Chemiluminescent Detection of Active Oxygen Species, Singlet Molecular Oxygen and Superoxide, Using *Cypridina* Luciferin Analogues

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In order to show the usefulness of *Cypridina* luciferin analogues, 2-methyl-6-phenyl- and 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-ones for the specific detection of active oxygen species, chemiluminescence rates, and intensities, including their quantum efficiencies were measured and determined by using various oxidants in aqueous conditions. The oxidant-specificity of their chemiluminescence to both singlet molecular oxygen and the superoxide anion radical was shown experimentally for the first time.

Both singlet molecular oxygen ($^1\text{O}_2$) and superoxide (O_2^-), the active oxygen species, play important roles in various biological and chemical processes. For detecting the former oxygen species ($^1\text{O}_2$), direct spectroscopic observation of the near-infrared emission at 1270 nm is one of the most reliable physical method.^{1,2)}

The direct observation of $^1\text{O}_2$ in biological systems, however, is still extremely difficult, in spite of recent advances in detection techniques for the active oxygen species by using sensitive detectors employing semiconductors, as a result of the low quantum yield of its emission ($\leq 10^{-6}$ einstein/mol).³⁾ On the other hand, there is no direct spectroscopic method for detecting the latter oxygen species (O_2^-) except for chemiluminescent methods using *Cypridina* luciferin analogues (CLAs).

Cypridina luciferin analogues, 2-methyl-6-phenyl- and 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-ones (CLA and MCLA, respectively) have been shown to be versatile tools for the specific detection of $^1\text{O}_2$ and O_2^- .⁴⁻⁸⁾ Previously, we have reported quenching constants of $^1\text{O}_2$ and reaction rate

constants of O_2^- by CLA, 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).⁷⁻⁹⁾ In this report, we describe a method of measurements of chemiluminescence (CL) rates and efficiency of CLA and MCLA in the cases of oxidation by various oxidants in aqueous conditions.

Hence, the oxidant-specificity of CL of the

Fig. 1. CLA, MCLA, and FCLA.

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Cypridina luciferin analogues to both $^1\text{O}_2$ and O_2^- was shown experimentally for the first time.

Materials and Methods

MCLA and CLA were purchased from Tokyo Chemical Ind. Luminol, hemin (bovine I), cumylhydroperoxide (cumylOOH), xanthine oxidase (XOD, from buttermilk, grade III, 25 units/1.7 ml), hypoxanthine, and bovine serum albumin were obtained from Sigma Chem. NaOCl, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, KH_2PO_4 , K_2CO_3 , $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, H_3BO_3 , and glycine were purchased from Wako Pure Chemical Ind. H_2O_2 was obtained from Santoku Chemical Ind, while *tert*-butylhydroperoxide (*t*-BuOOH) and $\text{K}_2\text{S}_2\text{O}_8$ were from Nacalai Tesque. O_2^- was generated from hypoxanthine and xanthine oxidase.^{10,11} 100mM Phosphate buffer (pH 7.0) and 50 mM borate buffer (pH 9.3) solutions were prepared from Na_2HPO_4 and KH_2PO_4 and boric acid and borax, respectively.

Chemiluminescent reactions were performed as follows: Two ml of 1 mM oxidant in the buffer (pH 7.0 or 9.3) was added to 20 μl of 6.7×10^{-14} M CLA, MCLA, or luminol in the same buffer in a 18 mm ϕ quartz cell placed on a photomultiplier tube (PM: R331, Hamamatsu Photonics, max sensitivity at 410 nm) at 25°C to give a final concentration of 1.0 mM, 2.0×10^{-6} mol) through an injection needle. Amounts of emitted CL was

measured through the bottom of the quartz cells by the usual single photon counting technique. In the case of O_2^- , a solution of 5 mM hypoxanthine (final concn. 9.9×10^{-3} M) was added to a mixture of CLAs (20 μl), the buffer solution (1.15 ml), an albumin solution (1 mg/ml; 0.5 ml), and 50 μl of XOD solution (7.4×10^{-2} unit). Singlet oxygen was generated by the addition of both 1 mM H_2O_2 and 1 mM NaOCl through the injection needles at once. For the examination of the effects of O_2 dissolved in the solution, the buffer employed was bubbled by N_2 and O_2 at 0°C for 30 min before use.

