BIOLOGICAL PROTECTION BY SUPEROXIDE DISMUTASE

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SUMMARY. - The toxicity of superoxide radicals, $O_2^{\cdot -}$, on various biological systems, both in vivo and in vitro, has been examined. These systems include bacteria and bacteriophage as well as enzymes such as ribonuclease and lysine tRNA ligase from yeast. In all cases, presence of superoxide dismutase conferred considerable protection. The results demonstrate the biological role and importance of superoxide dismutase.

Superoxide dismutases are enzymes which catalyse the destruction of superoxide radicals by dismutation

$$2\ H^+ + 2\ O_2^{\cdot -} \longrightarrow O_2 + H_2O_2$$

Extensive studies by Fridovich and collaborators (1) have shown that the dismutases are metallo-proteins which contain different metals. For example, in erythrocupreine from eucaryotes copper and zinc are present, whereas the enzyme isolated from certain procaryotes contains manganese(2). Certain marine bacteria such as Photobacterium leiognathi and Photobacterium sepia possess superoxide dismutases which contain iron as the metal component (3). As previously indicated by Fridovich et al (4) these enzymes, present in all aerobic cells, play an important evolutionary role with respect to protection against the toxic effects of oxygen and superoxide radicals. In this communication we report various studies which develop this hypothesis, and demonstrate the protective action of superoxide dismutases against oxidative degradation of biological macromolecules as well as of micro-organisms.

METHODS

Superoxide radicals were produced in the system either by photochemical reduction of flavin mononucleotide followed by withdrawal of one electron from oxygen by the dihydroflavin (5, 6) or enzymatically (1).

A. Reduction of oxygen by reduced FMN. A solution of 10^{-4} M FMN in 10^{-2} M phosphate buffer pH 7.0 containing 10^{-3} M EDTA was irradiated at 365 nm in a closed quartz cuvette in a Zeiss spectrofluorimetre (Filter M 365). Photoreduction occured rapidly and was followed by measurement of the decrease in intensity of fluorescent emission at 530 nm. At the end of reduction the solution was vigorously agitated with air to obtain complete reoxidation. This process can be repeated several times without significant structural degradation of the FMN.

Alternatively, the irradiation was performed with a much stronger source of light at 365 nm, a lamp B 100 A (Ultraviolet Products, Inc.), with continually stirred solutions. At the distances used, approximately 0.55 $\mu W/cm^2$ were incident on the container.

B. Enzymic production of $O_2^{\cdot-}$ by the system xanthine oxidase/xanthine or hypoxanthine/oxygen has been well established (1). In this work solutions containing 0.3 ml of 1 M phosphate buffer pH 7.8, 0.3 ml 10^{-3} M EDTA, 0.05 ml of a solution of xanthine oxidase (1 mg/ml ; Boehringer) and 2.1 ml of water, were used. The reaction was initiated by injection of 0.3 ml of 10^{-3} M hypoxanthine to give a final volume of 3 ml.

Superoxide dismutase. The enzyme was extracted from the luminous mushroom Pleurotus olearius and purified to homogeneity on acrylamide gel electrophoresis. This superoxide dismutase has a molecular weight of 78,000 and contains two functional atoms of manganese per mol of protein (7). The activity of the enzyme was measured by inhibition of the chemiluminescence of luminol produced by the system xanthine oxidase/hypoxanthine/oxygen (8). Reaction mixtures contained 0.3 ml 10^{-3} M luminol, 0.3 ml of 1 M glycine buffer pH 9, 0.3 ml 10^{-3} M EDTA, 5 μl of a solution of xanthine oxidase (1 mg/ml) and 1.1 ml of water, with injection of 1 ml of 0.33×10^{-4} M hypoxanthine to initiate light emission (3). The apparatus used for measurement of light emission (a steady state output occurs at least for several minutes under these conditions) has been described previously (9).

