Chemiluminescence of *Cipridina* luciferin analogues. Part 2. Kinetic studies on the reaction of 2-methyl-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one (CLA) with superoxide: hydroperoxyl radical is an actual active species used to initiate the reaction

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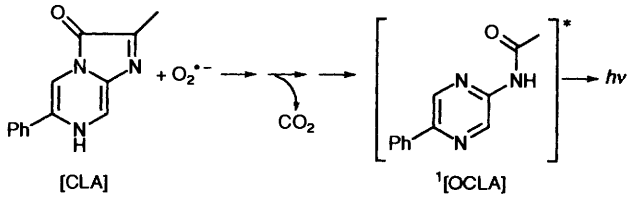
Using the xanthine–xanthine oxidase–O_2 system as a continuous superoxide radical anion supplying system, kinetic studies of the chemiluminescence reaction of the *Cypridina* luciferin analogue (CLA), 2-methyl-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one, with superoxide have been carried out. One mole of CLA appeared to consume two moles of superoxide to emit light in aqueous solution in a pH range 5.5–10.0 under aerobic conditions. Only when the supply of superoxide is very small while the concentration of molecular oxygen is high, was a little contribution of autoxidation of CLA by a chain reaction process observed. A substantial lag in the emission of light after the disappearance of CLA was observed, indicating that the initial reaction of CLA with superoxide is not solely the rate-determining step. Subsequent reactions of intermediates that lead to the final light emitter also contribute to the rate-determining step in this chemiluminescence reaction. The decay of CLA was found to be first order with respect to the concentration of CLA and superoxide from competitive kinetics with superoxide dismutase. From the effect of pH on the apparent second-order rate constant for the decay of CLA, the active species of superoxide that initiates the reaction was found to be the hydroperoxyl radical. The second-order rate constants have been determined to be (7.03 ± 0.59) × 10^5 dm^3 mol^{-1} s^{-1} for the reaction with CLA and (2.56 ± 0.40) × 10^8 dm^3 mol^{-1} s^{-1} for the reaction with the conjugate base of CLA at 20.0 °C.

Detection and quantification of superoxide radical anions ($O_2^{\bullet -}$) in living bodies have become quite important in connection with the recent explosive development of free-radical biochemistry and medical sciences.[1] Nakano and Goto and co-workers showed 2-methyl-6-phenylimidazo[1,2-*a*]pyrazin-3(7*H*)-one, *Cypridina* luciferin analogue (CLA), to be a good chemiluminescence probe for detecting $O_2^{\bullet -}$ in biological systems.[2,3a] Investigation of the kinetics and mechanisms of the reaction between CLA and $O_2^{\bullet -}$ are not only indispensable for this purpose, but also provides useful fundamental information to clarify the enzymatic reaction mechanisms for the bioluminescences of the *Cypridina* luciferin–luciferase system and related bioluminescence, *e.g.* jellyfish *Aquorea* aquorin.[4] The luciferin involved in the latter bioluminescence is coellenterazine, which is also a derivative of imidazo[1,2-*a*]pyrazin-3(7*H*)-one, and has recently been used for detection of traces of Ca^{2+} in biological systems.

As shown in Scheme 1, although the actual emitter of this chemiluminescence of CLA has been identified as the singlet excited 3-acetamido-6-phenylpyrazine, oxyluciferin analogue ^{1}(OCLA)*, which would be formed *via* a dioxetanone intermediate,[2,3] neither the stoichiometry nor the mechanism of the reaction of CLA with superoxide is known. Meanwhile two groups have reported the apparent second-order rate constants (k_{CLA}) for the reaction of CLA with superoxide to be 2.12 × 10^5 dm^3 mol^{-1} s^{-1} in 50 mmol dm^{-3} Tris–HCl buffer solution at pH 7.1 containing 0.1 mmol dm^{-3} ethylenediaminetetraacetate (EDTA) at 25 °C,[5] and 1.08 × 10^8 dm^3 mol^{-1} s^{-1} in 18 mmol dm^{-3} potassium phosphate buffer solution containing 0.17 mg albumin per cm^3 solution at pH 7.8 and 25 °C.[6] Both groups tried to determine k_{CLA} values from the rates of the emission of light during the reaction of CLA with superoxides. A few reasons are conceivable for this serious discrepancy between the two reports. One possibility is that the emission of light is the last step of the consecutive reaction processes in which intermediate(s) accumulate, as has been observed in the chemiluminescence of peroxyoxalates.[7] If this was the case, the rate of the light emission is not equal to k_{CLA}. The other is the effect of additives in the reaction mixtures, *e.g.* albumin[8] and EDTA.[9] Contamination with traces of transition metal ions such as iron may also change the reaction.[2] Thus, the kinetics of both the light emission and the disappearance of CLA have been investigated using the xanthine–xanthine oxidase (XO) enzymatic reaction system as a superoxide supplier in aqueous buffer solutions which were carefully treated with a special ion exchange resin to eliminate transition metal ions.



Scheme 1 Chemiluminescence of *Cypridina* luciferin analogue (CLA) with superoxide

Experimental

Materials

Distilled water was passed through an ion exchange resin before use. All buffer solutions were treated with Millipore Chelex 100 before use to eliminate a trace of transition metal ions. CLA hydrochloride was prepared by the known method.[10] Xanthine was purchased from Sigma. Superoxide dismutase (Cu–Zn type) from bovine erythrocyte was obtained from Wako.

