SINGLET MOLECULAR OXYGEN IN BIOLOGICAL SYSTEMS: NON-QUENCHING OF SINGLET OXYGEN-MEDIATED CHEMILUMINESCENCE BY SUPEROXIDE DISMUTASE*

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SUMMARY: Chemiluminescence measurements indicate that superoxide dismutase does not significantly quench singlet molecular oxygen. 1-Phospha-2,8,9-trioxaadamantane ozonide is used as a singlet oxygen source. However, superoxide dismutase effectively inhibits the chemiluminescence produced by xanthine-xanthine oxidase or potassium superoxide. It is, therefore, likely that superoxide dismutase produces triplet molecular oxygen from its copper-containing active site during the dismutation of superoxide anion radicals, unlike the non-enzymatic dismutation reaction which yields singlet oxygen.

Recent years have witnessed an increasing interest in the reactions of singlet molecular oxygen ($^1O_2$) with biological substances (1). These reactions appear to be related to carcinogenesis, aging processes, and oxygen toxicity.

The chemical dismutation of superoxide anion radicals ($O_2^{\cdot-}$) (eq. 1) generates molecular oxygen in the singlet state (2), which is high reactive to a variety of organic compounds. Chemiluminescence is subsequently produced by excited oxygen molecular pairs (3). Singlet oxygen has a half-life of 2 μsec in H_2O (3).

$$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow {}^1O_2 + H_2O_2 \qquad (1)$$

$$2\ {}^1O_2 \rightarrow 2\ {}^3O_2 + h\nu \qquad (2)$$

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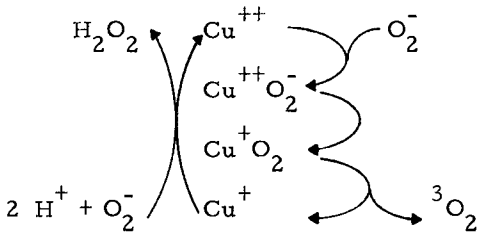
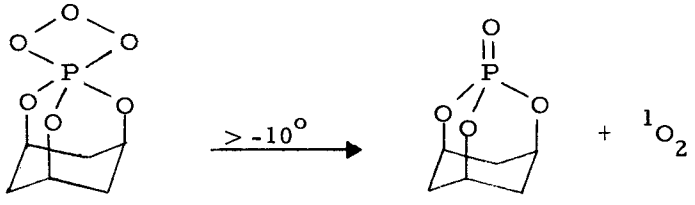
Abbreviations: SOD, superoxide dismutase; DABCO, diazobicyclo-(2.2.2)octane; BSA, bovine serum albumin.



Superoxide dismutase (SOD), which contains 2 g atoms of Cu and Zn each per mole of protein, catalyzes reaction 1. However, the resultant molecular oxygen is produced in the triplet state. It has, therefore, been suggested by several investigators that SOD may serve as a singlet oxygen quencher (4-6). Paschen and Weser (4) reported that SOD inhibits the chemiluminescence from a reaction mixture containing luminol and potassium peroxychromate (K_3CrO_8), a singlet oxygen source (7). From this observation, they concluded that the main physiological function of SOD is the scavenging of $^1O_2$, rather than catalyzing the dismutation reaction.

We have investigated this proposal using 1-phospha-2, 8, 9-trioxa-adamantane ozonide as a $^1O_2$ source (8). This ozonide is soluble in water and its thermal decomposition at ambient temperature in H_2O produces $^1O_2$ at a measurable rate (eq. 3). This reaction is accompanied by chemiluminescence which we attribute to the singlet oxygen dimol emission.



(3)

MATERIALS AND METHODS

1-Phospha-2, 8, 9-trioxaadamantane ozonide was prepared in CH_2Cl_2 by the addition of ozone to the phosphite (8). Removal of the CH_2Cl_2 under vacuum at -40^o gave the solid ozonide. The ozonide was then dissolved in dry tetrahydrofuran to give the solution used for this study. 1,4-Diazabicyclo[2.2.2]octane (DABCO) was purchased from Aldrich and recrystallized

Table I. Effect of SOD on Chemiluminescence from 1-Phospha-2,8,9-trioxaadamantane Ozonide.

