Steady-State Near-infrared Detection of Singlet Molecular Oxygen:
A Stern-Volmer Quenching Experiment with Luminol, Superoxide Dismutase, and Cypridina Luciferin Analogues

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A sensitive near-infrared detection system with improvements was used to study the quenching of the steady-state luminescence of singlet molecular oxygen at 1270 nm. Stern-Volmer plots which were linear up to 80% quenching by luminol of the $^{1}$O$_{2}$ generated by rose bengal yielded rate constants of $9.30 \times 10^{8}$ and $1.40 \times 10^{9}$ m$^{-1}$ sec$^{-1}$ for the quenching of $^{1}$O$_{2}$ in pH 7.1 and pH 10.1 buffer solutions (25 mM). Quenching rate constants of $^{1}$O$_{2}$ by superoxide dismutase and Cypridina luciferin analogues, 2-methyl-6-phenyl- and 2-methyl-6-[p-[2-[sodium 3-carboxylato-4-(6-hydroxy-3-xanthenon-9-yl)phenylthio]ureylene]ethylbenzenesulfonatophenyl]-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (CLA and FCLA) were measured similarly as $2.73 \times 10^{9}$, $6.30 \times 10^{8}$ and $8.00 \times 10^{8}$ m$^{-1}$ sec$^{-1}$ in the pH 7.1 buffer solutions, respectively.

Singlet molecular oxygen ($^{1}$O$_{2}$) is important in various biological and chemical processes. For detecting this active oxygen species, direct spectroscopic observation of near-infrared emission of $^{1}$O$_{2}$ at 1.27 µm is one of the best ways, the most reliable method physically.$^{1,2}$ However, direct observation of $^{1}$O$_{2}$ in biological systems is still extremely difficult in spite of recent advances of detection techniques for the active oxygen species using sensitive detectors made with semiconductors as a result of low quantum yields of its emission ($\lesssim 10^{-6}$ einstein/mol).$^{3}$

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) are versatile tools for specific detection of $^{1}$O$_{2}$ and superoxide ion ($^{1}$O$_{2}$).$^{4-6}$ We reported previously a rate constant for [MCLA][$^{1}$O$_{2}$] by measuring the quenching constant for $^{1}$O$_{2}$ ($^{1}$A$_{g}$) by MCLA together with that of [NaN$_{3}$][$^{1}$O$_{2}$].$^{7}$ In this

![Chemical Structures](attachment:image)

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report, we describe measurement of rate constants for [CLA][1O2], [luminol][1O2], and [superoxide dismutase (SOD)][1O2] together with the other Cypridina luciferin analogue, FCLA, by similar techniques.

Materials and Methods

Luminol was purchased from Sigma Chem. Co., (St. Louis, MO, U.S.A.). Superoxide dismutase (SOD III, 3,520 U/mg) was from Toyobo Co., Ltd., (Osaka, Japan). CLA and FCLA were from Tokyo Chem. Ind. Co. (Tokyo, Japan). Rose bengal (RB, certified grade) was from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). The concentration of RB in all the experiments was \(0.5 \times 10^{-4} M\).

Near-IR emission spectra were measured by a spectrometer system as shown in our former report\(^8\) using a flow cell mode in our laboratory (Fig. 1) at the flow rates of 2.5 ml/min.

To measure the rate constants \(k_q\) for quenching \({}^{1}\text{O}_2\) with the quenchers (CLA, FCLA, luminol, and SOD), the Stern–Volmer description of dynamic quenching is given in the following equation\(^9\)

\[
\frac{I_0}{I} = 1 + k_q \tau [Q]
\]

where \([Q]\), \(\tau\), and \(\frac{I_0}{I}\) are the concentration of the quencher, the lifetime of \({}^{1}\text{O}_2\), and the ratio of the emission intensity of \({}^{1}\text{O}_2\) in the absence and presence of the quencher, respectively. Equation 1 indicates that a plot of \(\frac{I_0}{I}\) vs. \([Q]\) gives a straight line with a slope equal to \(k_q \tau\).

From these values the rate constants \(k_q\) for quenching \({}^{1}\text{O}_2\) with the quenchers can be calculated if the lifetime of \({}^{1}\text{O}_2\) is known.

