

Rate of Spin-Trapping of Superoxide as Studied by Chemiluminescence

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A novel method was developed and applied to the measurement of the rate constants for the interaction of superoxide with various spin traps. This method is based on the effect of spin traps on the chemiluminescence intensity emitted from the interaction of superoxide with a Cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one.

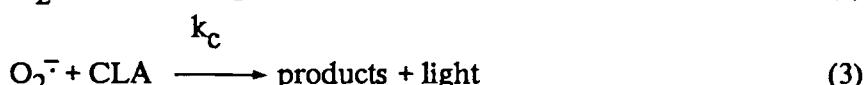
Superoxide anion radical, a one electron reductant of molecular oxygen, has received much attention in the fields of chemistry, biochemistry and medicine.¹⁾ The detection of superoxide has been usually performed by an electron spin resonance (ESR) spectrometry using a spin trapping technique.²⁾ Superoxide reacts covalently with a spin trap to form a stable radical which accumulates and can be observed by ESR. However, superoxide is not so reactive and a high concentration of spin trap is often required. There are only a few reports on the rate constant for the trapping of superoxide by a spin trap. For example, Finkelstein, Rosen, and Rauckman³⁾ obtained a rate constant $10 \text{ M}^{-1}\text{s}^{-1}$ for the reaction of superoxide with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) at pH 7.8. In the present study, we have developed a novel method where the absolute rate constant for the trapping of superoxide by a spin trap is measured by an inhibitory effect of the spin trap on 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]-pyrazin-3-one (CLA) dependent chemiluminescence induced by hypoxanthine (HX)-xanthine oxidase (XOD) system, a well known superoxide generating system. The use of CLA as a probe for superoxide has been developed by Goto⁴⁾ and Nakano.⁵⁾ The rate constants obtained by the chemiluminescence method were compared with those measured by nitro blue tetrazolium (NBT) reduction method, where, upon reduction by superoxide, NBT which is soluble and yellow in color becomes relatively insoluble blue-black formazon having a maximum absorption at 560 nm.⁶⁾ The increase in the formation of formazan was followed spectrophotometrically at 560 nm in 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA, 100 μM NBT, 200 μM HX, and 2.6 unit/l XOD.

Hypoxanthine, xanthine oxidase, and superoxide dismutase (SOD) were purchased from Sigma

Chemical Co. The final concentrations of HX, XOD, and CLA were determined as 500 μM , 0.22 unit/l, and 5.0 μM , respectively, in order to obtain a constant chemiluminescence intensity for at least the first 10 minutes. The aqueous solution contained 50 mM Tris-HCl buffer (pH 7.8) and 0.1 mM EDTA. The chemiluminescence intensity was measured with a single photon counting apparatus, type OX-7, manufactured by Tohoku Electric Industries.

The spin traps, α -phenyl-N-t-butyl nitrone (PBN), α -(4-pyridyl 1-oxide)-N-t-butyl nitrone (POBN), and 3,3,5,5-tetramethyl-1-pyrroline N-oxide (TMPO) were obtained from Sigma Chemical Co. and used as received. DMPO with high purity was obtained from Labo-Tech Co., Ltd.

When XOD was added into a solution of HX and CLA, a strong emission of light was observed instantaneously. The chemiluminescence intensity increased with an increase in XOD. Little chemiluminescence was observed when either XOD or HX was omitted from the medium. CLA, on the other hand, had appreciable emission of light by itself. When a spin trap such as DMPO was added to a system of HX-XOD-CLA, the chemiluminescence intensity decreased with increasing concentration of DMPO as shown in Fig. 1. This is apparently due to the scavenging of superoxide by DMPO, which reduces the amount of superoxide attacking CLA to emit light. When a compound X is added into the reaction medium of HX-XOD-CLA, X and CLA compete to interact with superoxide (reactions 2 and 3).



When the rate of superoxide flux is constant, the chemiluminescence intensity, I_x , in the presence of X is given by

$$I_x = I_0 \frac{k_c [\text{O}_2^{\cdot-}] [\text{CLA}]}{k_c [\text{O}_2^{\cdot-}][\text{CLA}] + k_x [\text{O}_2^{\cdot-}] [\text{X}]} \quad (4)$$

where I_0 is the chemiluminescence intensity in the absence of X, and k_x and k_c are the rate constants for the reactions 2 and 3, respectively. The Eq. 4 gives Eq. 5.