XO from butter milk was purchased from Wako and purified by the following procedure in a cold room according to the method reported by Nishino *et al.*[11] All buffer solutions used for purification and the stock solution of XO contained 1 mmol dm^{-3} salicylic acid as a stabilizer.[11] Commercial XO was dialysed against 10 mmol dm^{-3} Tris–HCl pH 8.0 buffer solution and applied to a column of Whatman DE52 diethylaminocellulose equilibrated with 10 mmol dm^{-3} Tris–HCl buffer solution of pH 8.0. After the column was washed with the same buffer solution, the activity was eluted by 0.2 mol dm^{-3} KCl containing 10 mmol dm^{-3} Tris–HCl buffer solution at pH 8.0. The activity of XO was measured by following the increasing absorbance at 295 nm due to uric acid in 50 mmol dm^{-3} sodium pyrophosphate buffer (pH 8.5) containing 0.5 mmol dm^{-3} xanthine at 25.0 °C. The fractions containing XO were combined, dialysed against 50 mmol dm^{-3} pyrophosphate buffer solution at pH 8.5 and stored in ice–water. Salicylic acid contained in the stock solution of XO was removed by gel filtration just before use since salicylic acid quenches singlet excited light emitter formed in this chemiluminescence.[9]

Methods

The kinetics of the emission of the light in the reaction of CLA with superoxide were performed by counting photons with an Aloka luminescence reader. The reaction was started by adding an aqueous solution of CLA hydrochloride to 3 cm^3 of buffer in a reaction tube mounted in the thermostatted luminescence reader. The rates of disappearance of CLA were measured by recording the decreasing absorbance of CLA on a JASCO UV–VIS spectrophotometer Ubest-50 equipped with a quartz UV cuvette with a water jacket to which water of 20.0 ± 0.1 °C was circulated from a NESLAB RTE-210 thermostatted bath. Chemiluminescence spectra were recorded on a Hitachi fluorescence spectrophotometer F4500. XO assay: the superoxide flux from the xanthine–XO system has been determined by following the increase of the reduced form of cytochrome c.[12]

Results and discussion

CLA, a nitrogen acid, is in an equilibrium with its conjugate base (CLA^-) in water as shown in eqn. (1) (pK_{CLA} = 7.64).[10]

$$CLA \underset{}{\overset{K_{CLA}}{\rightleftharpoons}} CLA^- + H^+ \quad (1)$$

Thus, CLA_t represents the sum of CLA and CLA^-. The

$$[CLA]_t = [CLA] + [CLA^-]$$

superoxide radical anion ($O_2^{\bullet -}$) is the conjugate base of the hydroperoxyl radical ($HO_2^{\bullet}$), an oxygen acid, as shown in eqn. (2) (pK_{SO} = 4.8).[13] Thus, the word 'superoxide' will be used in

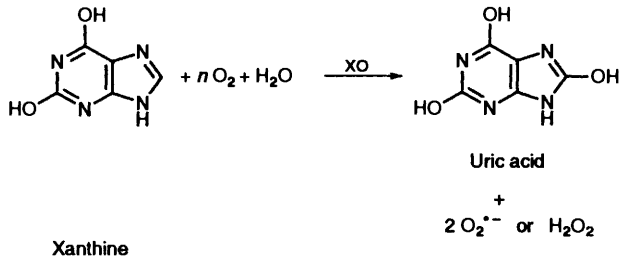
$$HO_2^{\bullet} \underset{}{\overset{K_{SO}}{\rightleftharpoons}} O_2^{\bullet -} + H^+ \quad (2)$$

this paper to designate the sum of superoxide radical anions and hydroperoxyl radicals.

$$[O_2^{\bullet -}]_t = [O_2^{\bullet -}] + [HO_2^{\bullet}]$$

Superoxide generating system

Superoxide radicals were generated by the xanthine–xanthine oxidase (XO)–O_2 system.[12] In this enzymatic reaction xanthine is dehydrogenated to give uric acid while the abstracted electrons are given to molecular oxygen affording either superoxide or hydrogen peroxide (Scheme 2). The rate of



Scheme 2 Generation of superoxide from the reaction of xanthine and molecular oxygen catalysed by xanthine oxidase

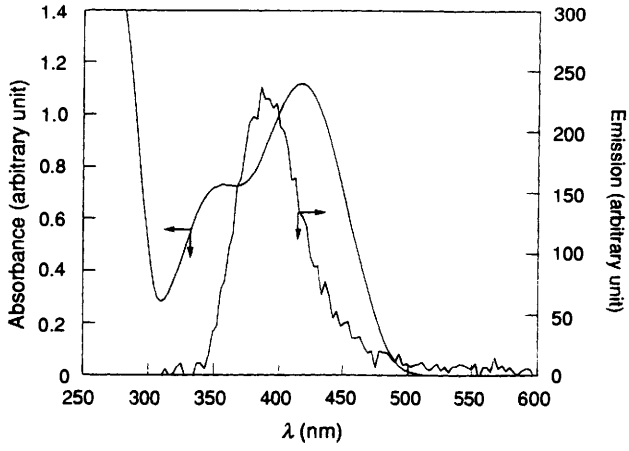


Fig. 1 Absorption spectrum of CLA with $[CLA]_t$ = 120 μmol dm^{-3} and cemiluminescence spectrum recorded in the reaction between CLA_t and superoxide at pH 7.00 ($[CLA]_{t0}$ = 4.47 μmol dm^{-3}; R_i = 43.1 nmol dm^{-3} s^{-1})

superoxide production (R_i; nmol dm^{-3} s^{-1}) was determined from the kinetics of the reduction of ferric- to ferrous–cytochrome c by superoxides in the same manner as previously reported, which was based on the value of ε_{550} (ferric–ferrous) = 19.6 dm^3 mol^{-1} cm^{-1}.[12] If the superoxide forming reaction shown in Scheme 2 proceeds quantitatively, the rate of superoxide production should be twice as large as that for uric acid formation. The actual yield of superoxide was found to depend on the pH of the solution, *e.g.* 36% at pH 7 and 100% at pH 10.

Rates of light emission

The reaction of CLA_t with superoxide in aqueous buffer solutions at pH < 7 gave the chemiluminescence spectrum emitted from singlet excited OCLA, ^{1}[OCLA]* (λ_{em} = 380 nm), whereas the spectrum of λ_{em} = 450 nm due to the singlet excited state of the conjugate base of OCLA, ^{1}[$OCLA^-$]*, was observed in a range pH > 8.5. This pH effect on the chemiluminescence spectra is essentially the same as the chemiluminescence of spontaneous autoxidation of CLA_t.[10] The absorption spectrum and chemiluminescence spectrum of CLA_t recorded at pH 7.0 are shown in Fig. 1.

