Existence of a New Reactive Intermediate Oxygen Species in Hypoxanthine and Xanthine Oxidase Reaction

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We investigated a hypoxanthine (HPX) and xanthine oxidase (XOD) reaction by using a luminol analog 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione sodium salt (L-012)-mediated chemiluminescence (CL) response. Addition of a high activity of superoxide dismutase (SOD), a potent $O_2^{-\cdot}$ scavenger, and of a high concentration of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), a potent spin trapping agent, diminished completely the CL response. Whereas a high concentration of dimethyl sulfoxide (DMSO), as a potent $^{\cdot}OH$ scavenger could not attain to the complete diminishment of the CL response. It has been reported that luminol monoanion reacts with $^{\cdot}OH$ to form luminol radical, and then resultant luminol radical reacts with $O_2^{-\cdot}$ to elicit CL response. Complete scavenging for $^{\cdot}OH$ is assumed to result in lack of luminol radical, which in turn induces lack of CL response. However, our results did not support the idea. Furthermore, we examined the effect of L-012 on the DMPO-OOH formation in the presence or absence of DMSO in the HPX-XOD system by applying an electron spin resonance (ESR)-spin trapping method. The DMPO-OOH formation was inhibited even in the presence of DMSO, and the rate constant (k_2) between L-012 and $O_2^{-\cdot}$ obtained in the presence of DMSO was 9.77×10^2 M^{-1} s^{-1} and the constant in the absence of DMSO was 2.97×10^3 M^{-1} s^{-1}. The data suggests that L-012 is converted to a radical form that reacts with $O_2^{-\cdot}$ even under the conditions of the absence of $^{\cdot}OH$. From these, we postulate that the existence of a reactive intermediate oxygen species in the HPX-XOD system.

Key words reactive intermediate oxygen species; hypoxanthine; xanthine oxidase

An electron spin resonance (ESR)-spin trapping method and a chemiluminescenece (CL) method are frequently used for quantitative determination of reactive oxygen species (ROS) such as $O_2^{-\cdot}$ and $^{\cdot}OH$. A major advantage of ESR-spin trapping method is to determine separately $O_2^{-\cdot}$ and $^{\cdot}OH$ according to the hyperfine coupling constants,[1] whereas ROS-selectivity by a CL method is poor. Although the luminol-CL reaction has been well documented,[2] reaction processes in the ROS generation system such as hypoxanthine (HPX) and xanthine oxidase (XOD) system are not clear.

Here we investigated the HPX-XOD system by using 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione sodium salt (L-012), a luminol analog, mediated CL response, and found a possibility of existence of a reactive intermediate oxygen species relevant to $O_2^{-\cdot}$ and $^{\cdot}OH$.

Experimental

Reagents were purchased from the following sources: 8-amino-5-chloro-7-phenylpyrido[3,4-*d*]pyridazine-1,4-(2*H*,3*H*)dione sodium salt (L-012) and dimethyl sulfoxide (DMSO) from Wako Pure Chemical Industries (Osaka, Japan); xanthine oxidase (XOD from cow's milk) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Labotec (Tokyo, Japan); hypoxanthine (HPX) and superoxide dismutase (SOD from bovine erythrocytes, product no. S5395) from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other reagents used were of analytical grade.

CL determinations of ROS generated by HPX-XOD cell-free system used in this study was essentially identical to those described in our previous papers.[3,4] A reaction mixture containing 10 μl of 2 μM L-012 solution, 50 μl of 0.6 mM EDTA in 0.2 M Tris–HCl buffer (pH 7.6), 10 μl of 20 mU/ml of XOD in 0.2 M Tris–HCl buffer containing 0.6 mM EDTA, 20 μl of pure water, and 10 μl of sample or solvent (50 mM Tris–HCl buffer) alone was dispensed into a ϕ 1.6 cm×1.0 cm petri dish. The samples were prepared by mixing 10 μl of different concentrations of SOD dissolved in 50 mM Tris–HCl buffer (pH 7.5), DMPO dissolved in pure water or DMSO diluted with pure water. After preincubation for 1 min at 25 °C, the reaction was triggered by the addition of 100 μl of 1 mM HPX, and the CL intensity was recorded continuously for 5 min using a CLA-210 Chemiluminescence analyzer (Tohoku Electronic Industrial Co., Ltd., Sendai, Japan), and the intensity, cumulative over 5 min, was expressed as total counts.