The activity of the enzyme is defined in arbitrary units. A decrease of 50 % of the maximal light output in the above system corresponds to 0.1

units. The specific activity of the pure enzyme is 7400 units per mg of protein.

Bacteriophage R 17 was conserved in a solution containing 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl and 10 ml of 0.01 M $CaCl_2$ per 1000 ml of water. The same solution was used for dilution. Infectivity was followed by incubation for 10 min at 38° C of 0.1 ml of the bacteriophage suspension with 0.2 ml of a culture (optical density 0.5 at 650 nm) of Escherichia coli KGY 306 (culture medium was 10 g trypticase, 5 g yeast extract and 10 g NaCl per litre of water). After pre-incubation, 3 ml of liquid agar (8 g Agar, 10 g trypticase, 1 g yeast extract, 8 g NaCl, 2 ml of 1 M $CaCl_2$ and 5 ml of 20 % glucose per litre) at 45° C was added and the mixture then poured into petri dishes containing 20 ml of solid agar (12 g Agar, 10 g trypticase, 1 g yeast extract, 8 g NaCl, 2 ml of 1 M $CaCl_2$ and 5 ml of 20 % glucose per litre) and the dishes maintained at 37° C.

Photobacterium leiognathi used for studies of protection by superoxide dismutase were cultivated at 28° C in a medium containing 8 g of nutrient broth, 10 g NaCl, 14 g Na_2HPO_4 and 2 g KH_2PO_4 per litre adjusted to pH 7.0. Bacterial counts were made by spreading 0.1 ml of bacterial suspension diluted with physiological saline in petri dishes containing solid agar (8 g nutrient broth, 10 g NaCl and 15 g Agar per litre, adjusted to pH 7). The dishes were incubated at 25° C.

Ribonuclease activity was measured by following the increase in optical density at 260 nm on a Cary 15 of a solution of crude tRNA (0.1 mg/ml) in 10^{-2} M phosphate buffer pH 7.0 containing 10^{-3} M EDTA.

Preparation of yeast tRNA ligases. Yeast (Saccharomycetes cerevisiae) was grown in a medium containing 30 g of glucose and 5 g of yeast extract per litre at 30° C. Cells were harvested in the middle of the logarithmic phase. 67 g of yeast (wet weight) were dispersed in 67 ml of 0.01 M Tris buffer pH 8.0 containing 0.01 M $MgCl_2$, 1 mM EDTA 2 mM phenylmethylsulphonium fluoride and 10 % glycerol, and passed three times in a French press. The suspension was then centrifuged for 30 min at 15,000 rpm and the supernatant recentrifuged in a Spinco for 2 h at 50,000 rpm. The supernatant was applied to a column of DEAE-cellulose DE 52 (36 x 2 cm) previously equilibrated with 0.02 M potassium phosphate buffer pH 7.5 containing 0.02 M mercaptoéthanol, 1 mM $MgCl_2$ and 10 % glycerol and the column washed with 370 ml of the same buffer. The fraction containing tRNA ligases was eluted with 0.25 M potassium phosphate buffer pH 6.5 in 0.02 M mercaptoéthanol 1 mM $MgCl_2$ and 10 % glycerol.

Active fractions were located by testing for lysine tRNA ligase and concentrated by ultracentrifugation.

Preliminary results indicate that the lysine tRNA ligase is a lipoprotein complex. Thus extraction with ether of the enzyme bound to DEAE-cellulose removed a fraction which chromatographically behaved as a cholesteryl lipid. This treatment although not accompanied by extensive protein denaturation results in a loss of activity of the protein which can be entirely recovered by addition of the lipid extract in a ratio corresponding to that originally present.

