Enhancement of Chemiluminescence from the Oxidation of Luminol with Hydrogen Peroxide Catalyzed by Mn(III)-microperoxidase 8

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Mn(III)-containing microperoxidase 8 (Mn(III)-MP8) is an effective catalyst for the cheminluminescent (CL) reaction involving the oxidation of luminol with hydrogen peroxide at high pH. The CL emission was dramatically enhanced by the presence of guanidine hydrochloride or sodium carbonate in the reaction mixture. The enhancement was strongly pH-dependent, with the most prominent pH at 10. Kinetic studies of the CL reaction have revealed that the enhancement was caused primarily by the significant acceleration of the CL cycle by the enhancer. The CL signal covers several orders of magnitude over a wide concentration range of luminol and hydrogen peroxide. The intense CL of the [Mn(III)-MP8]-luminol-H_2O_2 system, especially in the presence of the enhancer, will have a great potential for extremely sensitive CL assays.

Keywords: Chemiluminescence; Stopped-flow; [Mn(III)-MP8]-Luminol-H_2O_2; Guanidine hydrochloride; Sodium carbonate.

INTRODUCTION

Chemiluminescence (CL) based on luminol oxidation is one of the best known systems that has wide analytical applications for the determination of numerous substances as described in several reviews.[1-4] A variety of catalysts, including metal ions,[4] metal complexes,[5,6] and peroxidases,[7-9] have been used in this system. One of the very effective catalysts for the oxidation of luminol is microperoxidase (MP), which has been applied to the assay of peroxides,[11] antioxidants,[12] and catecholamines.[13] MP contains a heme moiety with a covalently linked oligopeptide (e.g., MP8 contains an octapeptide), which exhibits peroxidase-like activity.[14] Owing to the lack of the surrounding protein, MP can withstand much more severe experimental conditions in comparison with ordinary peroxidases.

The mechanism for the CL emission of the peroxidase-luminol-H_2O_2 system is well known.[15] Luminol is a diprotic acid (denoted as LH_2) with pK_a's of 6 and ~ 13.[16] Luminol exists mostly as LH^- in the range of pH 9 ~ 13 employed in this study. The mechanism of the MP-catalyzed luminol-CL generally involves the following steps:

$$MP + HO_2^- \rightarrow X + OH^- \quad (1)$$

$$X + LH^- + OH^- \rightarrow MP + {}^\bullet L^- + H_2O \quad (2)$$

$$2\ {}^\bullet L^- + H_2O \rightarrow L + LH^- + OH^- \quad (3)$$

$$L + H_2O_2 \rightarrow 3\text{-AP} + N_2 + \text{light} \quad (4)$$

where X, ${}^\bullet L^-$, L, and 3-AP stand for the intermediate of MP8, luminol radical, diazaquinone, and 3-aminophthalate, respectively. Any factors that facilitate the formation of the MP-intermediate and/or luminol radical will tend to increase the CL emission.

Replacement of Fe(III) in MP8 with Mn(III) yields Mn(III)-MP8, which also exhibits peroxidase and cytochrome P-450 activities.[17,18] The maximum peroxidase activity occurs at pH ~ 12, which is similar to the optimal pH for most CL reactions involving luminol. The presence of guanidine hydrochloride (GdnHCl) or sodium carbonate greatly accelerated the oxidation of substrate with H_2O_2 catalyzed by Mn(III)-MP8 (unpublished results). Thus the addition of these two reagents is expected to have a profound effect on the CL emission of Mn(III)-MP8-catalyzed oxidation of luminol.

In this paper, we have investigated the CL emission of luminol-H_2O_2 catalyzed by Mn(III)-MP8 using the stopped-flow technique. We found that Mn(III)-MP8 is an effective catalyst for the CL reaction involving luminol and that the presence of GdnHCl or sodium carbonate causes a tremendous enhancement (up to more than two orders of magnitude) of the CL emission. We have also examined the effects of the concentrations of guanidine hydrochloride, sodium carbon-

Dedicated to Professor Fa-Ching Chen on the occasion of his ninetieth birthday.

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ate, luminol, and hydrogen peroxide on the CL emission. The mechanism of the CL enhancement has also been discussed.

