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Chemiluminescence Reaction Rates of *Cypridina* Luciferin Analogues with Superoxide Using Quenching Experiments with Superoxide Dismutase*1

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Reaction rate constants of superoxide (O_2^-) with *Cypridina* luciferin analogues (CLAs), 2-methyl-6-phenyl-, 2-methyl-6-*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-ones (CLA and MCLA) and 2-methyl-6-[*p*-[2-[sodium 3-carboxylato-4-(6-hydroxy-3-xanthenon-9-yl)phenylthioureylene]ethyleneoxy]phenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (FCLA), were determined by using superoxide dismutase (SOD) as a quencher as 1.08×10^8, 2.54×10^8, and 8.5×10^7 M^{-1} s^{-1} in pH 7.1 buffer solutions at 25℃ for the first time.

1 Introduction

Both singlet molecular oxygen ($^1O_2$) and superoxide (O_2^-) play important roles in various biological and chemical processes. For detecting the former active oxygen species ($^1O_2$), direct spectroscopic observation of near-infrared emission at 1.27 μm is one of the best ways, which is the most reliable physical method.[1,2]

However, direct observation of $^1O_2$ in biological systems is still now extremely difficult in spite of recent advances of detection techniques for the active oxygen species by using sensitive detectors constructed using semiconductors as a result of low quantum yields of its emission (≦ 10^{-6} einstein/mol).[3] On the other hand, there is no direct spectroscopic way for detecting the latter oxygen species (O_2^-).

Cypridina luciferin analogues (CLAs), 2-methyl-6-phenyl- and 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-ones (CLA and MCLA) were shown to be versatile tools for specific detection of $^1O_2$ and O_2^-.[4~6] In the previous reports, we have reported rate constants for [CLA] [$^1O_2$], [MCLA] [$^1O_2$], and [FCLA] [$^1O_2$] by measuring the quenching constants for $^1O_2$ ($^1\Delta g$) by CLA, MCLA, and FCLA together with those of [luminol] [$^1O_2$], [superoxide dismutase (SOD)] [$^1O_2$], and [NaN_3] [$^1O_2$].[7,8] In this report, we describe measurement of rate constants for

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[CLA] $[O_2^-]$, [MCLA] $[O_2^-]$, and [FCLA] $[O_2^-]$ by a quenching experiment of chemiluminescence (CL) by SOD, for the first time.

2 Materials and Methods

Superoxide dismutase (SOD Ⅲ, 3,520 U/mg) was purchased from Toyobo Co. Ltd. CLA and 2 - methyl - 6 -[*p*-[2- [sodium 3 - carboxylato - 4 - (6-hydroxy- 3-xanthenon-9-yl)phenylthioureylene]-ethyleneoxy] phenyl]-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (FCLA) were from Tokyo Chem. Ind. Co. Hypoxanthine, xanthine oxidase (XOD), and albumin (bovine, fraction Ⅴ) were from Sigma Chem. Co. MCLA was prepared according to the method described in the literature.[5] Quenching experiments were performed as follows; A solution of 10 μl of CLA (final concn. 4.4×10^{-8} M), MCLA (1.1×10^{-8} M), or FCLA (4.8×10^{-8} M); (2.2−y) m*l* of 25 mM phosphate buffer (pH 7.1); 0.5 m*l* of 1 mg/m*l* albumin solution in the buffer solution; 50 μl of XOD (200 μl in 1.8 m*l* of the albumin soln); and y m*l* of SOD (2.54×10^{-9} M for CLAs and 1.03×10^{-10} M for luminol) in the buffer solution in a 18 mm ϕ quartz cell was placed on a photomultiplier tube (R331, Hamamatsu Photonics) at 25 ℃ in a dark cell chamber. Into the solution was added a 0.2 m*l* of 3 mM hypoxanthine[9,10] in the buffer solution through an injection needle. CL was measured through the bottom of the quartz cells in the single photon counting technique as usual. Reaction rates for O_2^- can be given as follows,

$$d[O_2^-]/dt = E - k_1[O_2^-]^2 - k_2[Q][O_2^-] - k_3[SOD][O_2^-]$$

where E, k_1, k_2, and k_3 are production rate of O_2^-, rate constants of disappearance and reactions with the quencher, and with SOD of O_2^-.

