Role of 3-Morpholino-1-propanesulfonic Acid as Energy Transferor in Chemiluminescence Reaction of Fluorescein Catalyzed by Horseradish Peroxidase

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The horseradish peroxidase (HRP)-catalyzed chemiluminescence (CL) reaction of fluorescein (FL) with hydrogen peroxide (H_2O_2) was carried out in aqueous media buffered by citrate, phosphate, imidazole, and 3-morpholino-1-propanesulfonic acid (MOPS) at pH 7.0. When the H_2O_2 concentration was below 2×10^{-5} M, one peak was observed in a CL response curve in each buffer component tested. In contrast, only MOPS gave two peaks in the response curve at a concentration higher than 2×10^{-5} M H_2O_2. The CL emission of the second peak was attributable to formation from an excited FL dianion. The total photon amounts of CL emission of the second peak were dependent on the MOPS concentration. Dissolved oxygen was consumed during the occurence of the second peak. These results could be explained by taking into account the role of MOPS as an energy transferor. That is, MOPS reacted successively with the FL radical, produced from the catalytic cycle of HRP, and the dissolved oxygen to form an excited MOPS compound. An energy transfer occurred from the excited MOPS compound to the FL dianion, resulting in the emission of light.

Analytical interest has recently been focused on chemiluminescence (CL) methods in view of their high sensitivity, wide dynamic range and simplicity regarding instrumentation.[1—3] Usually, the CL intensity is dependent on the CL reagent concentration, the catalyst concentration and the pH. However, only little attention has been paid to the influence of the buffer components on the CL intensity, since most CL reactions proceed effectively in non-aqueous media or basic solutions. A buffer component could be a potential factor which would seriously influence CL reactions, since such a buffer component as an amine derivative tends to be subject to oxidation. Some amine derivatives are likely to react with an intermediate produced in the CL reaction. It has been reported that the CL reaction of peroxyoxalate was affected by the buffer components.[4,5] However, their functions in the CL reactions were not mentioned in detail.

We previously studied the CL determination of hydrogen peroxide (H_2O_2) using fluorescein CL catalyzed by horseradish peroxidase (HRP).[6] In an additional study, the effect of the buffer components on the CL intensity was investigated in aqueous media involving several buffer components.[7] As a result, citrate, phosphate and imidazole yielded higher CL intensities; citrate and phosphate were superior to imidazole and MOPS in view of masking metal ions which interfered with the determination. In addition, a mechanism of fluorescein CL was proposed, in which fluorescein serves dual functions of producing singlet oxygen and of being an energy acceptor from singlet oxygen.

During a study concerning the effect of H_2O_2 at high concentrations upon the fluorescein CL reaction, we found that two peaks could be observed in the CL response curves only when MOPS was used for a buffer component. The CL intensity of the second peak was strongly dependent on the H_2O_2 and MOPS concentrations. In the present work, we investigated the function of the MOPS concerning the appearance of the second peak.

Experimental

Apparatus. All of the CL measurements were made by using a CL detector (TD-3A; Tohoku Densi Sangyo Co., Ltd.) equipped with an automatic injector (Model 500, Nichiryo Co., Ltd.). The absorption spectra and the absorbance were recorded on a spectrometer U-2000 (Hitachi Co., Ltd). The fluorescence spectrum of fluorescein was obtained by measurements with a fluorescence spectrometer (RF-520; Shimadzu Co., Ltd). The dissolved oxygen concentration was measured by using a portable dissolved oxygen meter (DO-11P; TOA Electronics Ltd.) equipped with an oxygen electrode (OE-2102).

Reagents. All reagents were used as purchased without any further purification. HRP (Type VI) was obtained from Sigma. All of the aqueous solutions were prepared with water purified using a Millipore Milli Q-II system.