EXPERIMENTAL SECTION

Materials

Cytochrome *c*, luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), guanidine hydrochloride, and sodium dihydrogen phosphate were purchased from Sigma (St. Louis, MO, USA). Sodium carbonate was obtained from Acros organics company (Geel, Belgium). Ultra high purity deionized water was produced from a Milli-Q purification system (18.2 MΩ cm^{-1}, Millipore, Tokyo, Japan). The stock solution of hydrogen peroxide (Fluka, Seelze, Germany) was prepared by volumetric dilution of 30% (v/v) H_2O_2 by deionized water. The concentration of H_2O_2 was determined daily by measuring the absorbance at 240 nm on a Hitachi U-3210 spectrophotometer (Hitachi, Tokyo, Japan) using ε_{240} = 39.4 $M^{-1}cm^{-1}$.[19] Fe(III)-MP8 was prepared from the proteolytic digestion of cytochrome *c* with pepsin and trypsin according to a procedure in the literature.[14] Mn(III)-MP8 was synthesized according to a known procedure.[15] The concentrations of MPs were determined spectrophotometrically using ε_{397} = 1.57×10^5 $M^{-1}cm^{-1}$ at pH 7.0 for Fe(III)-MP8[19] and ε_{368} = 1.1×10^5 $M^{-1}cm^{-1}$ at pH 2.0 for Mn(III)-MP8.[17]

Stopped-flow CL and Kinetic Measurements

All the CL and kinetic measurements were carried out with the stopped-flow spectrofluorimeter (Hitech SF-61DX2, Hitech Scientific, Salisbury, UK). The CL experiments were performed in the fluorescence mode with the light source switched off. In a typical stopped-flow CL measurement, Mn(III)-MP8 solution was stored in one syringe and the luminol-H_2O_2 mixture in the other. Both solutions also contained Na_2CO_3 (0-0.5 M) or GdnHCl (0-3 M). The CL intensity versus time profile was recorded after 1:1 mixing of the two solutions. For measuring the rate of formation of the intermediate of Mn(IIII)-MP8, change in absorbance at 404 nm was monitored continuously after mixing the two solutions. The kinetic experiments were operated in the absorption mode with the photomultiplier tube (PMT) voltage set at 220 V. The time-resolved absorption spectra were measured by using a photodiode array detector. For measuring the rate for the reaction of the intermediate of Mn(III)-MP8 with luminol, Mn(III)-MP8 and H_2O_2 solutions in two separate syringes were allowed to mix in a mixer channel. After an appropriate delay to attain a maximum amount of intermediate, the mixture was mixed with luminol in the third syringe and the change in absorbance at 404 nm was recorded continuously. All the experiments were carried out at 25 °C. The built-in software of the stopped-flow instrument allows multi-exponential fitting of the traces. Ten replicate measurements were performed for each CL and kinetic analysis.

Measurement of the CL Emission Spectrum

A schematic diagram of the continuous manifold for the determination of the CL emission spectrum is shown in Fig. 1. The flow solution in R1 contains 5.0 μM Mn(III)-MP8 and 100 μM luminol, while R2 contains 5.0 μM H_2O_2. Both solutions also contain 3.0 M GdnHCl (or 0.5 M Na_2CO_3) in 5 mM phosphate buffer at pH 12.0. The solutions were pumped by a peristaltic pump (Gilson minipuls) through a PEEK Tee (0.566 μL, model P.727, Upchurch Scientific, USA) at a rate of 1.1 mL min^{-1}. The PEEK tubing (0.0625" OD, 0.02" ID, Upchurch Scientific, USA) was used to connect the reagent streams to the Tee and from Tee to flow cell (10 cm). The CL emission spectrum was recorded with a fluorescence spectrophotometer (F-2000, Hitachi, Japan) via a LC Micro Flow Cell Unit (18 μL, model 650-0151, Hitachi, Japan), which permits a high-sensitivity, low-scattering beam level measurement and can be used in high-speed liquid chromatography.

RESULTS AND DISCUSSION

Effect of enhancers on the CL signal

Mn(III)-MP8 is an effective catalyst for the luminol CL at high pH. Fig. 2 compares the stopped-flow CL intensity versus time profiles for the oxidation of 50 μM luminol with 9.6 μM H_2O_2 catalyzed by Fe(III)-MP8 and Mn(III)-MP8 (2.4 μM each) in 5 mM phosphate buffer at pH 12.0. The CL