Exp. Number	Additions	Initial Chemiluminescence Intensity	%
I	None	2.34×10^4 cpm	100
	SOD (50 μg/ml)	3.67	157
	SOD (100 μg/ml)	5.87	251
	Boiled SOD (500 μg/ml)	2.55	109
	BSA (50 μg/ml)	2.11	90
	DABCO (3.9×10^{-3} M)	0.77	33
	DABCO (9.3×10^{-3} M)	0.65	28
	$CuSO_4$ (4.7×10^{-5} M)	1.83	78
	$CuSO_4$ (4.7×10^{-4} M)	1.97	84
	$ZnCl_2$ (1.7×10^{-4} M)	1.58	68
	$ZnCl_2$ (3.3×10^{-4} M)	1.60	68
II	None	1.34×10^4 cpm	100
	α-Lipoic acid (7.5×10^{-5} M)	0.23	17

The reaction mixture contained: Tris-glycine buffer, 38 mM as glycine (pH 8.3); the ozonide, 6.7 mM for Exp. I, and 3.8 mM for Exp. II. The total volume was 15.0 ml. The reaction was started by adding 0.1 ml of the tetrahydrofuran solution of the ozonide. SOD was dialyzed against 0.01 M Tris buffer (pH 7.4) for 20 hr. Boiled SOD was prepared by heating at 100° for 30 min. The initial chemiluminescence was recorded at 9.0 ± 0.5 sec after the addition of the ozonide.

from acetone and sublimed. SOD was obtained from Miles and was further purified by Sephadex gel filtration and ammonium sulfate fractionation. Bovine serum albumin (BSA) and milk xanthine oxidase were purchased from Sigma. Potassium superoxide was obtained from K and K Laboratories. The chemiluminescence was measured with a Beckman liquid scintillation counter in the out-of-coincidence mode and the signals were recorded by a Hitachi recorder.

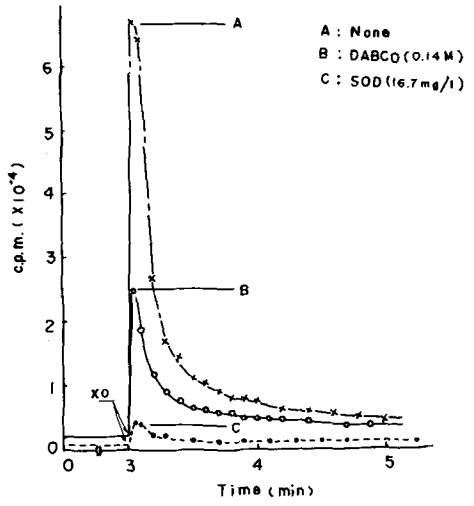


Figure 1. Effects of DABCO and SOD on Chemiluminescence Produced By Xanthine-Xanthine Oxidase. The reaction mixture contained: Tris-glycine buffer, 0.01 M as glycine (pH 8.3); xanthine, 0.27 mM; and catalyase, 13.3 μg/ml. The total volume was 15.0 ml. The reaction was started by adding 0.073 units of xanthine oxidase.

RESULTS

Effect of SOD on Chemiluminescence from 1-Phospha-2, 8, 9-Trioxaadamantane Ozonide. We have examined the effect of SOD, boiled SOD, BSA, DABCO, Cu^{2+}, and Zn^{2+} on the singlet oxygen-mediated chemiluminescence from the thermal decomposition of the ozonide in H_2O. The first-order rate constant for the decay of the chemiluminescence intensity was 6.6×10^{-2} min^{-1} at ambient temperature. Since the lifetime of $^1O_2$ is very short in H_2O (3), rate-limiting step of the chemiluminescence reaction must be the decomposition reaction of the ozonide. The effect of the various substrates on the chemiluminescence intensity was determined by noting the luminescence intensity 9 sec after the addition of a cold solution of ozonide in tetrahydrofuran to the aqueous solution of the substrate at ambient temperature.