A CL reagent solution containing 5.0×10^{-8} M (1 M=1 mol dm^{-3}) HRP and 6.6×10^{-4} M fluorescein was prepared with a 0.10 M MOPS solution (pH 7.0). An H_2O_2 solution was made daily by dilution of 31% H_2O_2 solution with water.

Procedure. CL measurements were performed by placing a 0.50 cm^3 portion of the CL reagent solution into a CL cuvette (22 mm i.d.×20 mm). A 0.50 cm^3 portion of an H_2O_2 solution was then added to the cuvette using the injector. The CL reaction was thus initiated, and the CL emission was monitored as a function of time with a photomultiplier tube. The resultant photocurrent was converted to a voltage, the value of which was displayed on a chart recorder. The CL intensity was defined as integrated numbers of emitted photons in a respective peak.

In the measurement of the absorbance-time course of fluorescein, a 0.20 cm^3 portion of the CL reagent solution and a 0.20 cm^3 portion of a H_2O_2 solution were mixed in a 0.1-cm quartz cell. Then, the absorbance at 490 nm (the maximum absorption wavelength of fluorescein) was monitored every 6 seconds from the initiation of the reaction.

The absorption spectra of fluorescein were measured by mixing a 1.0 cm^3 portion of the CL reagent solution and a 1.0 cm^3 portion of a H_2O_2 solution in a 1-cm quartz cell. The absorption spectra were measured every 30 s at a scan speed of 2400 nm min^{-1}.

The time profile of the dissolved oxygen concentration was determined with the oxygen electrode. A 10.0 cm^3 portion of the CL reagent solution was added into a glass vial (40 mm i.d.×65 mm). The oxygen electrode was then immersed in the solution. Subsequently, stirring with a magnetic stirrer was started. After readings had become constant, a 10.0 cm^3 portion of a H_2O_2 solution was injected into the vial, and the reaction was initiated. The dissolved oxygen concentration was measured from the initiation of the reaction. The vial was thermostated at 25±1 °C.

Results and Discussion

Effect of Buffer Components on CL Response. Typical CL response curves of fluorescein in a MOPS buffer solution at several levels of H_2O_2 concentrations are shown in Fig. 1. The CL always occurs immediately after mixing the CL reagent solution and a H_2O_2 solution; most of the CL emission ended within 3 min. When a 3.0×10^{-6} M H_2O_2 solution was employed, the CL reached a maximum value within a few seconds, and then decreased (curve 1). Meanwhile, the second peak was observed in the CL response curve at H_2O_2 concentrations higher than 2.0×10^{-5} M (curves 2—4). The CL intensity of the second peak was increased along with an increase in the H_2O_2 concentrations. In contrast, the use of other buffer components, such as citrate, phosphate, and imidazole did not give a second peak at all. The first peak seems to appear according to the same mechanism, independently of the buffer components. Since the second peak was only observed in the presence of MOPS, the CL mechanism for the second peak is probably different from that for the first peak.

The CL response curves at several concentrations of MOPS were obtained by using a 2.0×10^{-4} M H_2O_2 solution. As shown in Fig. 2, only one peak was observed at a 5.0×10^{-3} M MOPS concentration in the CL reagent solution, whereas two peaks appeared above 3.0×10^{-2} M MOPS. The CL intensity of the second peak was increased with increasing the MOPS concentration, while that of the first peak was constant, independent of the MOPS concentration. The appearance of the second peak was, thus, due to MOPS, itself, since the intensity of the second peak was dependent on the MOPS concentration.

Identification of Emitted Species. In order to investigate the luminous species for the second peak, the CL spectrum of the second peak was measured in a manner described previously.[7] As a result, the CL spectrum was found to have its maximum emission wavelength at about 530 nm, which is in good agreement with the maximum emission wavelength of the fluorescence spectrum of fluorescein under the same conditions. Therefore, the emission of the second peak was ascribable to the excited fluorescein dianion, as well as the emission of the first peak.[7] The mechanism of the occurrence of the second peak was then explored in the experiments described below.