Fig. 2 illustrates time courses for both the light emission intensity and the concentration of CLA_t observed in the reaction with $[CLA]_t$ = 4.00 μmol dm^{-3} and R_i = 116 nmol dm^{-3} s^{-1} at pH 7.0. If the initial reaction between CLA_t and superoxide is solely the rate-determining step, the time courses of the intensity of the light emission and the concentration of CLA_t should have the same pattern. However, this was not the case. The change in the intensity of light emission appeared to lag behind the disappearance of CLA_t.

This is further exemplified by the time courses of the rate of the light emission recorded at pH 7.90 shown in Fig. 3, in which, while keeping the initial concentration of CLA_t constant, 4.24

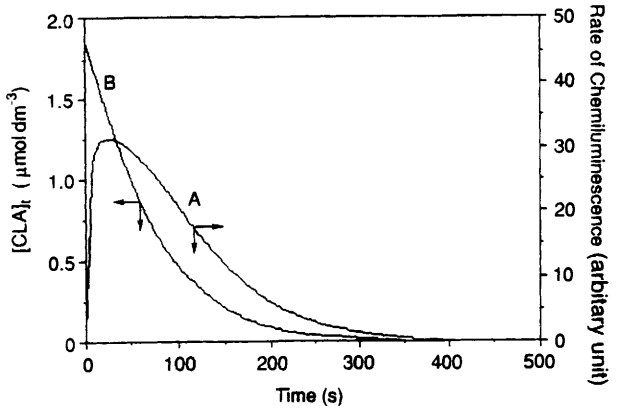


Fig. 2 Time courses of the rate of chemiluminescence and the concentration of remaining CLA in the reaction between CLA_t and superoxide at pH 7.00 with R_i = 116 nmol dm^{-3} s^{-1} $[CLA]_{t0}$ = 4.00 μmol dm^{-3} at 25 °C

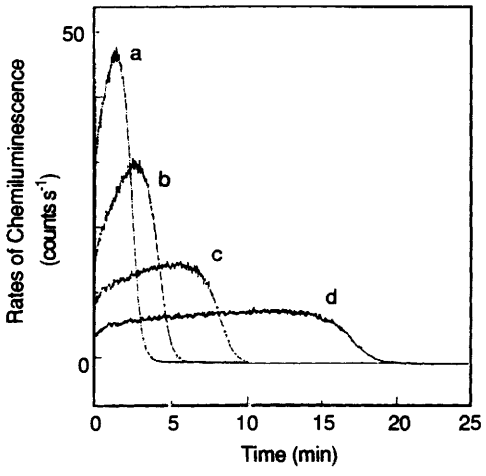


Fig. 3 Time courses of rate of chemiluminescence at pH 7.00 with various R_i values and constant $[CLA]_{t0}$ (4.24 μmol dm^{-3}) at 25 °C

μmol dm^{-3}, R_i was varied from 3.3 to 26 nmol dm^{-3} s^{-1}. When the initial reaction between CLA_t and superoxide is accelerated by the increase of R_i, the lag appears more clearly than with small R_i. These observations reveal that the reaction steps of some intermediates that intervene between the primary intermediate and ^{1}[OCLA]* contribute as rate-determining steps. The overlap of the absorption spectrum of the starting CLA_t and the emission spectrum from [OCLA]* should be partly responsible for the apparent lag of the light emission. Namely, CLA_t molecules act as an internal filter for the chemiluminescence. However, since the reaction was conducted at a very low initial concentration of CLA_t, such an internal filter effect on the absorbance of the chemiluminescence should be a minor factor. Thus, the rate of the emission is not identical to that of the reaction between CLA_t and superoxide. The chemiluminescence reaction of peroxyoxalate has been known as an example which proceeds by this type of sequential reaction kinetics.[7] An attempt to fit the time course of the intensity of the light emission to a consecutive kinetics equation was made assuming the intervention of a single intermediate which partly controls the rate of the light emission as discussed in the chemiluminescence of peroxyoxalate.[7] Unfortunately, the consecutive reaction mechanism involving the single intermediate did not simulate the actual data well. Thus, a precise second-order rate constant could not be obtained for the reaction between CLA_t and superoxide from the time courses of the chemiluminescence.

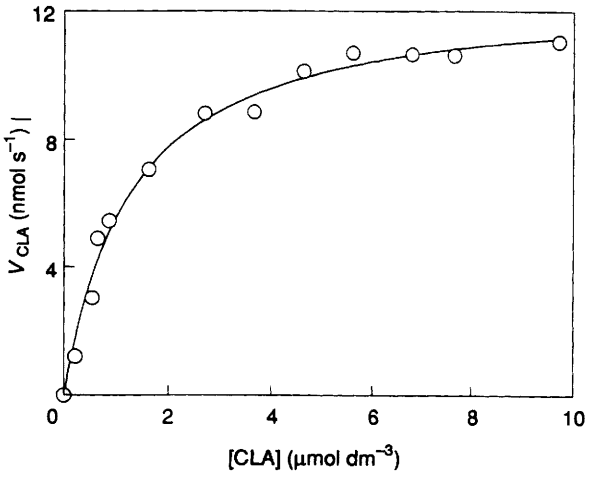
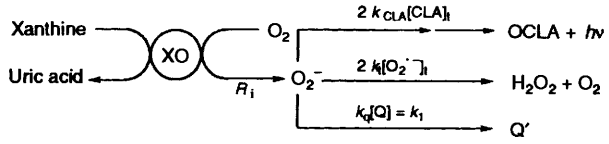


Fig. 4 Plots of initial rates as a function of [CLA] at pH 7.00 with R_i = 21.7 nmol dm^{-3} s^{-1} at 20 °C. The solid line is a theoretical curve calculated from eqn. (3).