CL spectra were measured by a fluorescence spectrometer (Otsuka Electronics, IMUC-7000).

The CL reaction rates were analyzed using the quasi-first order rate equation,

$$\log(a-x) = \log a - (k/2.303)t$$

where a is the initial concentration of CLAs, $(a-x)$ is its concentration after t s, and k is the quasi-first order rate constant. The value of $(a-x)$ is a difference between the total quantity of CL and the sum of the quantity of CL till that time. These values can be determined by the computer used. For comparison, the CL of luminol for the various oxidants was examined similarly. Luminol was known to give strong CL at high pH region, therefore CL of MCLA being examined also in such solutions of high pH. CLA was insoluble in solutions of high

Fig. 2. CL-time curves of MCLA at pH 7.0. (a) O_2^- (XOD/hypoxanthine), (b) $^1\text{O}_2$ ($\text{H}_2\text{O}_2/\text{NaOCl}$), (c) NaOCl, and (d) H_2O_2 .

Fig. 3. Quasi-first order plots of CL of MCLA at pH 7.0. (a) O_2^- (XOD/hypoxanthine), (b) 1O_2 ($H_2O_2/NaOCl$), (c) $NaOCl$, and (d) H_2O_2 .

pH, and therefore we were unable to measure CL of the substance.

Results and Discussion

Some CL emission curves and quasi-first order plots are shown in Figs. 2 and 3, respectively.

The k values and maximum values of the emission intensity (I_{max}) obtained for CLA, MCLA, and luminol in pH 7.0 and pH 9.3 buffer solutions are summarized in Tables 1–3. Quantum yields of CL (Φ) which were estimated by comparison with the Hastings' standard,^{12,13} are also shown in the Tables. The standard has its CL max at 410 nm. CLA showed CL max at 380 nm, but those of MCLA and luminol were shown at 424 and 460 nm, respectively. Suitable corrections were made for the PM sensitivity (max at 410 nm).

The results shown in Table 1–3 suggested that some enhancement occurred in the values of k , I_{max} , and/or Φ when the buffer solutions were bubbled by O_2 . H_2O_2 and t -BuOOH almost gave

no enhancement for the CL of CLAs, but cumyl OOH enhanced that of CLA in the k value. $NaOCl$ by itself, to our surprise, oxidized CLAs almost as fast as $H_2O_2/NaOCl$, especially in a buffer at pH 7.0 but not so much in a buffer at pH 9.3. However, the values of I_{max} and Φ were smaller than those obtained by $H_2O_2/NaOCl$ -oxidation.

Hypoxanthine was consumed in about 10 min under the conditions examined. This means roughly that dissolved O_2 was consumed at the rate of $1.65 \times 10^{-10} \text{ ms}^{-1}$ at pH 7.0. According to the Fridovich,^{10,11} about 20% of O_2 consumed was reduced to superoxide under similar conditions. Hence, O_2^- would be produced at the rate of $3.3 \times 10^{-11} \text{ ms}^{-1}$, which is fast enough for the oxidation of $6.7 \times 10^{-14} \text{ M}$ CLA, MCLA, or luminol. At pH 10, it was believed that there was no large difference in the circumstances.

The oxidant specificity for reactions of CLAs both to 1O_2 and O_2^- was evidenced experimentally for the first time, as suggested in the previous papers.^{4–6} The reaction rates of CLAs measured

Table 1. Chemiluminescence of CLA at pH 7.0 (25°C)

Oxidant	CLA		
	$k/10^{-2} \text{ (s}^{-1}\text{)}$	$I_{\text{max}}/10^3 \text{ (cps)}$	$\Phi/10^{-5} \text{ (hv/mol)}^{*1}$
Buffer soln ^{*2} (N ₂)	1.73	5.44	1.71
(O ₂)	10.5	9.11	1.79
H ₂ O ₂	1.56	32.33	17.1
NaOCl	35.2	111.44	4.70
H ₂ O ₂ /NaOCl (¹ O ₂)	40.0	302.33	11.4
O ₂ ⁻	4.09	101.11	44.6
CumylOOH	14.9	1.33	0.51
<i>t</i> -BuOOH	4.20	1.99	2.64
K ₂ S ₂ O ₈	3.24	5.44	2.96

*1 CL quantum yield (Φ) was estimated comparing with the Hastings' standard, which shows the CL max at CL max at 410 nm (see ref. 12, 13).