ESR-spin trapping determinations of ROS generated by HPX-XOD cell-free system used in this study was essentially identical to those described in our previous papers.[3—5] In the assay of effect of SOD, in brief, 50 μl of 2 mM HPX, 30 μl of DMSO, and 50 μl of different activity of SOD or solvent (50 mM Tris–HCl, pH 7.5) alone, 20 μl of 4.5 M DMPO, and 50 μl of 0.4 U/ml XOD were placed in a test tube and mixed. In the assay of effect of DMSO, in brief, 50 μl of 2 mM HPX, 30 μl of different concentrations of DMSO, and 50 μl of pure water, 20 μl of 4.5 M DMPO, and 50 μl of 0.4 U/ml XOD were placed in a test tube and mixed. Different concentrations of DMSO were prepared by diluting with pure water. In the assay, the concentration of XOD was about 100 times higher than in the CL method due to the high sensitivity of the CL method. The mixture was transferred to an ESR spectrometry cell, and the DMPO-OOH and DMPO-OH spin adducts were quantified 97 s after the addition of XOD. The measurement conditions for ESR (JES-FA-100, JEOL, Tokyo, Japan) were as follows: field sweep, 330.50—340.50 mT; field modulation frequency, 100 kHz; field modulation width, 0.07 mT; amplitude, 200; sweep time, 2 min; time constant, 0.1 s; microwave frequency, 9.420 GHz; microwave power, 4 mW.

Results and Discussion

Figure 1 shows the effect of SOD, a potent $O_2^{-\cdot}$ scavenger, on the L-012 mediated CL response in the HPX-XOD system. When HPX was added to the reaction mixture, a rapid CL response was observed, and the CL response was reduced by the presence of SOD in an activity dependent manner. When 500 mU/ml of SOD was added to the reaction mixture, CL response was reduced by 99% or more (data not shown). In addition, DMPO, a potent spin trapping agent, reduced the CL-response in concentration-dependent manner and achieved 99% or more reduction at a high concentration (data not shown). Figure 2a shows the effect of DMSO, a potent $^{\cdot}OH$ scavenger, on the L-012 mediated CL response in HPX-XOD system. Compared with the CL-response of sol-

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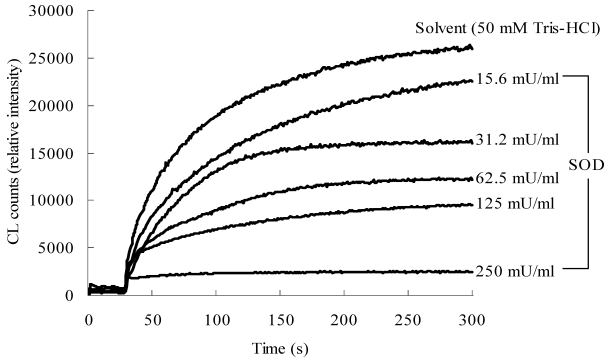

Fig. 1. Effect of SOD, a Potent $O_2^{-\cdot}$ Scavenger, on the L-012 Mediated CL Response in the HPX-XOD System

Each curve represents the mean of duplicate determinations.

