Role of Amino Thiols as Radical Quenchers in Delayed Chemiluminescence of Luminol Catalyzed by Horseradish Peroxidase†

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A horseradish peroxidase (HRP: EC 1.11.1.7) has been widely used as a catalyst in luminol chemiluminescence (CL). The HRP-catalyzed luminol CL provides a sound basis for an assay of hydrogen peroxide (H₂O₂) and enzymatically generated H₂O₂.¹ On the other hand, the Cu(II)-catalyzed oxidation of amino thiol with oxygen is focused on a model reaction for elucidating the mechanism of the biological oxidation with copper-bearing enzymes such as galactose oxidase and diamine oxidase.² In the catalytic oxidation, H₂O₂ is shown to be produced as an intermediate from oxygen.³

In the course of our studies on the application of the HRP-catalyzed luminol CL to the detection of H₂O₂ formed during the catalytic oxidation of cysteine, we have found that a CL flash suddenly appeared after a certain dark period from the initiation of the reaction.⁴ The delay time and the CL intensity were dependent on the concentrations of Cu(II). Based on this finding, we proposed a delayed luminol CL method for the determination of Cu(II).⁴ The purpose of the present study is to investigate the effects of amino thiols on CL intensity-time profiles and to clarify the function of the thiols in the appearance of the delayed CL of luminol.

Experimental

Reagents

HRP (type VI), L-cysteine methyl ester (CME), L-cysteine ethyl ester (CEE) and L-cystine dimethyl ester (CDME) were obtained from Sigma Chemical Co. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), cysteamine and L-cysteine were purchased from Kanto Chemical Co. All thiols obtained were hydrochlorides. All chemicals used were guaranteed-grade reagents and were used without further purification. HRP solution was prepared with Carmody's buffer (pH 8.0) involving 0.2 M (1 M=1 mol dm⁻³) boric acid, 0.05 M citric acid and 0.1 M tertiary sodium phosphate. The concentration of HRP was determined spectrophotometrically with an ε₄₂⁰ value of 1.02×10⁵ M⁻¹ cm⁻¹.⁵ A 1.0×10⁻² M stock solution of luminol was prepared by dissolving the compound with 0.1 M NaOH solution. Working solutions of luminol were prepared by serial dilution with Carmody's buffer solution. Standard solutions of thiols were made daily. All solutions used were prepared with water from a Millipore Milli-Q water purification system.

CL detection

All CL measurements were made using a luminometer constructed in this laboratory.⁶ A glass cuvette was placed on a magnetic stirrer in the luminometer. A 1.0 cm³ portion of a 1.2×10⁻⁴ M Cu(II) solution, a 1.0 cm³ portion of a 4.5×10⁻⁴ M HRP solution and a 0.5 cm³ portion of a 6.0×10⁻² M luminol solution were added into the cuvette. The whole solution was saturated with oxygen by bubbling. Next, a 0.5 cm³ portion of a 1.2×10⁻² M thiol solution was injected into the cuvette. The CL reaction was initiated, and the CL emission was detected. Bubbling of oxygen at 50 cm³ min⁻¹ and mixing by a magnetic stirrer were continued during the reaction. All CL measurements were made at 25°C. The maximum light emission was referred to as the CL intensity. The time period from the reaction initiation

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to the time at which CL intensity was maximal was defined as the delay time.

**Analytical procedure for thiol and H$_2$O$_2$**

The concentrations of thiol consumed and H$_2$O$_2$ formed during the catalytic oxidation were determined spectrophotometrically. A 0.1 cm$^3$ portion of the reaction mixture was pipetted into the cell which contained a 0.1 cm$^3$ portion of a 1.0×10$^{-3}$ M EDTA solution for stopping the catalytic reaction. Thiol was determined with 2,2'-dithiobis(5-nitro-pyridine) by measuring absorbance at 386 nm, and H$_2$O$_2$ with Fe(II) complex of 1,10-phenanthroline at 510 nm. The absorption spectra were measured with a Hitachi U-2000-type spectrophotometer.