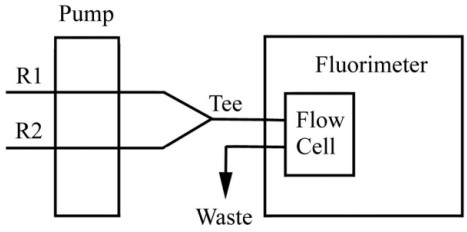

Fig. 1. Schematic diagram of the continuous flow manifold for the detection of the CL emission spectrum. R1: solution containing 5.0 μM Mn(III)-MP8 and 100 μM luminol, R2: solution containing 5.0 μM H_2O_2. Both solutions also contain 3.0 M GdnHCl (or 0.5 M Na_2CO_3) at pH 12.0.

profile for Mn(III)-MP8 showed a two-stage decay curve instead of one-step for Fe(III)-MP8. Moreover, the peak CL intensity for Mn(III)-MP8 was twice as large as that of Fe(III)-MP8. The effect of adding GdnHCl (0 ~ 3 M) on the CL intensity versus time profiles for the reactions of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, 50 μM luminol in 5 mM phosphate buffer (pH 12.0) is illustrated in Fig. 3a. The presence of an increasing amount of GdnHCl caused a drastic increase in CL signal and was accompanied by a progressive reduction in CL duration. The relative peak CL intensity (i.e., CL_r or I/I_o, where I and I_o are the CL intensity in the presence and absence of 3.0 M GdnHCl, respectively) was 13.2 ± 0.5. A slight decrease in CL intensity was observed when the concentration of GdnHCl was above 4 M (data not shown). The double-log plots for the relative peak CL intensities versus the concentration of GdnHCl (at pH 10.0 and 12.0) are shown in Fig. 3b. The plot was linear with a slope of 0.38 (0.1 ~ 3 M) at pH 12.0. At pH 10.0, two linear portions with slopes of 0.80 (0.1 ~ 2 M) and 0.23 (2 ~ 6 M) were observed. The CL enhancement at pH 10.0 was about an order of magnitude larger than that obtained at pH 12.0. The value of CL_r in 3.0 M GdnHCl at pH 10.0 was 165.3 ± 2.5 (Table 1). The presence of sodium carbonate also enhanced the CL emission significantly at pH 10.0 (CL_r = 56.3 ± 1.3), but only moderately at pH 12.0 (CL_r = 2.74 ± 0.08).

pH dependence of the CL intensity

Since the CL enhancement at pH 10 and 12 differ dramatically for both enhancers (GdnHCl and Na_2CO_3), it is important to examine the variation in CL intensity at different pH. Fig. 4 shows the CL intensity versus time profiles for the reactions of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, 50 μM luminol in 5 mM phosphate buffer at various pH. The CL intensity and total amount of emission (i.e., the area under the curve) increased rapidly as the pH increased from 10.0 to 12.5. Similar trends in the CL intensity profiles were also observed for the CL reactions containing GdnHCl or Na_2CO_3 (data not shown). The variations of the peak CL intensities at different pH in the absence or presence of GdnHCl (3 M) or Na_2CO_3 (0.5 M) are plotted in Fig. 5a. In the absence of enhancers, the peak CL intensity of the Mn(III)-MP8 system was higher at pH 12.0 but lower at pH 10.0, compared to the Fe(III)-MP8 system. Fig. 5b shows the plot of the enhanced factor (i.e., CL_r) at different pH. The maximum enhancement

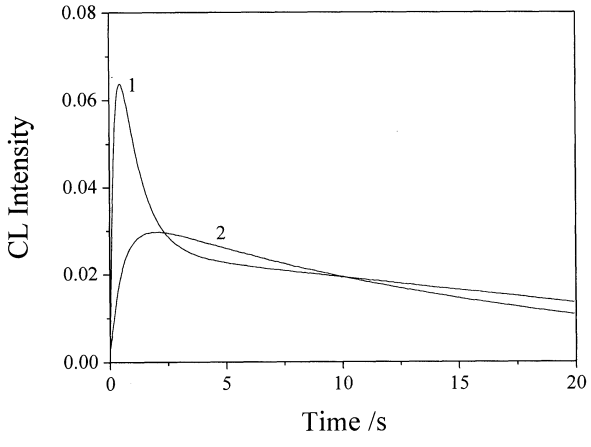

Fig. 2. Stopped-flow CL intensity versus time profiles for the oxidation of 50 μM luminol with 10 μM H_2O_2 catalyzed by 2.4 μM Mn(III)- (trace 1) and 2.4 μM Fe(III)-MP8 (trace 2) in 5.0 mM phosphate buffer at pH 12.0. The PMT voltage was set at 500 V.