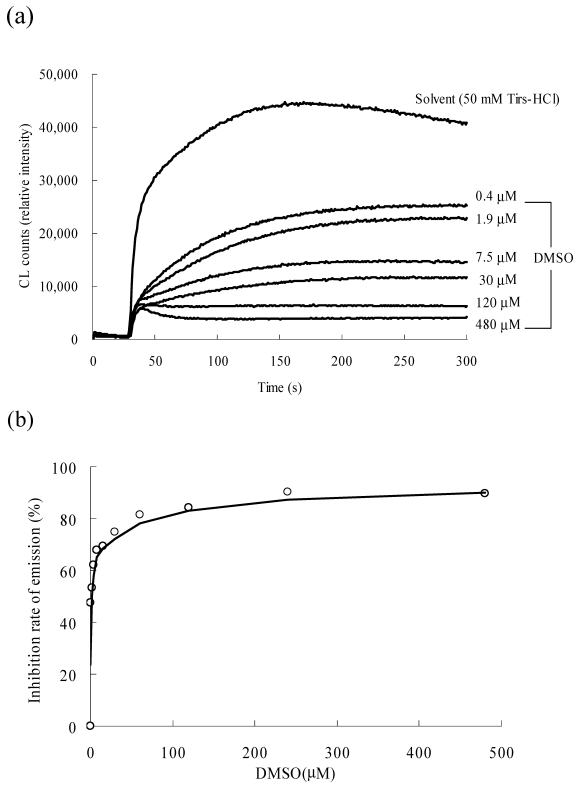

Fig. 2. Effect of DMSO, a Potent ·OH Scavenger, on the L-012 Mediated CL Response in the HPX-XOD System

(a) Representative CL responses, (b) inhibition rates of CL responses by the addition of different concentrations of DMSO. Each curve of CL responses and each value of inhibition rates represent the mean of duplicate determinations.

vent control in Fig. 1, the reaction rate was greater in Fig. 2a. Since the sensitivity of the CL method is very high, the control responses differ among assays. In the assay in Fig. 2a, DMSO reduced the CL-response in a concentration-dependent manner.

As shown in Fig. 2b, DMSO at concentrations of 240 μM or more reached the maximal reduction of CL-response. However, unlike the cases of SOD and DMPO, even a high concentration of DMSO reduced the CL-response by up to 90%.

It has been reported that a luminol-mediated CL response occurs by the following equations.[2)]

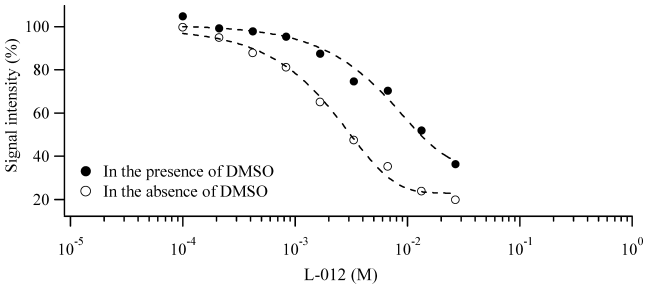

Fig. 3. Inhibition Curves on the DMPO-OOH Formation Obtained by the Addition of Different Concentrations of L-012 in the Presence or Absence of DMSO in the HPX-XOD System

Each value represents the mean of duplicate determinations.

Table 1. IC_{50}s and the Rate Constants (k_2) Obtained from the Inhibition Curves on the DMPO-OOH Formation (Fig. 3) Obtained by the Addition of Different Concentrations of L-012 in the Presence or Absence of DMSO in the HPX-XOD System

	IC_{50} (M)	k_2 ($M^{-1}s^{-1}$)
DMSO (+)	8.2×10^{-3}	9.77×10^{2}
DMSO (−)	2.7×10^{-3}	2.97×10^{3}

$$HPX+XOD+2O_2\rightarrow 2H^+ + 2O_2^{-\cdot} \quad \text{(Ia)}$$

$$2O_2^{-\cdot}+2H^+\rightarrow O_2+H_2O_2 \quad \text{(Ib)}$$

$$O_2^{-\cdot}+H_2O_2\rightarrow O_2+OH^-+{}^{\cdot}OH \quad \text{(A)}$$

$${}^{\cdot}OH+LH^-\rightarrow OH^-+H^++L^{-\cdot} \quad \text{(B)}$$

$$L^{-\cdot}+O_2^{-\cdot}\rightarrow LO_2^{2-}$$

$$LO_2^{2-}\rightarrow N_2+AP^{2-}\rightarrow AP^{2-}+h\nu \quad \text{(C)}$$

LH^-: luminol monoanion,
$L^{-\cdot}$: luminol radical,
AP^{2-}: aminophthallate dianion