**Determination of CDME by HPLC**

L-Cystine dimethyl ester (CDME) produced during the catalytic oxidation of CME was determined by high-performance liquid chromatography (HPLC). The HPLC apparatus consisted of a Shimazu LC-6A pump, a loop injector, a Merk Lichrosorb NH$_2$ column and a JASCO UVIDEC-100-IIW detector. A 0.1 cm$^3$ portion of the reaction mixture and then a phosphate buffer solution (pH 7.0) were submitted to HPLC at a flow rate of 1.0 cm$^3$ min$^{-1}$. The detection of CDME was made by measurement of absorption at 350 nm.

**Results and Discussion**

**Effects of amino thiols on the CL intensity-time profiles**

The H$_2$O$_2$ generated from oxidation of thiols was continuously determined by the HRP-catalyzed luminol CL method. The CL could be detected according to the consecutive reactions (1) and (2) under the same conditions.

$$2\text{RSH} + \text{O}_2 \xrightarrow{\text{Cuc}^{2+}} \text{RSSR} + \text{H}_2\text{O}_2$$

$$\text{luminol} + \text{H}_2\text{O}_2 \xrightarrow{\text{HRP}} \text{aminophthalate} + \text{N}_2 + \text{H}_2\text{O} + \text{hv}$$

Amino thiols were chosen by taking into account the acid dissociation constant ($pK_a$) of their mercapto groups. Chemical structures of these thiols (RSH) are shown in Fig. 1. The $pK_a$ values are 6.6 for CME, 6.7 for CEE, 8.3 for cysteine and 8.6 for cysteamine, respectively.

Typical CL response curves are shown in Fig. 2. A CL flash suddenly appeared after a dark period of 6.5 – 8.5 min from the initiation of the reaction in all thiols used. Cysteamine and CME gave shorter delay times than cysteine and CEE did. Cysteine showed the greatest CL intensity among the thiols. The delay time increased with an increase in concentration of each thiol.

**Time course of thiol consumption and H$_2$O$_2$ formation**

In order to elucidate the appearance of the delayed luminol CL, we determined the amounts of thiol consumed and H$_2$O$_2$ formed during the reaction. The results in the case of CME are shown in Fig. 3. When CME had been almost completely consumed, the decomposition of H$_2$O$_2$ was initiated. The time at the beginning of $H_2O_2$ decomposition was in accordance with the time at the beginning of the CL flash. Other thiols gave the same results as that of CME shown in Fig. 3. These results indicate that thiols inhibit the progress of the HRP-catalyzed luminol CL with H$_2$O$_2$ formed during the catalytic oxidation. Therefore, the HRP-catalyzed luminol CL is subsequently commenced after complete oxidation of thiols.

**Effect of Cu(II) on the oxidation rate of thiols**

The time until the beginning of the CL flash could be correlated to the oxidation rate of thiols. We then...
examined the oxidation of thiols by using a 1.2×10⁻⁵ M Cu(II) solution alone in the 5.0 - 10.0 pH range according to the procedure except that the buffer solution was employed in place of an HRP solution and a luminol solution. The concentrations of thiols in the reaction mixture were determined 6 min (cysteine esters), 4 min (cysteine) and 3 min (cysteamine) after the start of the reaction. The pH dependences of the oxidation rate of thiols are shown in Fig. 4. The oxidation rate exhibited a maximum at pH 6.0 for the cysteine esters, at pH 7.6 for cysteine and at pH 8.5 for cysteamine, respectively. The oxidation rate was maximal near the pKₐ,SH values of each thiol.

As shown in Fig. 4, the catalytic oxidation of cysteamine and cysteine by Cu(II) alone proceeded rapidly at pH 8.0. On the other hand, the oxidation rate of the cysteine esters by Cu(II) alone was remarkably slower than those of cysteine and cysteamine at pH 8.0. However, as can be seen in Fig. 2, the delay time observed in CME was about the same as that in cysteamine. These results suggest that other chemical species except Cu(II) could be involved in the oxidation of CME.

Effects of HRP and luminol on the oxidation rate of CME

HRP catalyzes the oxidation of amino thiols with oxygen.¹⁰ The catalytic ability of HRP for the oxidation of cysteamine with oxygen was equivalent to that of Cu(II).⁴ We then examined the oxidation of CME by using a 9.0×10⁻⁶ M HRP solution alone, according to the procedure, except that the buffer solution was employed in place of a Cu(II) solution and a luminol solution. However, no catalytic oxidation of CME occurred after 15 min from the start of the reaction as shown in Fig. 5 (curve 1).