[Q], $[O_2^-]$, and [SOD] represent concentration of quenchers used (CLA, MCLA, and FCLA), of superoxide and of SOD. At stationary state, it should be zero,

$$O = E - k_1[O_2^-]^2 - k_2[Q][O_2^-] - k_3[SOD][O_2^-]$$

$$E = k_1[O_2^-]^2 + k_2[Q][O_2^-] + k_3[SOD][O_2^-]$$

Quantum efficiency (Φ) of CL of Q (CLA, MCLA, and FCLA) is represented as

$$\Phi = k_2[Q][O_2^-] / E$$
$$= k_2[Q] / (k_1[O_2^-] + k_2[Q] + k_3[SOD])$$

When [SOD] = O, $\Phi = \Phi_0$,

$$\Phi_0 = k_2[Q] / (k_1[O_2^-] + k_2[Q])$$

Therefore, to determine the rate constants (k_2) for quenching O_2^- with the quencher, Q, the Stern-Volmer description of dynamic quenching is given in the following equation[11]

$$I_0 / I = \Phi_0 / \Phi$$
$$= (k_1[O_2^-] + k_2[Q] + k_3[SOD]) / (k_1[O_2^-] + k_2[Q])$$
$$= 1 + \{k_3 / (k_1[O_2^-] + k_2[Q])\}\,[SOD] \quad \text{(eq. 1)}$$

where I_0/I is ratio of the emission intensity of O_2^- in the absence and presence of the quencher. The equation 1 indicates that a plot of I_0/I vs [SOD] gives a straight line with a slope equal to $k_3/(k_1[O_2^-] + k_2[Q])$. From these values the rate constant (k_2) for quenching O_2^- with Q can be determined if the values of k_1,[12,13,*3] k_3,[14,15,*4] $[O_2^-]$, and [Q] are known. The term of $k_1[O_2^-]$ is negligible, because its value is much less than those of $k_2[Q]$ in these cases.

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*3 The reaction rate constant, k_1 for
$O_2^- + O_2^- \rightarrow O_2 + O_2^{2-}$
is reported as $k_1 = 10^2\ M^{-1}\ s^{-1}$ (refs. 12, 13).

*4 The rate constant k_3 for the reaction of SOD and O_2^-, $k_3 = 2\times10^9\ M^{-1}\ s^{-1}$ (refs. 14, 15).

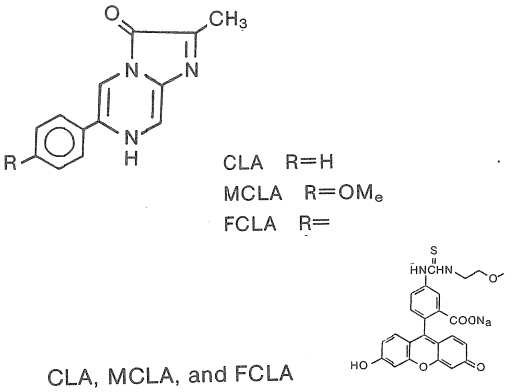
3 Results and Discussion

Figs. 1-3 shows the chemiluminescence-time curves of CLAs initiated by O_2^- in the absence and presence of SOD in pH 7.1 buffer solutions.

Values of I_0/I at various SOD concentration were plotted in Fig. 4. The straight lines were drawn by a least-squares fit of the data points. For reference, a similar graph for luminol at pH 7.1 and 10.1 is shown in Fig. 5. The correlation coefficients for the data points and the straight lines are 0.997 (CLA), 0.997 (MCLA), 0.997 (FCLA), 0.977 (luminol at pH 7.1), and 0.985 (luminol at pH 10.1), respectively.