RESULTS

Protection of bacteria. A culture of Photobacterium leiognathi (100 ml) in exponential phase (optical density at 600 nm of 0.3) was centrifuged at 10,000 rpm for 10 min at 3° C. The bacteria were then dispersed in 100 ml of physiological saline. This suspension (1 ml) was then diluted 10 fold in 5×10^{-5} M FMN containing 3 % NaCl. Each sample (with and without superoxide dismutase) was then irradiated with the Ultraviolet lamp B 100 A at 365 nm with constant agitation at 25° C in a shaker bath. At different time intervals aliquots were taken and the number of viable bacteria counted after dilution in physiological saline. The protection afforded by superoxide dismutase (13.5 units/ml) is shown in table 1.

Table 1

Time of irradiation in min	Viable cells	
	Control	Plus dismutase
0	1×10^7	1×10^7
30	4×10^6	7×10^6
60	5×10^5	3.9×10^6
120	8×10^3	6×10^4

Protection of bacteria against ultraviolet irradiation. A significant protection by superoxide dismutase was observed against ultraviolet irradiation (365 nm) in the absence of exogenous FMN. Presumably such irradiation can lead to superoxide radicals by indirect means such as photosensitization. Suspensions of Photobacterium leiognathi 665 in 10 ml of physiological saline were irradiated for 16 hours with the B 100 A ultraviolet lamp (0.55 $\mu w/cm^2$). The original suspension of 9.7×10^4 viable cells/ml was reduced to a count of 1.5×10^2 cells/ml in the absence of superoxide dismutase whereas in presence of 53 units of superoxide dismutase/ml the viable count was 5×10^2 cells/ml, that is, a 3.3 fold increase.

Bacteriophage R 17. a) Production of $O_2^{\cdot-}$ via photoreduction of FMN. A suspension of bacteriophage R 17 was diluted 10 fold in 10^{-4} M FMN, 10^{-2} M phosphate buffer pH 7, 10^{-3} M EDTA. The final volume of the suspensions used was 0.8 ml. This was photoreduced several times in the spectrofluorimetre as described in methods. The protective effect of 13.5 units/ml of superoxide dismutase is shown in table 2 as is the lack of effect acid denatured dismutase (pH 3) as control. After nine cycles of superoxide radical a four fold protection can be seen.

Table 2

	Infective particles/ml	
Number of cycles of photoreduction	Control (plus denatured dismutase)	Plus 13.5 units/ml of superoxide dismutase
0	4.1×10^8	4.1×10^8
3	4.9×10^7	7.5×10^7
6	5.0×10^6	1.5×10^7
9	5.0×10^5	2.0×10^6

b) Inhibition of the toxicity of xanthine oxidase/hypoxanthine/O_2. A suspension of 0.3 ml of bacteriophage R 17 was diluted ten fold with the xanthine oxidase system. After 15 min at room temperature the system was again initiated by addition of 0.05 ml of xanthine oxidase (1 mg/ml) and 0.3 ml of 10^{-3} M hypoxanthine. The viability of the bacteriophage in absence and in presence of superoxide dismutase (200 units/ml) is presented in table 3.

Table 3

	Infective particles/ml	
Number of enzymic cycles	Control	Plus superoxide dismutase
0	5.0×10^8	5.0×10^8
1	3.5×10^8	5.0×10^8
2	2.0×10^8	3.0×10^8

Inhibition of the oxidative degradation of ribonuclease. A solution of ribonuclease (0.1 mg/ml) in 10^{-2} M phosphate buffer pH 7.0, 10^{-3} M EDTA was diluted 10 fold in 10^{-4} M FMN, 10^{-2} M phosphate buffer pH 7.0, 10^{-3} M EDTA to give a final volume of 0.8 ml. The solutions, in absence of dismutase, in presence of 67.5 units/ml of superoxide dismutase, and in presence of acid denatured dismutase, were submitted to several cycles of photoreduction.