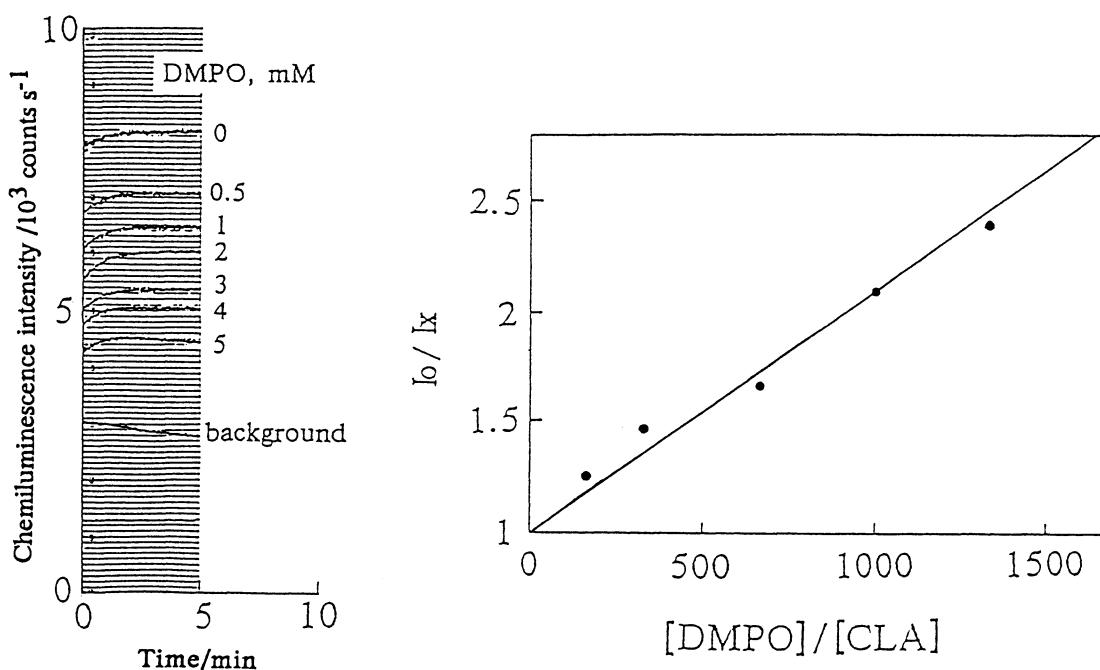


Fig. 1. (left) Chemiluminescence from CLA by its interaction with O_2^- and its inhibition by DMPO at 25 °C. [HX] = 500 μ M; [XOD] = 0.65 unit/l; [CLA] = 3 μ M.

Fig. 2. (right) Plot of I_0/I_x as a function of $[DMPO]/[CLA]$ for the system HX-XOD-CLA-DMPO. Condition are the same as those in Fig. 1.

$$\frac{I_0}{I_x} = 1 + \frac{k_x [X]}{k_c [CLA]} \quad (5)$$

Hence, the plot of I_0/I_x as a function of $[X]/[CLA]$ should give a straight line with an intercept of 1 for the vertical axis. Figure 2 shows an example for DMPO. The slope of this line is k_x/k_c , and if k_c is known, k_x is obtained.

NBT reacts with superoxide to give diformazan. Thus, the addition of 2.6 unit/l of XOD into an aqueous solution containing 200 μ M HX, 100 μ M NBT, 100 μ M EDTA and 50 mM Tris-HCl buffer (ph 7.8), the absorption at 560 nm due to diformazan increased linearly with time. The addition of a spin trap decreased the rate of increase in the absorption at 560 nm in a dose-dependent manner. By using a similar equation as Eq. 5 and from the reported⁷⁾ rate constant $k = 5.88 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ for the interaction between superoxide and NBT, the rate constant for the interaction of superoxide with CLA was obtained as $k_c = 2.12 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ at 25 °C. CLA did not reduce NBT by itself.

Table 1. Rate constants for the interaction of O_2^- with spin traps at 25 °C ($M^{-1}s^{-1}$)

PBN	POBN	DMPO	TMPO
18	67	2.3×10^2	8.2

Table 1 summarizes the rate constants for the scavenging of superoxide by several spin traps obtained by the chemiluminescence method. The agreement of the rate constants obtained by the chemiluminescence and NBT methods was fair. It shows that superoxide reacts with the spin traps slowly, much slower than other oxygen radicals such as hydroxyl, alkoxy and peroxy radicals. In other words, it is not easy to scavenge superoxide quantitatively unless quite a high concentration of spin trap is used.

The above results show that this chemiluminescence method is applicable to the measurement of rate constants for the interactions of superoxide with various substrates. This is quite important since it makes it possible to study the role and action of superoxide, which are not clearly understood and often controversial. It may be worth pointing out that the chemiluminescence method devised in the present work has several advantages: that is, it has high sensitivity and quick response and it can be applied to even a heterogeneous system. The rate constants for the reactions of superoxide with a number of compounds in various systems are now being measured in our laboratory.

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