Mechanism of Appearance of Second Peak. The color tones of the final solutions in the CL reac-

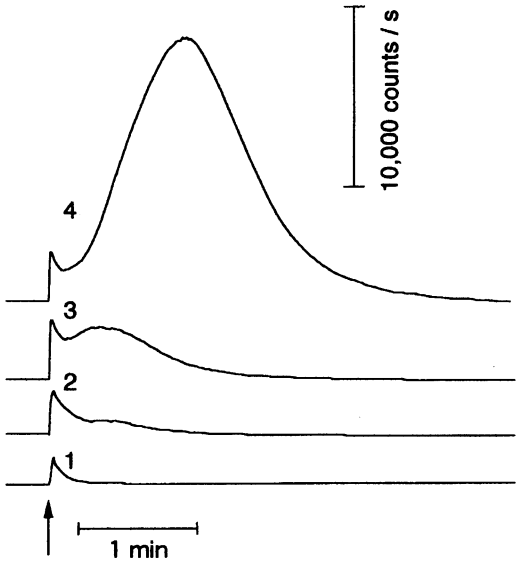

Fig. 1. Effect of the H_2O_2 concentrations on the fluorescein CL. CL reagent; [HRP]=5.0×10^{-8} M, [fluorescein]=6.6×10^{-4} M, [MOPS]=0.10 M, pH 7.0. [H_2O_2]; 1 : 3.0×10^{-6} M, 2 : 2.0×10^{-5} M, 3 : 5.0×10^{-5} M, 4 : 2.0×10^{-4} M. At the arrow, H_2O_2 solution was injected.

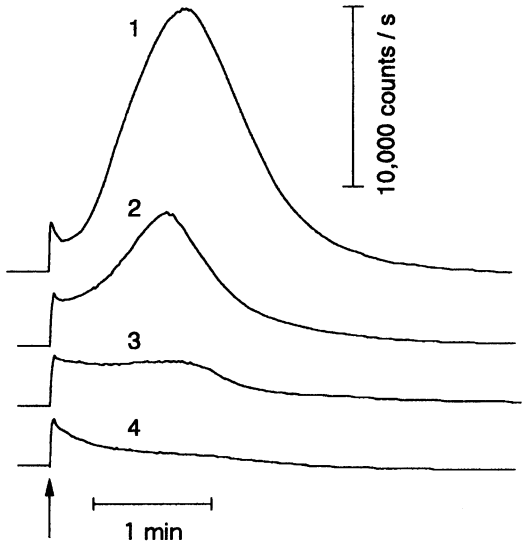

Fig. 2. Effect of the MOPS concentrations on the fluorescein CL. [H_2O_2]=2.0×10^{-4} M. CL reagent; [HRP]=5.0×10^{-8} M, [fluorescein]=6.6×10^{-4} M, pH 7.0. [MOPS]; 1 : 0.10 M, 2 : 0.05 M, 3 : 0.03 M, 4 : 0.005 M. At the arrow, H_2O_2 solution was injected.

tion were different from each other, depending on the MOPS and H_2O_2 concentrations. These results indicate that the concentrations of fluorescein in the final solutions depend on the MOPS and H_2O_2 concentration. Thus, the change in the fluorescein concentration during the CL reaction was investigated by monitoring the absorbance of fluorescein with time. Fluorescein was present about 80% in its dianion form (Fl^{2-}) at pH 7.0; the other was monoanion (FlH^-). The results are given in Fig. 3, in which the H_2O_2 concentration was 2.0×10^{-4} M at several MOPS concentrations. Apparently, the absorbance decreased instantaneously upon initiation of the reaction. This was previously interpreted as meaning that FlH^- as a reducing substrate forms a radical in the reaction of the HRP cycle {Eqs. 1, 2, and 3}, and decomposes in the following reaction {Eq. 4}.[7]

$$\text{HRP} + H_2O_2 \longrightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + HFl^- \longrightarrow \text{Compound II} + Fl^{\cdot -} \quad (2)$$

$$\text{Compound II} + HFl^- \longrightarrow \text{HRP} + Fl^{\cdot -} \quad (3)$$

$$Fl^{\cdot -} + \text{dissolved oxygen} \longrightarrow \text{singlet oxygen} + \text{decomposed fluorescein} \quad (4)$$