Reaction rates (k_2) for [CLAs] [O_2^-] obtained from the equation 1 were summarized in Table 1. The results suggest that the CLAs react and give CL with O_2^- as fast as SOD does[13].

According to our previous papers,[8,9] the



CLAs react with $^1O_2$ at the rates 6.30×10^8 (CLA), 2.94×10^9 (MCLA), and 8.00×10^8 (FCLA) $M^{-1}\ s^{-1}$ (k_q values), respectively. The reaction rates obtained for O_2^- seem to parallel those for $^1O_2$. As similarly as in the previous

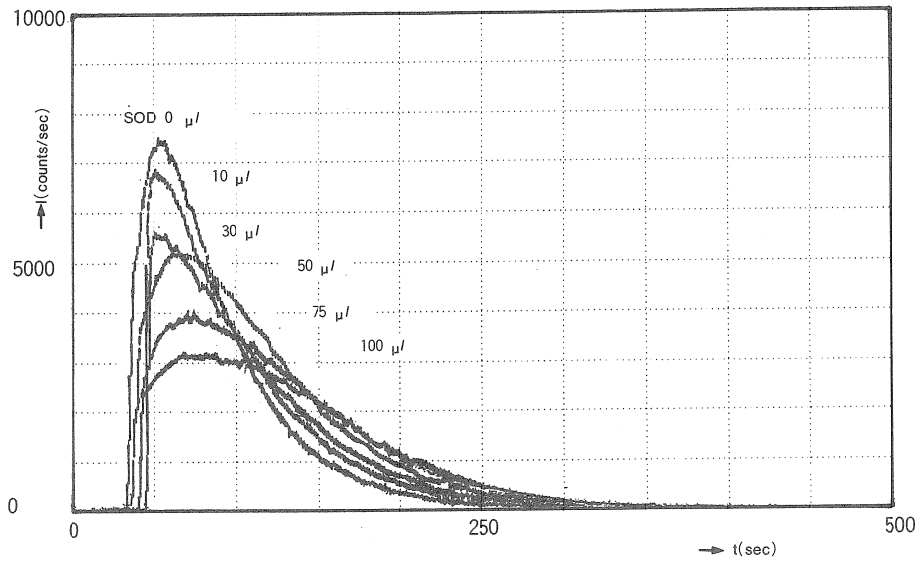


Fig. 1. Chemiluminescence-time curves of CLA with or without SOD.
[SOD] means amounts of SOD (μl of 7.53×10^{-8} M solution) added. The final concentration was 2.54×10^{-9} M at [SOD] = 100 μl.

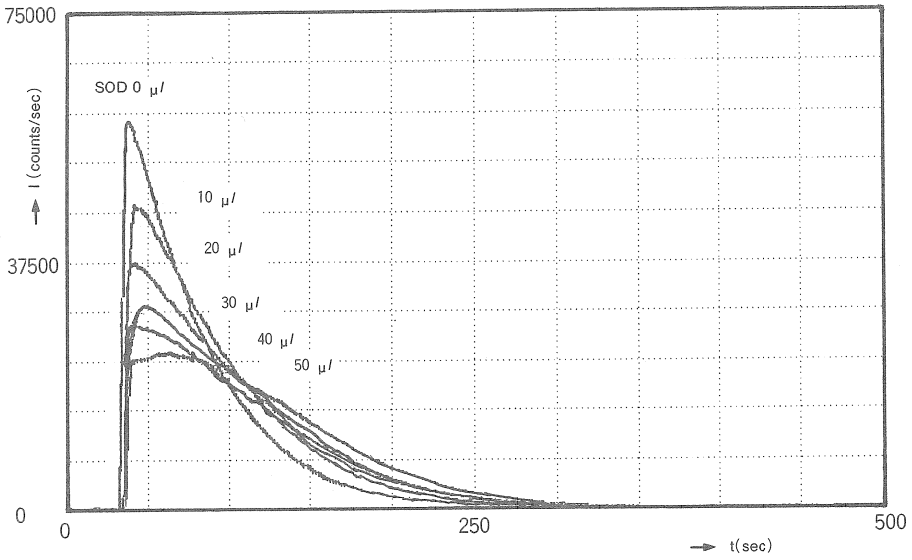


Fig. 2. Chemiluminescence-time curves of MCLA with or without SOD.
[SOD] are as same as those shown in Fig. 1.