Stoichiometry and rates for disappearance of CLA_t

The time course of the disappearance of CLA_t (Curve B in Fig. 2) is characterized as follows. In the initial stage of the reaction, the rate is approximately zero-order with respect to $[CLA]_t$, while the last stage of the reaction obeys approximately first-order kinetics with respect to $[CLA]_t$. An increase in R_i shortens the zero-order kinetic range and finally it disappears if R_i is sufficiently large. In order to obtain precisely the second-order rate constant for the reaction between CLA_t and superoxide, the initial rates for disappearance of CLA_t (V_{CLA}) have been measured with various initial concentrations of CLA_t with constant R_i. The experimental results obtained at pH 7.00 are illustrated in Fig. 4, in which the initial rates for disappearance of CLA_t are plotted against $[CLA]_{t0}$. The rate obeys a first-order kinetic equation if $[CLA]_t$ is small. However, the reaction becomes zero-order in the reaction with large $[CLA]_t$. These observations are in accordance with curve B in Fig. 2 and suggest that the reaction of CLA_t in the presence of the xanthine–XO superoxide generating system takes place as shown in Scheme 3.



Scheme 3 Fates of superoxides generated by the xanthine–XO system in the presence of CLA

There are three fates for superoxide, *i.e.* dismutation to give molecular oxygen and hydrogen peroxide (k_t),[14] reactions with some unknown superoxide quenchers (k_q) other than CLA_t and the reaction with CLA_t (k_{CLA}). Assuming a steady state for $[O_2^{\cdot-}]_t$ and that one mole of CLA_t consumes two moles of superoxide, one gets the kinetic equation (3). If $[CLA]_t$ is very

$$C_{CLA} = \frac{k_{CLA}[CLA]_t(\sqrt{(2k_{CLA}[CLA]_t + k_t)^2 + 8k_tR_i} - 2k_{CLA}[CLA]_t - k_t)}{4k_t} \quad (3)$$

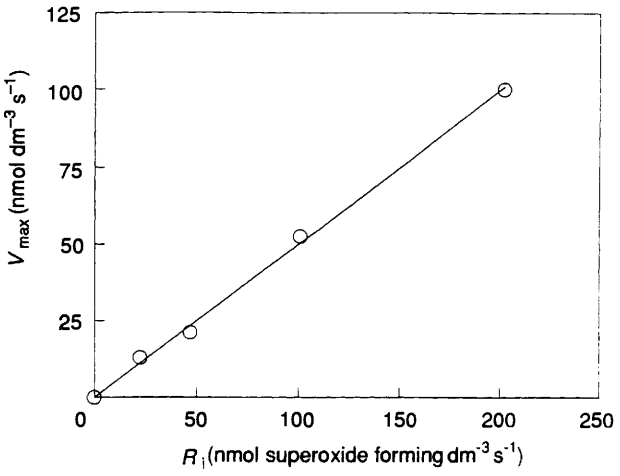


Fig. 5 Plots of V_{max} against R_i at 20.0 °C (pH 7.00). Slope = 0.499 ± 0.008.

small, the dismutation of superoxide predominates over the reaction with CLA to give eqn. (4). Here, $[CLA]_t$ and $[O_2^{\cdot -}]_t$

$$V_{CLA} = \sqrt{\frac{R_i}{k_t}} k_{CLA}[CLA]_t \quad (4)$$

denote concentrations of CLA_t and superoxide, respectively. If $[CLA]_t$ is large enough, the reaction with CLA_t exceeds the dismutation and whole superoxides are trapped by CLA_t and therefore the rate of disappearance of CLA_t is determined by the rate of superoxide generation from XO to give V_{max} [eqn. (5)].

$$V_{CLA} = \frac{R_i}{2} \quad (5)$$

Values of apparent second-order rate constants for the reaction between CLA_t and superoxide (k_{CLA}) and R_i are found to be (4.15 ± 0.32) × 10^5 dm^3 mol^{-1} s^{-1} and (21.7 ± 0.52) nmol dm^{-3} s^{-1}, respectively, by treating the kinetic data with eqn. (3) by a non-linear least squares method. The value of R_i obtained kinetically is well in accordance with the R_i value determined by the kinetics of the reduction of cytochrome c, *i.e.* 21.7 nmol dm^{-3} s^{-1}. The nice consistency of the two R_i values determined by the two different methods reveals that one mole of CLA_t consumes exactly two moles of superoxides under the conditions [eqn. (6)].

$$CLA + 2O_2^{\cdot -} + 2H^+ \longrightarrow OCLA + CO_2 + h\nu \quad (6)$$

Usually, radical-initiated oxidations of organic molecules under aerobic conditions proceed by a chain reaction; *e.g.* superoxide-initiated autoxidation of the reduced form of nicotine adenine dinucleotide (phosphate), NAD(P)H.[12] Such a radical chain autoxidation process, initiated by hydrogen abstraction of CLA with the superoxide radical anion [eqns. (7)–(11)], does not occur in the reaction of CLA_t with superoxide. In order to investigate the effect of R_i on the stoichiometry of the reaction, maximum rates were measured by the same procedure as shown in Fig. 5 with various R_i values. Values of V_{max} in the solution of pH 7.00 are plotted against R_i in Fig. 5.

A linear relationship with slope 0.5 clearly shows that the contribution of the autoxidation involving chain process is nil. The same was true in the pH range 4.5–10. When R_i is small while [CLA] is large, a tendency for V_{max} to exceed $R_i/2$ slightly was observed. Under such conditions, the reaction process of chain autoxidation [eqns. (7)–(11)] is significant.

$$CLA + O_2^{\cdot -} + H^+ \longrightarrow CLA^{\cdot} + H_2O_2 \quad (7)$$

$$CLA^{\cdot} + O_2 \longrightarrow CLA\text{–}O_2^{\cdot} \quad (8)$$

$$CLA\text{–}O_2^{\cdot} + CLA \longrightarrow CLA\text{–}O_2H + CLA^{\cdot} \quad (9)$$

$$CLA^{\cdot} + O_2^{\cdot -} \longrightarrow CLAO_2^{\cdot -} \quad (10)$$

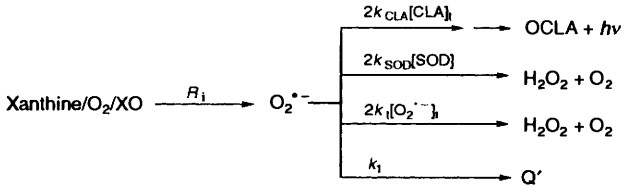
$$2CLA^{\cdot} \longrightarrow (CLA)_2 \quad (11)$$