According to the equations, LH^- reacts with ·OH to form $L^{-\cdot}$, and then resultant $L^{-\cdot}$ reacts with $O_2^{-\cdot}$. Therefore, maximal scavenging for ·OH is assumed to result in the lack of CL response to the full. However, addition of DMSO could not achieve near 100% reduction of the CL response, so that we speculate the existence of an intermediate reactive to LH^-.

To further confirm this, we examined the effect of L-012 on the DMPO-OOH formation in the presence or absence of DMSO in the HPX-XOD system by applying the ESR-spin trapping method. Figure 3 shows the inhibition curves obtained by the addition of different concentrations of L-012, and Table 1 summarizes the IC_{50}s (the concentrations which inhibit the formation of the spin adduct by 50%) and the rate constants. We calculated the rate constant (k_2) by using the equation and k_1 (the rate constant between DMPO and $O_2^{-\cdot}$) as reported previously.[6)]

$$O_2^{-\cdot}+\text{DMPO}\xrightarrow{k_1}\text{DMPO-OOH}$$

$$O_2^{-\cdot}+\text{L-012}\xrightarrow{k_2}\text{L-012-OOH}$$

$k_2=k_1\cdot[\text{DMPO}]/IC_{50}$
$k_1=1.8\times10\ M^{-1}s^{-1}$ (from ref. 7)
$[\text{DMPO}]=4.45\times10^{-1}$ M

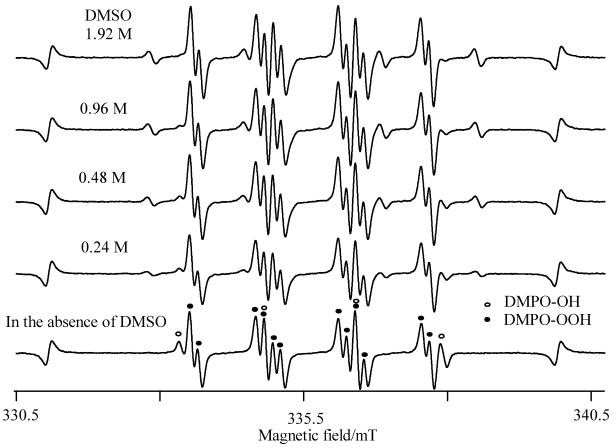

Fig. 4. Representative ESR Spectra Showing the Effect of DMSO, a Potent ·OH Scavenger, on the DMPO-OOH Formation in the HPX-XOD System

$IC_{50}=8.2\times10^{-3}$ M or 2.7×10^{-3} M
$k_2=9.77\times10^{2}$ M^{-1} s^{-1} or 2.97×10^{3} M^{-1} s^{-1}

To explain the decreased rate constant by DMSO, there are two possibilities. One is that the rate of $L^{-\cdot}$ formation was reduced to about 1/3 because of decreased amount of ·OH, which reacts with LH^- to form $L^{-\cdot}$. The rate constant of DMSO and ·OH is 10^9 order,[8] whereas the rate constant of $LH^{-\cdot}$ and ·OH is smaller than that of mannitol and ·OH (10^8 order)[8,9] as shown by our preliminary study (unpublished data). Thus, a high concentration of DMSO assumed to scavenge ·OH to the full, so that $L^{-\cdot}$ formation was likely reduced to the full. The other is the existence of the intermediate reactive to L-012 in the presence of ·OH and $O_2^{-\cdot}$, and/or direct reaction of L-012 and $O_2^{-\cdot}$.