In Fig. 3, the decrease in the absorbance was suppressed in a MOPS buffer depending on its concentration. Concomitantly, the decomposition time of fluorescein was shortened. It is plausible that MOPS works as a reductant of $Fl^{\cdot -}$. Namely, considerable amounts of $Fl^{\cdot -}$ were reduced into FlH^- by MOPS, thus decreasing the amount of the decomposed fluorescein. Since the CL intensity of the second peak increased along with an augmentation of the reduced fluorescein, oxidized products from MOPS may contribute to the appearance of the second peak.

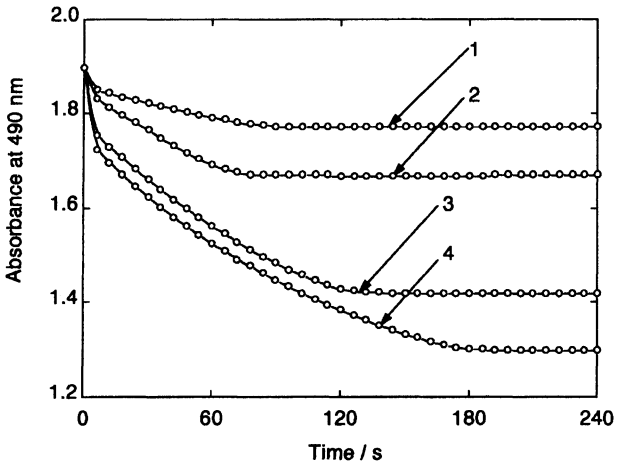

Fig. 3. Change in the absorbance during fluorescein CL catalyzed by HRP. $[H_2O_2]=2.0\times10^{-4}$ M. CL reagent; $[HRP]=5.0\times10^{-8}$ M, [fluorescein]$=6.6\times10^{-4}$ M, pH 7.0. [MOPS]; 1:0.10 M, 2:0.05 M, 3:0.03 M, 4:0.005 M.

At a concentration of 0.10 M MOPS, the decomposition of fluorescein was completed within about 70 s. This is not in agreement with the time which is required for leveling off the CL at the second peak. Hence, the CL reaction of the second peak proceeds without the decomposition of fluorescein.

We investigated whether HRP participated in the appearance of the second peak, according to absorption spectrum of HRP after completing fluorescein decomposition. In order to measure the spectral changes of both HRP and fluorescein, a 1.0 cm^3 portion of the CL reagent solution containing 1.0×10^{-5} M fluorescein and 1.4×10^{-6} M HRP was mixed with a 1.0 cm^3 portion of a 5.0×10^{-5} M H_2O_2 solution. In Fig. 4, the absorption band of 380—420 nm corresponds to the Soret band of HRP, while the absorption band at 490 nm is for fluorescein. The absorption spectra of several oxidation states of HRP are different from each other.[8] The native state of HRP has an absorption maximum at 403 nm; Compound II, an intermediate during the HRP cycle, has a maximum at 420 nm. In Fig. 4, immediately after the initiation of the CL reaction, the absorption band of fluorescein begins to diminish. Simultaneously, the HRP absorption band shifts from 403 to 420 nm, indicating the formation of compound II. The decomposition of fluorescein ended within 60 s, and the HRP absorption band concurrently returned to 403 nm. This fact implies that HRP forms a native state after the termination of the decomposition of fluorescein. Meanwhile, the CL reaction of the second peak proceeds even after the termination; thus, HRP probably does not take part in the second CL reaction as a catalyst.