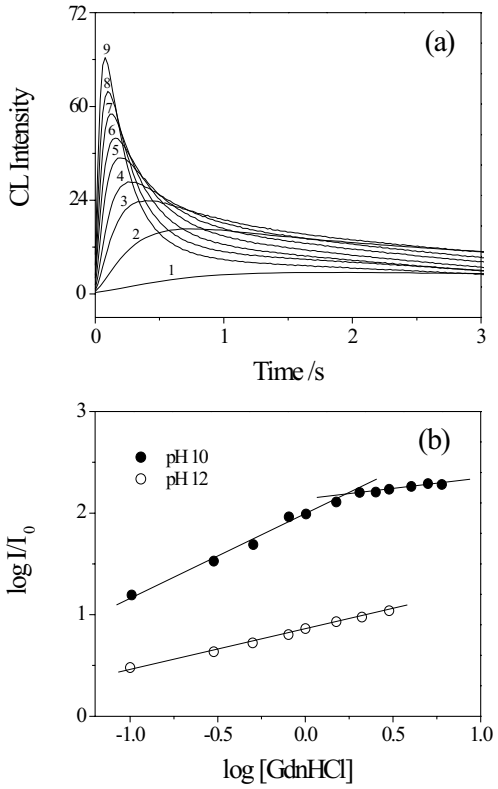

Fig. 3. (a) Stopped-flow CL intensity vs. time profiles for the reactions of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, 50 μM luminol in 5 mM phosphate buffer (pH 12.0) at various concentration of GdnHCl (from trace 1 to 9): 0, 0.1, 0.3, 0.5, 0.8, 1.0, 1.5, 2.1 and 3.0 M. The PMT voltage is 700 V. (b) The double-log plot of the relative peak CL intensity versus the concentration of GdnHCl at pH 10.0 (closed circles) and 12.0 (open circles).

Table 1. Effect of GdnHCl and Na_2CO_3 on the Values[a] R_{1r},[b] R_{2r},[c] $R_{1r} \times R_{2r}$,[d] and CL_r[e]

	0.5 M Na_2CO_3	3 M GdnHCl
R_{1r}	1.74 ± 0.04	16.19 ± 0.28
R_{2r}	1.94 ± 0.04	2.73 ± 0.05
$R_{1r} \times R_{2r}$	3.38 ± 0.10	44.2 ± 1.1
CL_r	56.3 ± 1.3	165.3 ± 2.5

[a] The value in each entry was the average of 10 replicate measurements.
[b] R_{1r} is the relative rate for the formation of O=Mn(IV)-MP8 obtained in the presence and absence of an enhancer.
[c] R_{2r} is the relative rate for the reaction of O=Mn(IV)-MP8 with luminol obtained in the presence and absence of an enhancer.
[d] $R_{1r} \times R_{2r}$ is the product of R_{1r} and R_{2r}.
[e] CL_r is the relative CL intensity obtained in the presence and absence of an enhancer.

occurs at pH 10.0 for both enhancers. Departure of the pH from 10.0 caused a sharp drop in the CL enhancement. Despite the large CL enhancement, the peak CL intensity at pH 10.0 in the presence of 3 M GdnHCl was still an order of magnitude smaller than that observed at pH 12.0 (Fig. 5a). Therefore, the most sensitive CL assays involving Mn(III)-MP8 are achieved by carrying out the reaction in the presence of 3 M GdnHCl at pH 12.0.

Effect of the concentrations of H_2O_2 and luminol on the CL signal

Since the maximum CL intensity occurs at pH 12.0, the dependence of CL signal on the concentrations of H_2O_2 and luminol were performed at this pH. Typical CL intensity versus time profiles for the reactions of 2.4 μM Mn(III)-MP8, 50 μM luminol, in the presence of 3 M GdnHCl at various concentrations of H_2O_2 (1.92 μM ~ 39.2 μM) are shown in Fig. 6a. The CL intensity increased rapidly and was accompanied by a progressive reduction in CL duration as the concentration of H_2O_2 increased. High concentration of H_2O_2 will accelerate the CL cycle, leading to a striking burst of CL emission over a short period of time. The double-log plot for the peak CL intensity versus the concentration of H_2O_2 is shown in Fig. 6b. The CL signal increased in three linear stages with slopes of 0.29 ($10^{-8} \sim 10^{-7}$ M), 1.76 ($2.4 \times 10^{-7} \sim 2.4 \times 10^{-6}$ M), and 1.00 ($4.9 \times 10^{-6} \sim 4.9 \times 10^{-4}$ M), respectively. The CL intensity covered five orders of magnitude over a wide range of H_2O_2 concentration. Similar double-log plots for the CL reactions obtained in the absence of enhancer and in the presence of 0.5 M Na_2CO_3 are also shown in Fig. 6b. In the presence of 0.5 M Na_2CO_3, the CL intensity also increased in three stages