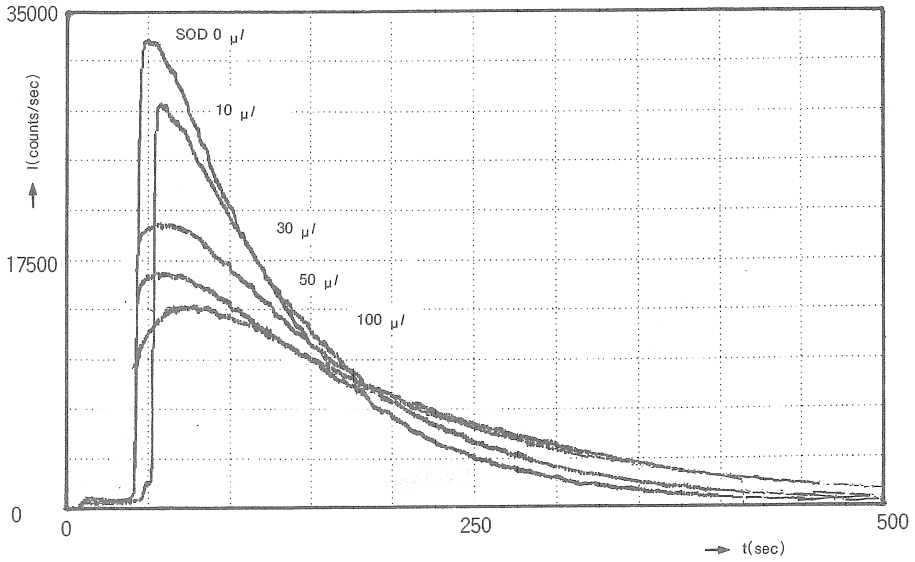


Fig. 3. Chemiluminescence-time curves of FCLA with or without SOD.
[SOD] are as same as those shown in Fig. 1.

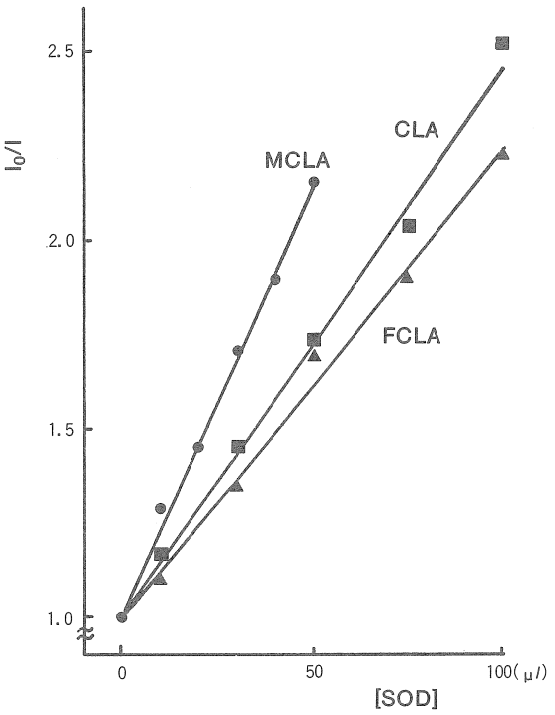


Fig. 4. I_0/I – [SOD] plots for CLA, MCLA, and FCLA. [SOD] are as same as those shown in Fig. 1.

