the chemically defined medium was incubated in the presence of 6×10^{-5} M EDTA, 5×10^{-4} M xanthine, 3.67×10^{-8} M xanthine oxidase, 1.25% glucose, and 0.002 M potassium phosphate, pH 9.0. At definite time intervals samples were withdrawn, diluted, and plated on nutrient agar and incubated overnight at 37° in the dark. The number of colonies was counted to determine the number of viable cells.

Photochemical reduction of nitrofurantoin. 3 ml of incubation system containing 5×10^{-5} M riboflavin, 1.5×10^{-3} M methionine, 10 μg/ml of nitrofurantoin, and 0.05 M potassium phosphate, pH 7.8, was equilibrated with air and incubated at 37° in fluorescent light, as mentioned earlier.

Enzymic reduction of nitrofurantoin. Air equilibrated reaction mixture (3 ml) containing 1×10^{-4} M xanthine, 6×10^{-8} M xanthine oxidase, 10 μg/ml of nitrofurantoin, and 0.05 M potassium phosphate, pH 7.8, was incubated at 37°. Nitrofurantoin reduction in each case was measured by determining the decrease in OD

at 365 nm in a Beckman DU–2 spectrophotometer.

RESULTS

The inhibition pattern of xanthine oxidase/xanthine-mediated and oxygen-dependent reduction of ferricytochrome *c* by the cell-free extracts of *Vibrio el tor* and *Vibrio* NFT indicates the presence of superoxide dismutase in these extracts as shown in Fig. 1. The specific activities of superoxide dismutase in *Vibrio el tor* and *Vibrio* NFT cell-free extracts were found to be 10.4 and 8.4 units/mg protein, respectively. Densitometric scans of polyacrylamide gels indicated the presence of only one type of enzyme in both strains. The enzyme activity appears as an achromatic zone against a dark blue background (Fig. 2). Cell-free extracts of the superoxide dismutase-induced cells gave the same type of scans with a single peak at the same position.

Upon exposure to hyperbaric oxygen (20 atm) for 3 hr the levels of superoxide dismutase in deep still cultures of *Vibrio el tor* and *Vibrio* NFT were, 9.5 to 14.7 units/mg protein and from 8.8 to 13.1 units/mg protein, respectively. Under the same condition, nitrofurantoin at a concentration of 10 μg/ml totally inhibited the induction in *Vibrio el tor* whereas in *Vibrio* NFT the induction was not affected at

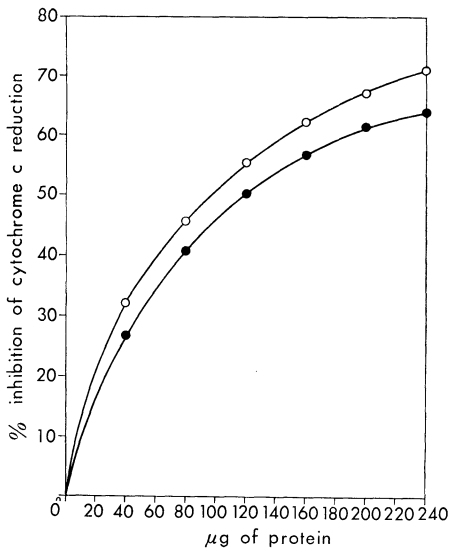

Fig. 1.

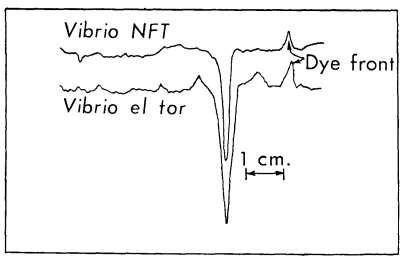

Fig. 2.

Fig. 1. Inhibition pattern of oxygen-dependent and xanthine oxidase-mediated reduction of ferricytochrome *c* by cell-free extracts of *Vibrio el tor* and *Vibrio* NFT. ○, *Vibrio el tor*; ●, *Vibrio* NFT.

Fig. 2. Densitometric scans of polyacrylamide gels at 560 nm stained for superoxide dismutase activity.

all by nitrofurantoin even at a concentration of 25 μg/ml in the medium. The specific activities of superoxide dismutase were found to be 8.6 and 13.0 units/mg protein, respectively, in *Vibrio el tor* and *Vibrio* NFT when the cells were grown under hyperbaric oxygen (20 atm) for 3 hr in the presence of nitrofurantoin at the concentrations mentioned above. However, chloramphenicol, a well-known inhibitor of protein synthesis, could inhibit this induction process in both strains. The specific activities of superoxide dismutase were found to be 9.2 and 8.2 units/mg protein in *Vibrio el tor* and *Vibrio* NFT, respectively, in the cell-free extracts of the cells grown under 20 atm oxygen for 3 hr, in the presence of 0.5 mg/ml of chloramphenicol.