Goto and co-workers, reported that the coupling of CLA$^{\cdot}$ takes place in the single electron oxidation of CLA [eqn. (11)]. Since the dimer, $(CLA)_2$, forms under aerobic conditions, the reaction of CLA$^{\cdot}$ with molecular oxygen is slow compared with the dimerization. $(CLA)_2$ has an intense characteristic visible light absorption band at a longer wavelength than CLA.[15] However, careful examination of the time-dependent visible spectra of the reaction mixture failed to detect any formation of the dimer. These observations indicate that the reaction of CLA$^{\cdot}$ with the second superoxide radical exceeds the other competitive reactions, *i.e.* reactions (8) and (11). If R_i is very small, *i.e.* at low $[O_2^{\cdot -}]_t$ and high $[O_2]$, reaction (8) can compete with reaction (10) initiating the radical chain autoxidation process.

In the procedure for obtaining k_{CLA} by means of the plot in Fig. 4, values of V_{CLA} with the low $[CLA]_t$ are very critical; however, under such conditions the reproducibility of the rate of CLA_t consumption is poor, since the rate for reaction of CLA_t with superoxide becomes comparable or less than the other superoxide reactions, *e.g.* disproportionation and the reactions with unidentified quenchers of superoxide other than CLA_t such as xanthine and uric acid.[14] On the other hand, since reproducibility of V_{max} is much better than the rates measured with low $[CLA]_t$, values of the apparent second-order rate constant (k_{CLA}) have been determined by the competitive method with superoxide dismutase (SOD) under the V_{max} conditions as mentioned below.

Determination of k_{CLA} by competitive kinetics

In the presence of SOD and CLA_t, superoxides generated with rate R_i disappear by four pathways as shown in Scheme 4.



Scheme 4 Fates of superoxides generated by xanthine–XO system in the presence of SOD and CLA under the conditions of $R_i = (k_{CLA} + k_{SOD})/2$

Under the conditions $2k_{CLA}[CLA]_t \gg (2k_t[O_2^{\cdot -}]_t + k_1)$, namely, $V_{CLA} \simeq V_{max}$, the rate of CLA consumption ($V_{max/SOD}$) is given by eqn. (12). Second-order rate constants for the

$$V_{max/SOD} = \frac{k_{CLA}R_i[CLA]_t}{k_{SOD}[SOD] + 2k_{CLA}[CLA]_t} \quad (12)$$

reaction of SOD with superoxide (k_{SOD}) at various pHs have been reported by Klug *et al.* at 20 °C.[16] The values of k_{CLA} were obtained by fitting $V_{max/SOD}$ values obtained at various concentrations of SOD into eqn. (12). The results obtained at pH 7.00 are shown in Fig. 6.

Fig. 6(*a*) illustrates $V_{max/SOD}$ as a function of [SOD]. From the fitting of $V_{max/SOD}$ values shown in Fig. 6(*a*) into eqn. (10), k_{CLA} = (2.39 ± 0.17) × 10^5 dm^3 mol^{-1} s^{-1} was obtained. The solid line in Fig. 6(*a*) is a theoretical curve obtained by substituting k_{CLA}

Table 1 Apparent second-order rate constants (k_{CLA}) for the reaction of CLA_t with superoxide at 20 °C

pH	k_{CLA}/dm^3 mol^{-1} s^{-1}	k_{SOD}/10^{-9} dm^3 mol^{-1} s^{-1} [a]	R_i/nmol dm^{-3} s^{-1}	R_i/nmol dm^{-3} s^{-1}	[CLA]/μmol dm^{-3}	[SOD]/nmol dm^{-3}
3.5	1.01 ± 0.02 × 10^6	0.48	*d*	18.0	151	0–80[e]
4.4	6.08 ± 0.30 × 10^5	1.54	*d*	17.6	136	0–80[e]
5.2	6.29 ± 0.09 × 10^5	2.9	*d*	47.6	145	0–50[e]
6.1	4.46 ± 0.15 × 10^5	3.25	*d*	27.3	132	0–40[e]
7.0	2.39 ± 0.17 × 10^5	2.3	102	111	45.2	0–14[e]
7.0	2.76 ± 0.12 × 10^5	2.3	85.3	84.0	22.9	0–7[e]
7.0	2.05 ± 0.83 × 10^5	2.3	0–120[f]	—	26.2	3.98
8.2	6.90 ± 0.34 × 10^4	1.9	130	125	40.6	0–8
8.2	6.53 ± 0.29 × 10^4	1.9	122	138	38.5	0–7[e]
9.0	3.28 ± 0.26 × 10^4	1.8	145	202	78.3	0–10[e]
9.7	9.98 ± 0.75 × 10^3	1.6	150	156	79.6	0–7[e]
9.7	9.18 ± 0.26 × 10^3	1.6	0–160[f]	—	84.1	9.56

[a] Ref. 10. [b] Determined by the reduction of cytochrome c (nmol dm^{-3} s^{-1}). [c] Determined from CLA decay by eqn. (12) (nmol dm^{-3} s^{-1}). [d] Value of R_i could not be determined at this pH by reduction of cytochrome c. [e] With variable [SOD] and fixed R_i. [f] With variable R_i and fixed [SOD].