Antioxidants such as glutathione are accepted to be quenching agents for radicals.¹¹ Similarly, CME could react with luminol radicals (L⁻) to yield luminol (LH⁻) and thiyl radicals. Formation of thiyl radicals is followed by subsequent reactions involving the formation of disulfide (RSSR) and H₂O₂ (reaction (3)).¹²

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2L⁻ + 2RSH + 2H⁺ + O₂ \rightarrow 2LH⁻ + RSSR + H₂O₂
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(3)

We then tested the oxidation of CME in the presence of HRP and luminol according to the procedure, except
that the buffer solution was employed in place of a Cu(II) solution. The time course for CME consumption is indicated in Fig. 5 (curve 2). The oxidation rate by the combined use of luminol and HRP increased remarkably compared to those by Cu(II) or HRP alone. Therefore, these results indicate that CME reacts with luminol radicals to produce disulfide of CME and H$_2$O$_2$.

This idea suggests that the luminol concentrations give rise to significant effects on the formation of CDME, since the amounts of CDME formed may be dependent on the concentration of luminol radical produced. We then examined the effects of luminol concentrations on the formation of CDME. The concentration of CDME was determined by HPLC 12 min from the start of the reaction. Figure 6 shows the influence of the luminol concentration on the formation of CDME. The concentration of CDME formed increased with increasing luminol concentrations between 1.0X$\times$10$^{-5}$ M and 1.0X$\times$10$^{-4}$ M luminol, after which the concentration of CDME was constant. The increase of the amount of CDME formed is probably attributable to the increase of the luminol radical concentration generated from the enzymatic cycle of HRP increasing luminol concentration.

Mechanism of the appearance of the delayed luminol CL

Next, we examined the oxidation of CME in the presence of HRP, luminol and Cu(II). The time course of CME consumption is shown in Fig. 5 (curve 3). The oxidation rate increased remarkably by the addition of Cu(II). The increase of the oxidation rate could be explained by taking into account the H$_2$O$_2$ formed in the reaction (3). That is, the oxidation rate of CME catalyzed by Cu(II) in the presence of H$_2$O$_2$ increased remarkably compared to that with oxygen alone [reaction (1)] (results not shown). On the other hand, the time at which CME was almost completely oxidized was almost the same for the time at the beginning of the CL flash, as shown in Fig. 2.

From these results, the appearance of the delayed luminol CL could be explained by the proposed scheme illustrated in Fig. 7. Native HRP reacts with H$_2$O$_2$ produced by the Cu(II)-catalyzed oxidation of thiol and then catalyzes the oxidation of luminol (LH-) through the enzymatic cycle.$^{13,14}$ Most of luminol (LH$_2$) exists as LH$^-$ at pH 8.0. Numbers in parentheses in Fig. 7 show the effective oxidation level of iron in native HRP and its intermediates. Luminol reacts with such HRP-intermediates as compounds I and II to form luminol radical (L$^-$. In the absence of thiol, the luminol radicals react with oxygen to yield endoperoxide (LO$_2^-$). Endoperoxide then decomposes to yield an electronically excited 3-aminophthalate dianion (AP$^{2-}$$^*$) which returns to a ground state to emit light. On the other hand, in the presence of thiol, thiol competes with oxygen for luminol radicals. The luminol radical react with thiol more rapidly than with oxygen, thereby preventing luminol radicals oxidation to aminophthalate and subsequent light emission. Therefore, after complete oxidation of thiol, the HRP-catalyzed luminol CL is subsequently commenced using accumulated H$_2$O$_2$ as an oxidizing agent, and a CL emission suddenly appears after a dark period.
The oxidation of thiol proceeds by the reaction with luminol radicals in reaction (3) as well as by the Cu(II)-catalyzed oxidation in reaction (1). In particular, in the case of the cysteine esters, the reaction with luminol radicals influences the consumption rate of the cysteine esters.

In conclusion, quenching of luminol radicals by thiol caused a CL delay in the HRP-catalyzed luminol CL reaction when coupled to the Cu(II)-catalyzed oxidation of thiol with oxygen. The delay time was correlated to the oxidation rate of thiols. Thiols are oxidized by the reaction with luminol radicals as well as by the Cu(II)-catalyzed oxidation. The delay time and CL intensities are dependent on the concentrations of thiols. In this respect, the HRP-catalyzed CL-delay of luminol will be promising in the determination of thiols.

References


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