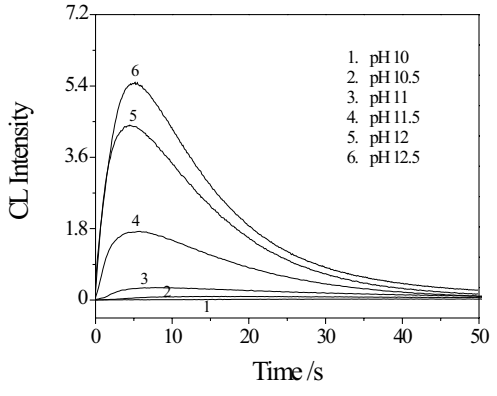

Fig. 4. Stopped-flow CL intensity vs. time profiles for the reactions of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, and 50 μM luminol in 5 mM phosphate buffer at various pH (from trace 1 to 6): 10.0, 10.5, 11, 11.5, 12 and 12.5. The PMT voltage is 700 V.

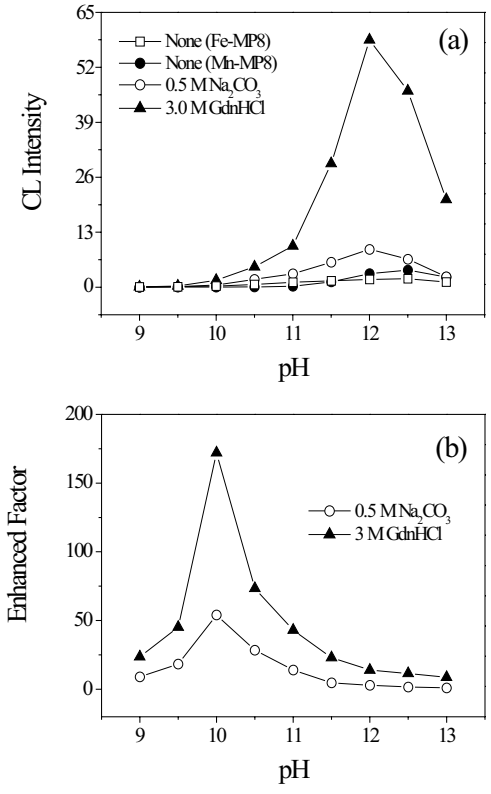

Fig. 5. (a) pH dependence of the peak CL intensities for the reactions of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, and 50 μM luminol in 5 mM phosphate buffer in the presence of (□) Fe(III)-MP8 (●) Mn(III)-MP8 (○) Mn(III)-MP8 and 0.5 M Na_2CO_3, (▲) Mn(III)-MP8 and 3.0 M GdnHCl. (b) pH dependence of the enhanced factor obtained by using the data in (a).

with slopes of 0.30 (10^{-8} ~ 10^{-7} M), 1.18 (2.4×10^{-7} ~ 9.8×10^{-6} M), and 0.96 (2.4×10^{-5} ~ 4.9×10^{-4} M). Without the enhancer, the plot contained only two linear portions with slopes of 0.29 (1 ~ 4.9×10^{-8} M) and 1.13 (4.9×10^{-8} ~ 2.4×10^{-3} M), respectively. The presence of GdnHCl exhibited a prominent CL enhancement (CL_r ranging from 4.5 ~ 14.8) in the entire range of the concentration of H_2O_2, whereas Na_2CO_3 caused a significant enhancement only at very low concentration (10^{-8} ~ 10^{-7} M) as seen in Fig. 6b. At moderate concentration (10^{-7} ~ 10^{-4} M), the enhanced factor is only ~ 2. At high concentration (> 2.4×10^{-4} M), a slight reduction in CL intensity was observed when Na_2CO_3 was present.