The dissolved oxygen concentration during the CL reaction was next examined as a function of time. The

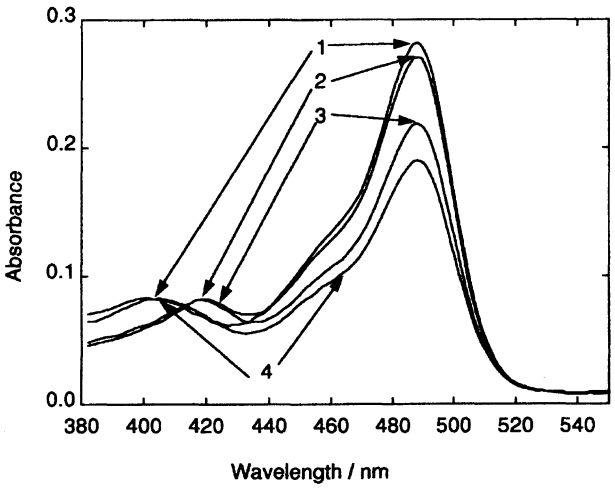

Fig. 4. Absorption spectra during the oxidation of fluorescein catalyzed by HRP in MOPS solution. 1:blank (no H_2O_2), 2:immediately after injection of the CL reagent solution, 3:after 30 s, 4:after 40 s. CL reagent; $[HRP]=1.4\times10^{-6}$ M, [fluorescein]$=1.0\times10^{-5}$ M, [MOPS]$=0.10$ M (pH 7.0), $[H_2O_2]$ 5.0×10^{-5} M.

results are shown in Fig. 5. The oxygen concentration decreased when the MOPS buffer was used (curve 1), while the oxygen concentration was constant in the phosphate buffer solution (curve 2). Accordingly, dissolved oxygen was consumed during the CL reaction in the MOPS buffer solution. The oxygen-consuming reaction in the MOPS buffer continued for about 3 min. The reaction time of the oxygen-consuming reaction was in good agreement with that of the second CL reaction. In the mixture of MOPS, HRP and H_2O_2, the oxygen concentration did not change; however, the addition of fluorescein to the mixture immediately produced a decrease in the oxygen concentration. Fluorescein is therefore necessary for the oxygen-consuming reaction. Since the oxygen-consuming reaction lasts after the termination of the fluorescein decomposition, the reaction of the oxidized MOPS with dissolved oxygen proceeds slowly.

From the results mentioned above, the CL mechanism for the appearance of the second peak in the MOPS buffer solution was speculated. A $Fl^{\cdot -}$, produced in the HRP cycle {Eqs. 1, 2, and 3}, reacted with MOPS so that FlH^- and oxidized MOPS were generated {Eq. 5}.

$$Fl^{\cdot -} + \text{MOPS} \longrightarrow FlH^- + \text{oxidized MOPS} \quad (5)$$

Following this, the oxidized MOPS reacted with dissolved oxygen to form an excited product P*; {Eq. 6}.

$$\text{oxidized MOPS} + \text{dissolved oxygen} \longrightarrow P^* \quad (6)$$

Subsequently, by an energy transfer from P*, Fl^{2-} was excited {Eq. 7}. The excited Fl^{2-} emitted photons to return to the groud state {Eq. 8}.

$$P^* + Fl^{2-} \longrightarrow (Fl^{2-})^* + P \quad (7)$$

$$(Fl^{2-})^* \longrightarrow Fl^{2-} + \text{Light} \quad (8)$$

In Eqs. 1, 2, 3, and 5, the production of both $Fl^{\cdot -}$ and the oxidized MOPS were dependent on H_2O_2 concentration. Thus, the formation of P* was anticipated to rely on the H_2O_2 concentration. Since P* was possibly generated slowly, which was expected from the results of the measurement of oxygen consumption, a high H_2O_2 concentration was necessary for the occurence of a clear second peak.