Figures 3 and 4 demonstrate that the superoxide radicals, whether produced photochemically or enzymically, are lethal to *Vibrio el tor* and *Vibrio* NFT, and the lethality of microorganisms toward the free radical is aggravated by nitrofurantoin in these strains. This enhancement of the deleterious effect of superoxide radicals by nitrofurantoin is found to be prevented by superoxide radicals in both strains.

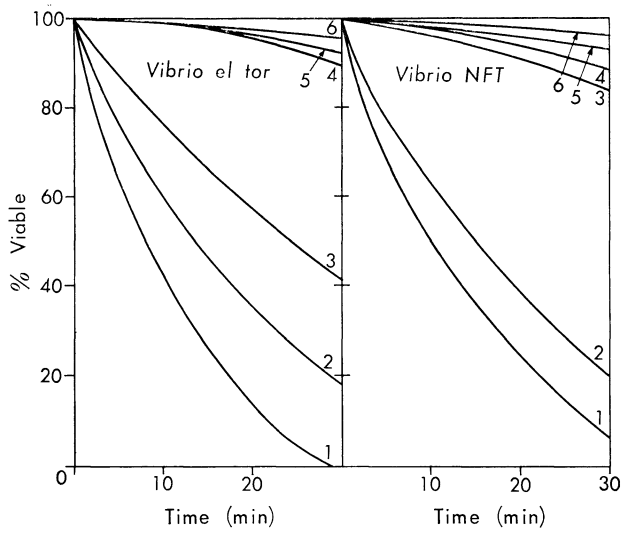

Fig. 3. Effect of photochemically generated superoxide radicals on *Vibrio el tor* and *Vibrio* NFT.

Cells were grown in chemically defined medium as described under MATERIALS AND METHODS unless otherwise mentioned.

1, cells incubated in the light with 10 μg/ml and 20 μg/ml of nitrofurantoin in the case of *Vibrio el tor* and *Vibrio* NFT, respectively; 2, cells incubated in light; 3, cells incubated in light with 20 μg/ml of bovine superoxide dismutase and 10 μg/ml and 20 μg/ml of nitrofurantoin in the case of *Vibrio el tor* and *Vibrio* NFT, respectively; 4, cells incubated in light with 20 μg/ml of bovine superoxide dismutase; 5, cells grown in peptone-saline medium under hyperbaric oxygen, incubated in light; 6, cells incubated in the dark.

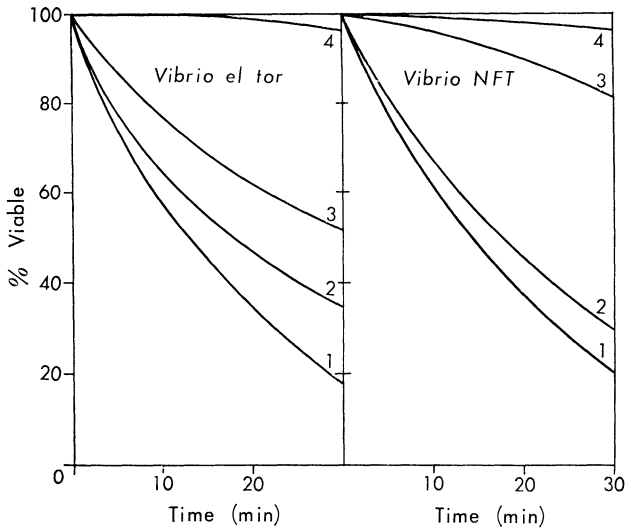

Fig. 4. Effect of enzymically generated superoxide radicals on *Vibrio el tor* and *Vibrio* NFT.

Cells were grown in the chemically defined medium as described under MATERIALS AND METHODS. 1, cells in presence of 10 μg/ml and 20 μg/ml of nitrofurantoin in the case of *Vibrio el tor* and *Vibrio* NFT, respectively; 2, cells without any effector; 3, cells in presence of nitrofurantoin (concentrations as in 2) and 20 μg/ml of bovine superoxide dismutase; 4, cells in presence of 20 μg/ml of bovine superoxide dismutase.

Table 1. Photochemical reduction of nitrofurantoin.

No.	Time of incubation (min)	Amount of superoxide dismutase (μg/ml)	Decrease in OD at 365 nm	Inhibition of nitrofurantoin reduction (%)
1	15	—	0.18	—
2	15	20	0.09	50

Table 2. Enzymic reduction of nitrofurantoin.