DABCO, an effective $^1O_2$ quencher (9), reduced the chemiluminescence to background levels immediately after addition. α-Lipoic acid which reacts with $^1O_2$ (10), also inhibited the chemiluminescence. These results strongly suggest that the chemiluminescence observed is largely due to the dimol emission of $^1O_2$, presumably from the $^1\Delta g\,^1\Delta g$ or $^1\Sigma g\,^1\Delta g$ pairs (11). In contrast to the experiments with $^1O_2$ quenchers, SOD does not quench the

Table II. Effect of SOD on Chemiluminescence from Potassium Superoxide.

Exp. Number	Additions	Initial Chemiluminescence Intensity	%
I	None	6440 ± 140 cpm	100
	SOD (0.3 μg/ml)	3740 ± 100	58
	SOD (3.3 μg/ml)	2430 ± 100	38
	Boiled SOD (3.3 μg/ml)	5500 ± 440	85
II	Catalase (13.3 μg/ml)	6240 ± 450	100
	SOD (3.3 μg/ml) plus Catalase (p3.3 μg/ml)	2390 ± 250	38

The reaction mixture contained: Tris-glycine buffer, 0.33 M as glycine (pH 8.3). The reaction was started by adding 2.0 ml of saturated potassium superoxide in dimethylsulfoxide. The total volume was 15.0 ml. The initial chemiluminescence was recorded at 9.0 ± 0.5 sec after the addition of potassium superoxide. The values were the average of three experiments. A separate experiment for cytochrome c reduction with xanthine oxidase revealed that SOD was catalytically active under these conditions. Boiled SOD was prepared by heating at 100°C for 30 min.

chemiluminescence. Rather, SOD enhanced the chemiluminescence intensity by increasing the rate of decomposition of the ozonide. BSA or boiled SOD had a negligible effect on the chemiluminescence. At 10-100 times higher concentrations compared with SOD, Cu^{2+} and Zn^{2+} showed slight quenching. These results are summarized in Table I.

In addition, we have examined the effect of SOD on the reaction of $^1O_2$ with α-lipoic acid and 9,10-diphenylanthracene-2,3-dicarboxylic acid (12). For these experiments, $^1O_2$ was generated photochemically by the heterogeneous sensitizer, (P)-Rose Bengal or by the ozonide. The conversion of the α-lipoic acid and 9,10-diphenylanthracene-2,3-dicarboxylic acid to their corresponding products was unaffected by SOD.

These two different lines of evidences led us to conclude that SOD does not quench $^1O_2$.

Effect of SOD on Chemiluminescence from Xanthine-Xanthine Oxidase and Potassium Superoxide. As shown in Figure 1, SOD or DABCO quenches the chemiluminescence from xanthine-xanthine oxidase. It has been reported that this enzyme system produces superoxide anion radicals with subsequent formation of singlet oxygen via the dismutation reaction (13, 14). SOD acts to remove $O_2^{\cdot-}$ by catalyzing the dismutation reaction. These results suggest that SOD produces $^3O_2$ rather than $^1O_2$ upon the dismutation of $O_2^{\cdot-}$. This is further evidenced by the following experiments. The effect of SOD was examined when potassium superoxide was used as the $O_2^{\cdot-}$ source (Table II). The results show that SOD is an effective quencher of the chemiluminescence produced via $O_2^{\cdot-}$.

DISCUSSION

From these results, we have concluded that at concentrations of 10^{-5} to 10^{-7} M, SOD does not significantly quench $^1O_2$. However, SOD does catalyze the dismutation of $O_2^{\cdot-}$ to O_2 and H_2O_2. In the catalyzed reaction the resultant O_2 is formed in the triplet state in contrast to the non-enzymatic dismutation reaction which yields singlet oxygen. This difference may be accounted for by the presence of catalytically active Cu^{2+} ions in the dismutase molecule (13). We speculate that $Cu^{2+}O_2^-$ is an intermediate in the dismutation reaction, and upon its decomposition, $^3O_2$ is spontaneously formed without the formation of $^1O_2$. Therefore, under physiological conditions, SOD would serve to prevent the formation of $^1O_2$ from $O_2^{\cdot-}$ and thereby inhibit damage by $^1O_2$ to biological systems.

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