Results and Discussion

To measure the generation of \({}^{1}\text{O}_2\) and the quenching ability by CLA and FCLA for \({}^{1}\text{O}_2\) as well as luminol and SOD for comparison, the near-IR emission spectra were measured in the absence and presence of the quenchers (Figs. 2a, b, c, and d). All the spectra had an emission peak around 1.27 \(\mu\text{m}\), indicating that \({}^{1}\text{O}_2\) was generated and the emission peak intensity of \({}^{1}\text{O}_2\) was reduced by the quenchers. These spectra also showed background signals which increased at shorter wavelengths owing to fluorescence of the photosensitizer. The absence and presence of the quenchers, however, did not affect the emission of the photo-
Quenching Constants of Singlet Molecular Oxygen

Fig. 2. $^{1}\text{O}_2$ Emission Spectra in the Near-IR Region
Sensitized by 50 μM RB in the Absence and Presence of
(a) CLA (148 μM), (b, c) Luminol (250 μM) at pH 10.1 and
pH 7.1, and (d) SOD (18.4 μM).

The Stern–Volmer plots for the quenchers
are shown in Figs. 3a, b, c, and d. Each data
data point was plotted from the peak intensity
around 1.27 μm and corrected for background.
Straight lines were drawn by a least-squares fit
of the data points. The correlation coefficients
for the data points and the straight lines are
0.9557 (CLA), 0.9871 (FCLA), 0.9981 (lumi-

The rate constants for the quenching of $^{1}\text{O}_2$
with the quenchers were calculated from Eq.
Table I. Quenching Constants of Singlet Oxygen by Some Quenchers

<table>
<thead>
<tr>
<th>Quencher</th>
<th>$k_q/10^7$ (M$^{-1}$s$^{-1}$)</th>
<th>Solv.</th>
<th>pH</th>
<th>Method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLA</td>
<td>63.0</td>
<td>A</td>
<td>7.1</td>
<td>E, G</td>
<td></td>
</tr>
<tr>
<td>MCLA</td>
<td>294</td>
<td>B</td>
<td>—</td>
<td>E</td>
<td>7</td>
</tr>
<tr>
<td>FCLA</td>
<td>80.0</td>
<td>A</td>
<td>7.1</td>
<td>E, G</td>
<td></td>
</tr>
<tr>
<td>Luminol</td>
<td>140</td>
<td>A</td>
<td>7.1</td>
<td>E, G</td>
<td></td>
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<tr>
<td>93.0</td>
<td>C</td>
<td>10.1</td>
<td>E</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>~3</td>
<td>D</td>
<td>11.8$^d$</td>
<td>F</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>SOD</td>
<td>273</td>
<td>A</td>
<td>7.1</td>
<td>E, G</td>
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<td>D</td>
<td>—</td>
<td>F</td>
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<td>11</td>
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</table>

$^a$ A, 25 mM phosphate buffer; B, distd. water; C, 25 mM glycine buffer; D, D$_2$O.
$^b$ E, Near-IR emission spectra of ¹O$_2$; F, ¹O$_2$-oxidation of bilirubin.
$^c$ G, our results.
$^d$ pD.

(1), using both the slope of the straight lines and the reported lifetime of ¹O$_2$ in water ($\tau = 4.2 \mu s$).$^{10}$ The $k_q$ values obtained were $6.30 \times 10^8$ M$^{-1}$s$^{-1}$ for CLA, $8.00 \times 10^8$ M$^{-1}$s$^{-1}$ for FCLA, $9.30 \times 10^8$ M$^{-1}$s$^{-1}$ (pH 10.1) and $1.40 \times 10^9$ M$^{-1}$s$^{-1}$ (pH 7.1) for luminol, and $2.73 \times 10^9$ M$^{-1}$s$^{-1}$ for SOD (Table I).

The value obtained for SOD is very close to that given by Bellus ($2.60 \times 10^9$ M$^{-1}$s$^{-1}$).$^{11}$ The value for luminol quenching of ¹O$_2$ obtained was larger than that measured by Matheson and Lee ($\sim 3 \times 10^7$ M$^{-1}$s$^{-1}$) in D$_2$O by observing the inhibition of ¹O$_2$-oxidation of bilirubin at pH 11.8.$^{12}$ However, this can be understood because ¹O$_2$-oxidation of luminol is faster in lower pH region than in higher pH region as observed in this work.

The values for CLA and FCLA were reasonably smaller than that for MCLA ($2.94 \times 10^9$ M$^{-1}$s$^{-1}$)$^{7}$ as expected by the chemical structure. The electron-donating substituent MeO$-$ on the phenyl group of CLA seems to accelerate the reaction of MCLA to electrophilic singlet oxygen. In the case of FCLA, the electron withdrawing thioketone, carboxylic, and quinoid groups might cancel the electrophilicity.

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References