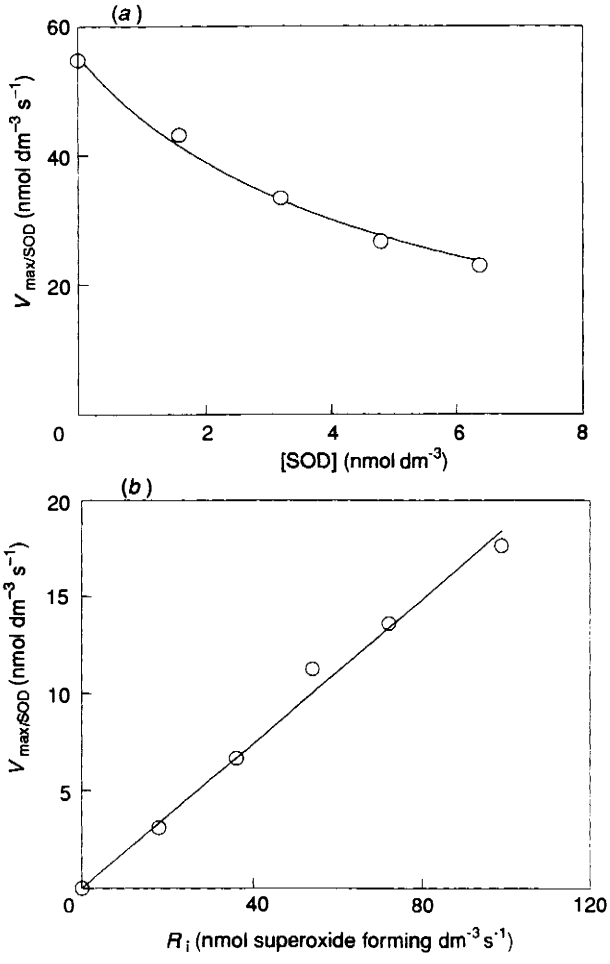


Fig. 6 (*a*) Plots of $V_{max/SOD}$ as a function of [SOD] at pH 7.00 with R_i = 111 nmol dm^{-3} s^{-1} at 20 °C. The value of k_{CLA} was obtained as 2.39 ± 0.17 × 10^5 mol^{-1} dm^3 s^{-1}. (*b*) Plots of V_{CLA} as a function of R_i at pH 7.00 with [SOD] = 3.98 nmol dm^3 at 20 °C. k_{CLA} was obtained as 2.05 ± 0.83 × 10^5 mol^{-1} dm^3 s^{-1}. Solid lines are theoretical, calculated from eqn. (12) and (O) are experimentally obtained k_{CLA} values.

in eqn. (12) with this experimental value. According to eqn. (12), if both [SOD] and $[CLA]_t$ are constant, $V_{max/SOD}$ should correlate linearly with R_i. In fact, a linear relationship between $V_{max/SOD}$ and R_i was observed, as shown in Fig. 6(*b*). From the slope of Fig. 6(*b*), k_{CLA} was calculated to be (2.05 ± 0.83) × 10^5 dm^3 mol^{-1} s^{-1}. Thus, the validity of eqn. (12) has been established not only by the co-incidence of experimental points

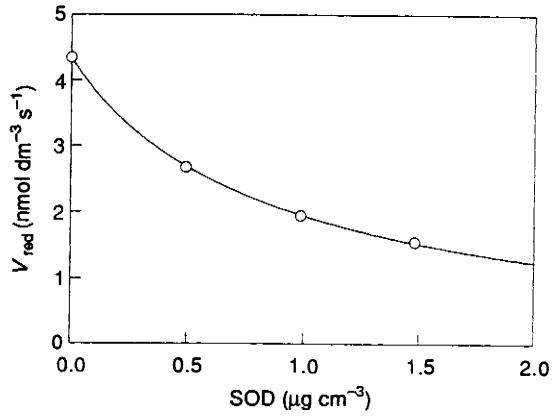
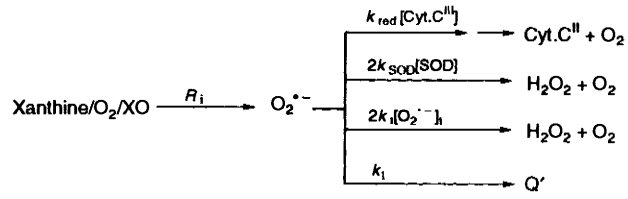


Fig. 7 Plots of the initial reduction rates of cytochrome c (V_{red}) as a function of [SOD] at pH 7.00 with [cytochrome c^{III}] = 11.6 μmol dm^{-3} at 20 °C. The line is a theoretical curve calculated from eqn. (13).

with the theoretical lines in Fig. 6(*a*) and (*b*), but also by a good agreement of k_{CLA} values calculated from the two different sources of $V_{max/SOD}$ values in Fig. 6(*a*) and (*b*). By the same procedure, k_{CLA} values have been obtained at various pH and are listed in Table 1.

In this competitive kinetic method, it is very important to determine the concentration of SOD precisely. Commercially available SOD is not a pure protein. This was accomplished by the similar competitive kinetic method at pH 7.00 [Scheme 5,



Scheme 5 Fates of superoxides generated by the xanthine–XO system in the presence of SOD and cytochrome c

eqn. (13) and Fig. 7], based on reported values of the second-

$$V_{red} = \frac{k_{red}R_i[\text{cyt.C}^{III}]}{k_{red}[\text{cyt.C}^{III}] + k_{SOD}[\text{SOD}]} \quad (13)$$

order rate constant for the dismutation of superoxide by SOD, k_{SOD} = (2.30 ± 0.17) × 10^9 dm^3 mol^{-1} s^{-1} and that for the reaction of superoxide with cytochrome c, k_{red} = 1.04 × 10^6 dm^3 mol^{-1} s^{-1} at 20 °C.[16,17] Scheme 5 illustrates the reaction pathways of superoxide in the presence of SOD and cytochrome

c. Under the V_{max} conditions, the rate of reduction of cytochrome c (V_{red}) is expressed by eqn. (13) as a function of concentration of the ferric form of cytochrome c ([Cyt.C^{III}]), [SOD] and R_i. Values of V_{red} have been determined with various concentrations of SOD with constant [Cyt.C^{III}] and R_i. Values of the latter two were determined based on ε_{550} (ferric–ferrous) = 19.6 dm^3 mol^{-1} cm^{-1} for cytochrome c.[12] 1 μg dm^{-3} [SOD] was thus determined to be 43.4 nmol dm^{-3} by fitting the experimental data shown in Fig. 7 into eqn. (13).