The RO_3H species (R=H, alkyl) have been believed to be key intermediates in the natural and polluted atmosphere,[10,11] and in the low-temperature ozonation of various organic substances.[12] As a case of biological system, it has been reported that all antibodies are capable of catalyzing the oxidation of water by singlet oxygen ($^1O_2$) to generate H_2O_2 and probably O_3. The authors postulated that these antibodies carried the reaction through H_2O_3 (HOOOH) as a key intermediate in the following way.[13]

$$2\,^1O_2+2H_2O\rightarrow 2HOOOH\rightarrow H_2O_4+H_2O_2\rightarrow H_2O_2+\,^3O_2+H_2O_2$$

It has been reported that the HPX-XOD system consists of following equations.[14]

$$HPX+XOD+2O_2\rightarrow 2H^++2O_2^{-\cdot} \quad \text{(Ia)}$$

$$2O_2^{-\cdot}+2H^+\rightarrow O_2+H_2O_2 \quad \text{(Ib)}$$

$$H_2O_2+O_2^{-\cdot}\rightarrow OH^-+{}^{\cdot}OH+\,^1O_2$$

Therefore, we postulated that one of the candidate intermediates in the HPX-XOD system was HOOOH that was formed by the reaction of $2\,^1O_2+2H_2O$. $^1O_2$ is known to emit luminescence at 1269 nm.[15] However, in our preliminary experiment by using a high performance chemiluminescence analyzer CLD-30 (Tohoku Electronic Industrial Co., Ltd., Sendai, Japan), we did not detect the luminescence at 1269 nm in the HPX-XOD system. To further confirm this, another method such as the oxidation of 1,3-diphenyl-isobenzofuran as measured at 420 nm will be applied for the detection of $^1O_2$.[16,17] The other hypothesis for the possibility of HOOOH formation is as follows;

$$O_2^{-\cdot}+H^++{}^{\cdot}OH\rightarrow 2HOOOH$$

However, the equation is controversial, because the addition of DMSO, a potent scavenger for ·OH, likely abolishes the formation of HOOOH. Therefore, we postulate that L-012 reacts with $O_2^{-\cdot}$ to form $L^{-\cdot}$ as in the following way.

$$O_2^{-\cdot}+3LH^-\rightarrow OH^-+H_2O+3L^{-\cdot}$$

This was supported by the fact that DMPO-OOH formation was increased two to three fold by the addition of DMSO (Fig. 4). HOOOH is also thought to be regarded as hydrated ozone and we surmise that it is susceptible to light and heat as is the case with HOCl and H_2O_2. The resultant HOOOH may contribute to the luminol-mediated CL response by the following way.

$$2HOOOH+2{}^{\cdot}OH\rightarrow 2HOOO^{\cdot}+2H_2O$$

$$HOOO^{\cdot}+LH^-\rightarrow L^{-\cdot}+HOOOH$$

$$L^{-\cdot}+O_2^{-\cdot}\rightarrow h\nu$$

All things considered, the equations proposed by Hodgson and Fridovich[2] are amended as follows;

$$HPX+XOD+2O_2\rightarrow 2H^++2O_2^{-\cdot}$$

$$2O_2^{-\cdot}+2H^+\rightarrow O_2+H_2O_2$$

$$O_2^{-\cdot}+H_2O_2\rightarrow O_2+OH^-+{}^{\cdot}OH$$

$${}^{\cdot}OH+LH^-\rightarrow OH^-+H^++L^{-\cdot}$$

$$O_2^{-\cdot}+3LH^-\rightarrow OH^-+H_2O+3L^{-\cdot}$$

$$O_2^{-\cdot}+H^++{}^{\cdot}OH\rightarrow 2HOOOH$$

$$2HOOOH+2\cdot OH\rightarrow 2HOOO^{\cdot}+2H_2O$$

$$HOOO^{\cdot}+LH^-\rightarrow L^{-\cdot}+HOOOH$$

$$L^{-\cdot}+O_2^{-\cdot}\rightarrow LO_2^{2-}$$

$$LO_2^{2-}\rightarrow N_2+AP^{2-}\rightarrow AP^{2-}+h\nu$$

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