No.	Time of incubation (min)	Amount of superoxide dismutase (μg/ml)	Decrease in OD at 365 nm	Inhibition of nitrofurantoin reduction (%)
1	15	—	0.29	—
2	15	20	0.14	50

Photochemical and enzymic reduction of nitrofurantoin and its inhibition by superoxide dismutase are presented in Tables I and II.

The cells used in the study on toxicity of superoxide radicals in the presence or absence of superoxide dismutase were grown in a chemically defined medium. As compared to the peptone-water grown cells, cell-free extracts of which ex-

hibited superoxide dismutase activities of 10.4 and 8.4 units/mg protein in *Vibrio el tor* and *Vibrio* NFT, respectively, the cell-free extracts of the synthetic medium-grown cells have very low levels of this enzyme (0.9 and 0.8 unit/mg protein in *Vibrio el tor* and *Vibrio* NFT, respectively) due to the absence of the metal ions used as co-factor of this enzyme.

DISCUSSION

The importance of superoxide dismutase as a protecting agent against the toxicity of oxygen in oxygen metabolising cells has already been demonstrated (*3*). However, the enzyme has not yet been studied in *Vibrio el tor* strains. The ability of the cell-free extracts of *Vibrio el tor* and *Vibrio* NFT to inhibit the oxygen-dependent reduction of ferricytochrome *c* by xanthine oxidase/xanthine system indicates the presence of superoxide dismutase in these two strains. The specific activity of superoxide dismutase is decreased to a certain extent after acquisition of resistance towards nitrofurantoin. The presence of superoxide dismutase is further confirmed by the densitometric scanning of the polyacrylamide gels (Fig. 2) which shows the presence of only one type of superoxide dismutase in *Vibrio el tor* and *Vibrio* NFT. *E. coli* K12 also possesses one type of superoxide dismutase when grown anaerobically but exposure to oxygen gives rise to two new isozymes of superoxide dismutase (*7*). *Vibrio el tor* and *Vibrio* NFT, however, do not give rise to any new isozyme of superoxide dismutase on exposure to hyperbaric oxygen, as mentioned earlier.

Hyperbaric oxygen could augment the level of superoxide dismutase in *Vibrio el tor* and *Vibrio* NFT to the extent of 5.2 and 4.3 units/mg protein, respectively, over the basal level. The induction was completely blocked by chloramphenicol, a potent inhibitor of protein synthesis, which indicates that new protein synthesis is necessary for the induction process. In *Vibrio el tor*, however, the induction was also inhibited by nitrofurantoin, whereas *Vibrio* NFT is completely insensitive to nitrofurantoin. A similar type of inhibition of tryptophanase induction in *Vibrio el tor* and *Vibrio* NFT by nitrofurantoin and chloramphenicol was observed by DASTIDAR *et al.* (*13*).

The induction of superoxide dismutase by hyperbaric oxygen and the increased resistance of these induced cells towards photochemically generated superoxide radicals further confirm the involvement of superoxide dismutase towards oxygen toxicity in these strains. Superoxide radicals are extremely lethal to both *Vibrio el tor* and *Vibrio* NFT grown in minimal media because of very low levels of superoxide dismutase in these cells. The reason for this low level of the enzyme is that the chemically defined medium does not contain any metal ion to be used as a co-factor of superoxide dismutase. The viability of *Vibrio el tor* and *Vibrio* NFT is further decreased if nitrofurantoin is present along with superoxide radical-generating system. The enhancement of the detrimental effect of nitrofurantoin

in the presence of superoxide radical-generating systems on *Vibrio* NFT, which is resistant to nitrofurantoin due to its inability to reduce nitrofurantoin, indicates that nitrofurantoin is somehow reduced during the generation of superoxide radicals. Xanthine oxidase/xanthine system, another potent source of superoxide radicals, was reported to be able to carry out the reduction of nitrofurantoin and thereby activate the drug (*8*). Photochemical reduction of nitrofurantoin has not so far been reported. The inhibition of enhanced lethality of superoxide radicals on *Vibrio* NFT in the presence of nitrofurantoin and of the photochemical and enzymic reduction of nitrofurantoin by bovine superoxide dismutase confirmed the involvement of superoxide radical in nitrofurantoin reduction. Thus *Vibrio* NFT, when grown in the presence of nitrofurantoin, a portion of superoxide radicals produced inside the cells are consumed continuously by nitrofurantoin itself and a lower level of superoxide radicals is thereby maintained in these cells in comparison to the normal cells, *Vibrio el tor*. This lower level of superoxide radicals was actually reflected on the lower level and the lower extent of induction of superoxide dismutase in *Vibrio* NFT.

The authors thank the Indian Council of Medical Research, for financial support.

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