*2 Bubbled by N₂ and O₂ for 30 min at 0°C before use for testing the effects of dissolved O₂ in solution.

Table 2. Chemiluminescence of MCLA at pH 7.0 and 9.3 (25°C)

Oxidant	pH 7.0			pH 9.3		
	$k/10^{-2} \text{ (s}^{-1}\text{)}$	$I_{\text{max}}/10^3 \text{ (cps)}$	$\Phi/10^{-5} \text{ (hv/mol)}^{*1}$	$k/10^{-2} \text{ (s}^{-1}\text{)}$	$I_{\text{max}}/10^3 \text{ (cps)}$	$\Phi/10^{-5} \text{ (hv/mol)}^{*1}$
Buffer soln ^{*2} (N ₂)	1.44	2.0	0.12	1.88	1.5	0.66
(O ₂)	1.00	7.9	2.51	1.25	7.3	1.88
H ₂ O ₂	0.93	8.2	16.0	0.32	7.6	91.0
NaOCl	50.1	99.9	3.12	10.4	23.8	1.36
H ₂ O ₂ /NaOCl (¹ O ₂)	44.5	274.5	5.48	51.7	203.6	18.6
O ₂ ⁻	3.63	335.0	151	2.50	270.2	193
CumylOOH	2.43	4.4	1.25	0.07	2.3	29.2
<i>t</i> -BuOOH	2.50	2.1	1.54	0.61	2.4	6.90
K ₂ S ₂ O ₈	0.824	1.6	3.43	3.40	44.6	21.6

*1,2 See footnotes of Table 1.

Table 3. Chemiluminescence of Luminol at pH 7.0 and 9.3 (25°C)

Oxidant	pH 7.0			pH 9.3		
	$k/10^{-2} \text{ (s}^{-1}\text{)}$	$I_{\text{max}}/10^3 \text{ (cps)}$	$\Phi/10^{-5} \text{ (hv/mol)}^{*1}$	$k/10^{-2} \text{ (s}^{-1}\text{)}$	$I_{\text{max}}/10^3 \text{ (cps)}$	$\Phi/10^{-5} \text{ (hv/mol)}^{*1}$
Buffer soln ^{*2} (N ₂)	—	—	—	—	—	—
(O ₂)	—	—	—	—	—	—
H ₂ O ₂	5.67	0.13	0.075	3.72	1.0	0.45
NaOCl	60.2	3.8	0.10	249	19.3	3.36
H ₂ O ₂ /NaOCl (¹ O ₂)	61.0	6580.8	125	55.3	15237.8	530
O ₂ ⁻	2.72	1.3	0.73	0.84	45.7	157
CumylOOH	38.5	0.07	0.004	—	—	—
<i>t</i> -BuOOH	—	—	—	—	—	—
K ₂ S ₂ O ₈	54.7	0.07	0.004	14.5	1.11	0.15

*1,2 See footnotes of Table 1.

in this method were pseudo-first-order with singlet molecular oxygen being larger than those with superoxide in 1-order of magnitude. This relation nearly parallels the relation between second-order rates of CLAs with both singlet molecular oxygen and superoxide (6.30 and $1.08 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for CLA, respectively, and 29.4 and

$2.54 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for MCLA, respectively⁽⁷⁻⁹⁾), which were determined by quenching experiments. The other oxidants such as NaOCl, H₂O₂, or dissolved oxygen in some cases, show large k and Φ values. These results suggest that special precautions to the existence of oxidants other than singlet molecular oxygen and superoxide are

necessary for interpretation of the experimental results.

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