Number of Photoreduction cycles	% Original activity: Control	Plus dismutase denatured at pH 3	Plus 67.5 units/ml of superoxide dismutase
0	100	100	100
3	67	56	89
6	45	45	89
9	22	22	67

Inhibition of the autoxidation of lysine tRNA ligase (yeast). This ligase appears to be a lipoprotein complex for which the enzymic activity is a function not only of the presence but also of the integrity of the lipid moiety. Autoxidation of the lipid is extremely rapid (probably due to trace metals such as Fe, Cu, Co and Ni which react with dissolved oxygen) and results in loss of enzymic activity. Solutions of 0.2 mg of the ligase in 0.3 ml of 0.5 M phosphate buffer pH 7.5 as control, or with 0.1 % ascorbate or 90 units of superoxide dismutase, were left at 4° C. Aliquots (10 μl) were taken at different time intervals and enzymic activity for charging tRNA measured. The incubation mixture (100 μl) contained 0.05 M N-morpholino-3-propane/H_2SO_4 buffer pH 6.5, 0.01 M $MgCl_2$, 0.002 M ATP, lysine ^{14}C (400 pM) and 10 optical density units (260 nm) of total yeast tRNA. After incubation at 30° C for 10 min. 50 μl were spotted on Whatman DE-81, dried and then eluted for 1 1/2 hours with 8.7 % acetic acid, 2.5 % formic acid. The paper was then dried and the areas corresponding to precipitated lysyl tRNA cut out and counted in a scintillation counter, to give the results shown below. Replacement of active superoxide dismutase by heat denatured dismutase gave results identical with the control, showing no protection whatsoever. Reduced glutathione also showed no protective action.

Days	% Activity: Control	+ 0.1 % ascorbate	+ 90 units superoxide dismutase
0	100	100	100
2	81	31	97
4	62	19	96
7	35	2.8	72
9	27	1.2	67

CONCLUSIONS

The results described above demonstrate the toxic character of superoxide radicals and the protection afforded by superoxide dismutase. Because of the relatively long life time of $O_2^{\cdot-}$ compared with other radicals derived from oxygen (10) superoxide can diffuse through the bacterial cell

wall and provoke a lethal alteration at the level of DNA (11) when the internal concentration becomes too high to be controlled by the endogenous cellular enzyme. In the case of bacteriophage R 17 the damage caused by superoxide radicals on this ribonucleoprotein is more rapidly produced and in fact a simple oxidative enzymic system which produces low yields of $O_2^{\cdot -}$ as a by product induces a marked diminution of the infectivity of the virus. Although in the present work only lethality has been measured, it would be of extreme interest to study the mutational effects (if any) induced by $O_2^{\cdot -}$ and the protection afforded by superoxide dismutase.

Striking results were also obtained with simple proteins such as ribonuclease. It is known that $O_2^{\cdot -}$ radicals can oxidize thiol groups (12) and tryptophane residues (6, 13) in proteins thus inducing a loss of enzymic activity. The protection afforded by superoxide dismutase against the autoxidation of lipoproteins (in the absence of an added system producing $O_2^{\cdot -}$) such as lysine tRNA ligase from yeast demonstrates not only the mechanism of lipid autoxidation (14) but also practical application of $O_2^{\cdot -}$ dismutases, particularly in comparison with the marked enhancement of loss of activity caused by ascorbic acid, which can indeed act as a pro-oxidant due to chain reactions involving free radicals (14, 15). The toxicity of superoxide radicals can be amplified by secondary reactions which lead to hydroxyl radicals which are likewise extremely reactive (16).

$$O_2^{\cdot -} + H_2O_2 \longrightarrow {}^{\cdot}OH + {}^{-}OH + O_2$$

Indeed, in certain experiments with unfortunately somewhat low reproducibility, we have observed a striking increase in protection when catalase is present as well as superoxide dismutase.

The present results indicate possible biological and medical applications of superoxide dismutases. It is evident that such enzymes, which are extremely rapid in action (17) must control very strictly the concentration of cellular $O_2^{\cdot -}$ radicals in order to avoid extensive damage to the various biological structures such as nucleic acids, proteins and lipid membranes.

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