Effect of pH on k_{CLA}

As mentioned at the beginning, both CLA_t and superoxide in water are mixtures of acid and conjugate base forms. Therefore, the reaction of CLA_t and 'superoxide' in water consists of the following four kinds of second-order reactions shown in eqns. (14)–(17).

$$CLA + HO_2^{\bullet} \xrightarrow{k_{aa}} CLA^{\bullet} + H_2O_2 \quad (14)$$

$$CLA^- + HO_2^{\bullet} \xrightarrow{k_{ba}} CLA^{\bullet} + HO_2^- \quad (15)$$

$$CLA + O_2^{\bullet -} \xrightarrow{k_{ab}} CLA^{\bullet} + HO_2^- \quad (16)$$

$$CLA^- + O_2^{\bullet -} + H_2O \xrightarrow{k_{bb}} CLA^{\bullet} + HO_2^- + HO^- \quad (17)$$

In order to determine these four second-order rate constants from the kinetic results that have been obtained in this work, log k_{CLA} values are plotted against pH, as shown in Fig. 8. As mentioned in an earlier context, the rate of CLA decay is first-order with respect to $[CLA]_t$ and $[O_2^{\bullet -}]_t$, respectively [eqn. (18)]. Thus, the apparent second-order rate constant, k_{CLA}, is

$$V_{CLA} = k_{CLA}[CLA]_t[O_2^{\bullet -}]_t \quad (18)$$

connected with the above four second-order rate constants and the two dissociation constants as a function of $[H^+]$ by eqn. (19).

$$k_{CLA} = \frac{k_{aa}[H^+]^2 + k_{ba}K_{CLA}[H^+] + k_{ab}K_{SO}[H^+] + k_{bb}K_{CLA}K_{SO}}{(K_{CLA} + [H^+])(K_{SO} + [H^+])} \quad (19)$$

Fitting the experimentally observed k_{CLA} values into eqn. (19) yields k_{aa} = (7.03 ± 0.59) × 10^5 dm^3 mol^{-1} s^{-1}, k_{ba} = (2.56 ± 0.40) × 10^8 dm^3 mol^{-1} s^{-1}, k_{ab} = (6.02 ± 8.08) × 10^3 dm^3 mol^{-1} s^{-1} and k_{bb} = (5.45 ± 5.78) × 10^4 dm^3 mol^{-1} s^{-1} at 20.0 °C. The theoretical curve is illustrated by a solid line in Fig. 8. The fact that values of both k_{ab} and k_{bb} have large errors reveal that these two are very minor contributors to k_{CLA}. The contribution of k_{bb} is practically zero and that of k_{ab} is only 0.1% or less at pH 4 and 1.6% or less at pH = 7–10. Over the whole pH range examined, experimentally observed k_{CLA} values agree well with the sum of k_{aa} and k_{ba}. Reaction (14) predominates over reaction (15) in the low pH region, while reaction (15) predominates over reaction (14) at pH > 5. For example, k_{aa} accounts for 92.2% of k_{CLA}, while k_{ba} accounts for 7.7% of k_{CLA} at pH 4; k_{aa} is responsible for 1.2% of k_{CLA} while k_{ba} accounts for 97.2% of k_{CLA} at pH 7. In this way, the active species of superoxide which initiates the reaction with CLA has been identified as the hydroperoxyl radical rather than superoxide radical anion.

The value of k_{CLA} determined at pH 7 in this work, (2.40 ± 0.24) × 10^5 dm^3 mol^{-1} s^{-1} (20 °C) is consistent with the value 2.12 × 10^5 dm^3 mol^{-1} s^{-1} (25 °C) at pH 7.1 reported by Goto and Niki.[5] On the other hand, the k_{CLA} value at pH 7.8 calculated from the theoretical curve shown in Fig. 6, 2.5 × 10^5 dm^3 mol^{-1} s^{-1} (20 °C), is very different from the value, 1.08 × 10^8 dm^3 mol^{-1} s^{-1} (25 °C), reported by Suzuki *et al.*[6]

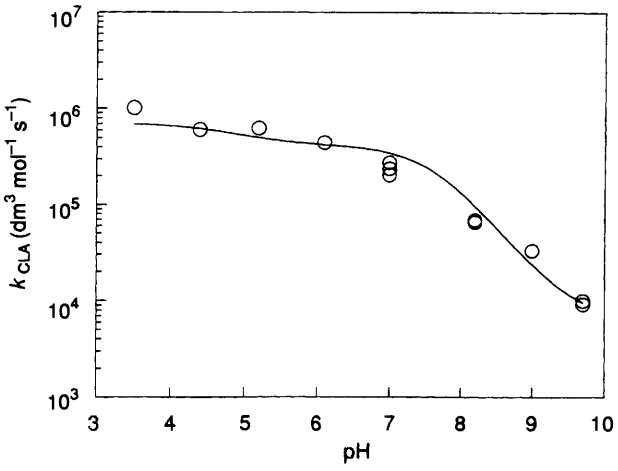
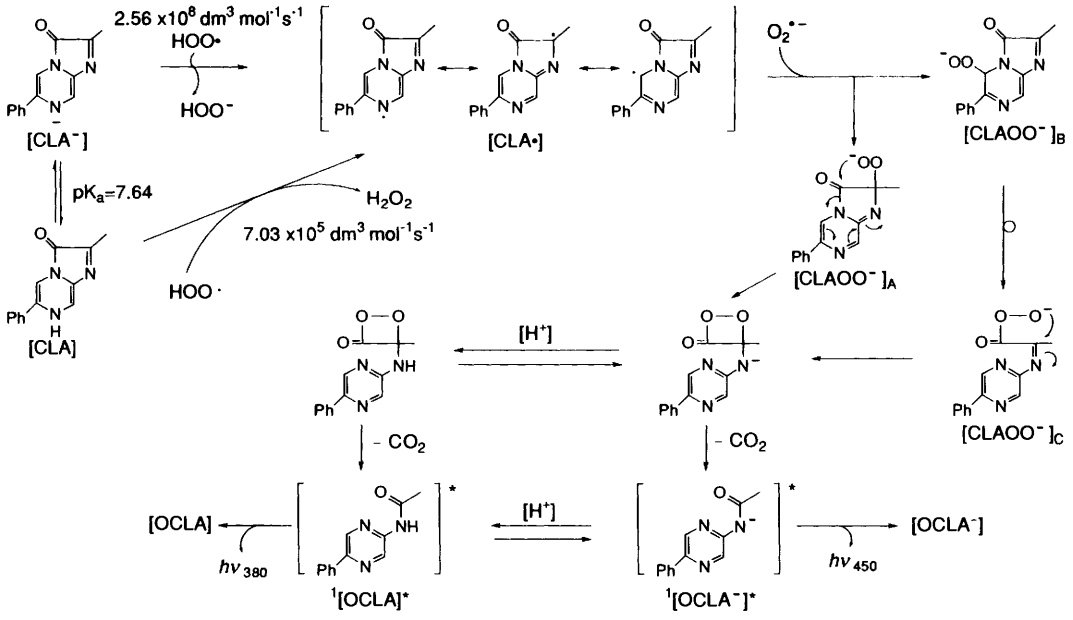