Finally, the validity of the mechanism was studied. If a MOPS molecule reacts with $Fl^{\cdot -}$, the same reaction can occur in the phosphate buffer in the presence of MOPS. We injected a 0.50 cm^3 portion of a 0.30 M MOPS solution to the phosphate buffer solution in which the fluorescein CL reaction was in progress. Figure 6 represents the CL response curves thus obtained. The CL, whose response resembles the second peak of the CL in the MOPS buffer, appears upon the addition of MOPS. The shorter is the time lag between the initiation of CL and the injection of the MOPS solution, the higher is the CL intensity.

The $Fl^{\cdot -}$ concentration is anticipated to be maximum immediately after initiation of the reaction, and then to gradually decrease. The higher concentrations of $Fl^{\cdot -}$ should give higher CL intensities. Thus, this mechanism seems to be reasonable.

Consequently, the appearance of the second peak in the presence of MOPS was probably due to the formation of the excited compound (P*), which was produced by a reaction of dissolved oxygen with the oxidized MOPS; the oxidized MOPS was generated in the

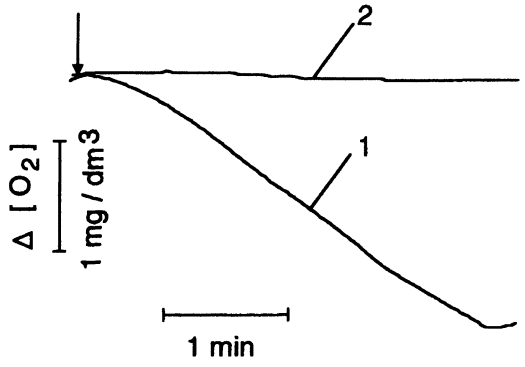

Fig. 5. Dissolved oxygen concentration as a function of time during the oxidation of fluorescein catalyzed by HRP. 1: MOPS, 2: phosphate. At the arrow, the H_2O_2 solution was injected.

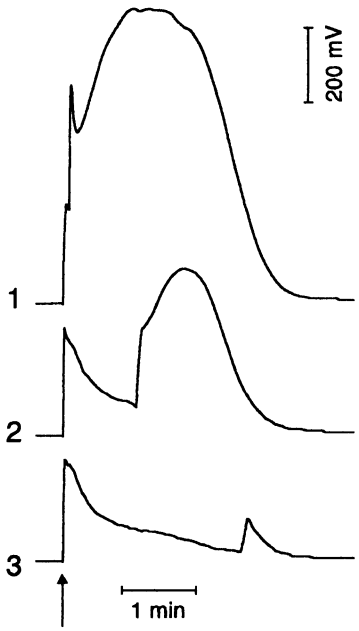

Fig. 6. Effect of the injection of MOPS on the time profile of CL during the fluorescein CL reaction. CL reagent; $[HRP] = 5.0 \times 10^{-8}$ M, [fluorescein] = 6.6×10^{-4} M, buffered by 0.10 M phosphate at pH 7.0. At the arrow, a 0.50 cm^3 portion of the CL reagent solution and a 0.50 cm^3 portion of 2.0×10^{-4} M H_2O_2 solution were mixed and the CL reaction was started. Then, a 0.50 cm^3 portion of 0.30 M MOPS solution (pH 7.0) was injected to the mixture after 6 s (curve 1), 60 s (curve 2), and 150 s (curve 3) from the initiation of the CL reaction.

reaction between MOPS and $Fl^{\cdot -}$. A Fl^{2-} was possibly excited by an energy transfer from the excited compound, and then emitted light.

In conclusion, it was found that MOPS functions not only as a buffer component, but also as an energy transferor in a HRP-catalyzed FL CL reaction. Therefore, the selection of a suitable buffer component is important when a HRP-catalyzed reaction is used for an indicator reaction.

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