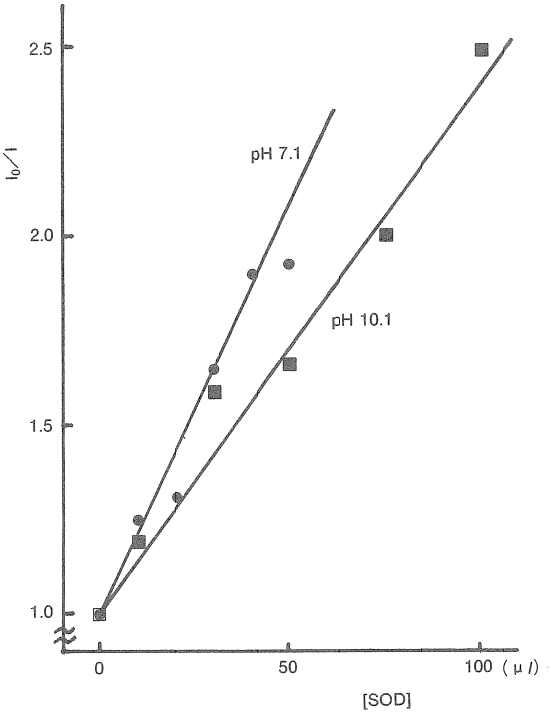


Fig. 5. I_0/I – [SOD] plots for luminol at pH 7.1 and pH 10.1. [SOD] means amounts of SOD (μl of 3.04 $\times$ 10^{-8} M solution) added. The final concentration was 1.03 $\times$ 10^{-10} M at [SOD] = 100 μl.

Table 1. Quenching constants of $^{-}O_2$ and $^1O_2$ by some quenchers in 25 mM buffer soln. (pH 7.1) at 25℃[7-9]

Quencher	$^{-}O_2$ (10^8 M^{-1} s^{-1})	Φ_{CL}[a]	$^1O_2$ (10^8 M^{-1} s^{-1})	Φ_{CL}[a]
CLA	1.08	3.8×10^{-4}	6.30	
MCLA	2.54	8.7×10^{-4}	29.4 (water)	
FCLA	0.85	22.1×10^{-4}	8.00	
Luminol	0.0156	1.8×10^{-7}	14.0	2.3×10^{-6}
	0.0151		9.30(pH 10.1)	3.8×10^{-8}
	(pH10.1)	2.7×10^{-4}	~0.3 (pD 11.8)	
			27.3	
SOD	20		26.0	

a) Einstein/mol; relative to the Hastings standard and are corrected both to the sensitivity of the photomultiplier tube (Hamamatsu Photonics, R331: max at 420 nm) and the CL max of the standard (λ_{max} 420 nm) [J. W. Hastings and G. Weber: *Photochem. Photobiol.*, **4**, 1049 (1965) ; *J. Opt. Soc. Am.*, **53**, 1410 (1963)].

paper, the electron donating substituent MeO- on the phenyl group of CLA seems to accelerate the reaction of MCLA to electrophilic superoxide anion radical. In the case of FCLA, the electron withdrawing thioketone, carboxylate, and quinoid groups might cancel the electrophilicity.

We are also conscious that antioxidation ability of antioxidants can be measured quantitatively when SOD is substituted by them in the present quenching experiment using O_2^-, as well as using $^1O_2$, as oxidants. Results of the application will appear in due course.

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スーパーオキシドジスムターゼを用いた消光実験によるウミホタルルシフェリン誘導体とスーパーオキシドの化学発光速度

鈴木喜隆・益子信郎・高須康介

ウミホタルルシフェリン誘導体（CLA, MCLA, FCLA）とスーパーオキシドの緩衝溶液中，25℃における化学発光の反応速度定数を，スーパーオキシドジスムターゼ（SOD）による消光を観測することによって測定した。測定値はCLAで1.08×10^{7}，MCLAで2.54×10^{8}，FCLAで8.5×10^{7}（それぞれ$M^{-1}s^{-1}$）であった。

これらの値は，一重項酸素との消光速度定数とほぼパラレルな値を示しており，双方の反応機構における類似性を示唆するものである。