Fig. 8 Plots of k_{CLA} as a function of pH at 20 °C. The line is a theoretical curve calculated from eqn. (19).

Reaction mechanisms

From all of these observations and the reaction mechanism proposed for the spontaneous autoxidation of CLA, the mechanism shown in Scheme 6 has been suggested for the reaction of CLA with superoxides. From the following observations, the reaction between CLA and superoxide is initiated by a single electron oxidation of CLA with hydroperoxyl radical to generate the CLA radical, which then reacts with a second superoxide very quickly. (*i*) The rate of CLA decay is first-order with respect to the concentrations of CLA and superoxide, respectively. (*ii*) CLA^- is 360 times more reactive than CLA toward $HO_2^{\bullet}$. This is consistent with the oxidation potential of CLA^- that has been observed to be 540 mV more cathodic than that of CLA.[18] (*iii*) $HO_2^{\bullet}$ is 100 times more reactive than $O_2^{\bullet -}$ toward CLA. (*iv*) One mole of CLA consumes two moles of superoxides. The coupling of $CLA^{\bullet}$ with superoxide [eqn. (8)] exceeds the coupling reaction with molecular oxygen, which initiates the chain autoxidation reaction in air [eqns. (6) and (7)]. The cyclic voltammetric studies of CLA also support this mechanism, *i.e.* it suggests that the reaction between $CLA^{\bullet}$ and molecular oxygen is very slow.[18] However, the coupling reaction with molecular oxygen cannot be negligible under a pure oxygen atmosphere where one mole of superoxide consumes more than 0.5 mole of CLA. This is the feature that is different from the uncatalysed spontaneous autoxidation of CLA where no chain reaction takes place even under pure oxygen, since the reaction of CLA^- with molecular oxygen affords an intimate pair of $CLA^{\bullet}$ and $O_2^{\bullet -}$ which is ready to couple.[10] On the other hand, since $CLA^{\bullet}$ and superoxide are born separately in the reaction conditions of this work, both species have to drift to encounter. Thus, superoxide and molecular oxygen are an equal distance from $CLA^{\bullet}$, and therefore, the autoxidation of CLA by the chain mechanism takes place more easily in the reaction with superoxide than in the spontaneous autoxidation.

There are two possibilities for the coupling of superoxide with $CLA^{\bullet}$, *i.e.* at the 2-position to afford $(CLAO_2H)_A$ and at the 5-position forming $(CLAO_2H)_B$ which rearranges to a peroxyacid $(CLAO_2H)_C$ through the similar mechanism for the rearrange-



Scheme 6 Mechanisms for reactions of CLA with superoxides

ment of acyl carbonates.[19] Although we preferred the latter possibility in a previous paper, the recent investigation of spontaneous chemiluminescence of CLA in acetonitrile suggests that the reaction *via* $(CLAO_2H)_A$ is a major process while $(CLAO_2H)_B$ is a minor contributor.[20] The reaction is followed by cyclization intramolecular nucleophilic substitution to give a dioxetanone derivative,[3b] which upon decarboxylation affords singlet excited light emitter as illustrated in Scheme 6.[10]

Appendix

Derivation of eqn. (3)

One mole of CLA_t consumes two moles of superoxide. Under the steady-state kinetics conditions, the rate of superoxide supply from XO is equal to the total rate of superoxide consuming reaction paths shown in Scheme 3 [eqn. (A1)].

$$R_i = \{2k_{CLA}[CLA]_t + k_1 + 2k_t[O_2^{\bullet -}]_t\}[O_2^{\bullet -}]_t \quad (A1)$$

Thus, the steady-state concentration of superoxide is given by eqn. (A2).

$$[O_2^{\bullet -}]_t = \frac{\{\sqrt{(2k_{CLA}[CLA] + k_1)^2 + 8k_tR_i} - 2k_{CLA}[CLA] - k_1\}}{4k_t} \quad (A2)$$

The rate for the decay of CLA_t is first order with respect to $[CLA]_t$ and $[O_2^{\bullet -}]_t$. Eqn. (3) is obtained from eqns. (A2) and (A3).

$$V_{CLA} = -d[CLA]_t/dt = k_{CLA}[CLA]_t[O_2^{\bullet -}]_t \quad (A3)$$

Derivation of eqn. (19)

Since CLA_t is consumed by the four reaction pathways in eqns. (14)–(17), one gets eqn. (A4).

$$V_{CLA} = k_{aa}[CLA][HO_2^{\bullet}] + k_{ba}[CLA^-][HO_2^{\bullet}] + k_{ab}[CLA][O_2^{\bullet -}] + k_{bb}[CLA^-][O_2^{\bullet -}] \quad (A4)$$

From eqn. (1),

$$[CLA] = [H^+][CLA]_t/(K_{CLA} + [H^+]) \quad (A5)$$

$$[CLA^-] = K_{CLA}[CLA]_t/(K_{CLA} + [H^+]) \quad (A6)$$

From eqn. (2),

$$[HO_2^{\bullet}] = [H^+][O_2^{\bullet -}]_t/(K_{SO} + [H^+]) \quad (A7)$$

$$[O_2^{\bullet -}] = K_{SO}[O_2^{\bullet -}]_t/(K_{SO} + [H^+]) \quad (A8)$$

One obtains eqn. (19) from eqns. (A3), (A4) and (A5)–(A8).

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