The CL signal also depends strongly on the concentration of luminol as illustrated in Fig. 7a for the reaction of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, in the presence of 3 M GdnHCl at various concentrations of luminol (0.1 ~ 100 μM). The CL signal and the total amount of CL emission increased rapidly as the concentration of luminol increased. At low concentration of luminol, the CL emission was limited by the depletion of the luminogenic material. Thus a prolonged and enhanced CL emission was observed as the concentration of luminol increased until the saturation level was achieved. The double-log plot for the peak CL intensity versus the concentration of luminol (Fig. 7b) shows two linear portions with slopes of 0.62 (10^{-7} ~ 10^{-5} M) and 0.19 (2×10^{-5} ~ 10^{-4} M), respectively. Also shown in Fig. 7b are the double-log plots for the CL reactions obtained in the absence of enhancer and in

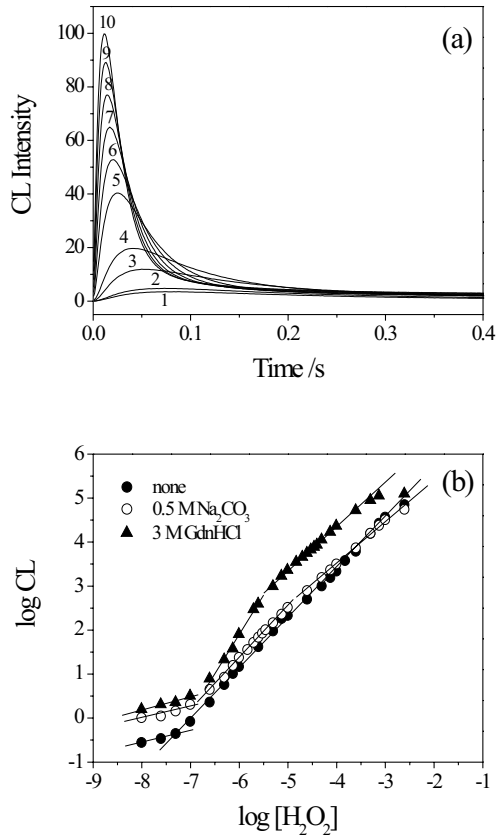

Fig. 6. (a) Stopped-flow CL intensity vs. time profiles for the reactions of 2.4 μM Mn(III)-MP8, 50 μM luminol, in 3.0 M GdnHCl and 5 mM phosphate buffer (pH 12.0) at various concentrations of H_2O_2 (from trace 1 to 10): 1.96, 2.45, 4.9, 7.35, 9.8, 19.6, 24.5, 29.4, 34.3, 39.2 μM. The PMT voltage was set at 500 V. (b) The double-log plot of the peak CL intensity versus the concentration of H_2O_2 in the presence of (●) none, (○) 0.5 M Na_2CO_3, and (▲) 3.0 M GdnHCl.

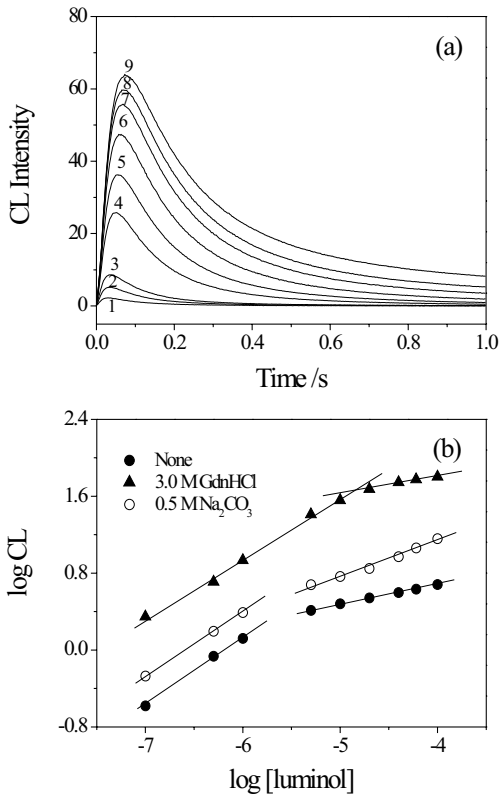

Fig. 7. (a) Stopped-flow CL intensity vs. time profiles for the reactions of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2 in 3.0 M GdnHCl and 5 mM phosphate buffer (pH 12.0) at various concentrations of luminol (from trace 1 to 9): 0, 0.1, 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 60, 100.0 μM. The PMT voltage was set at 700 V. (b) The double-log plots of the peak CL intensity versus the concentration of luminol in the presence of (●) none, (○) 0.5 M Na_2CO_3, and (▲) 3.0 M GdnHCl.

the presence of 0.5 M Na_2CO_3. Both plots consisted of two linear segments with slopes of 0.66 (10^{-7} ~ 10^{-6} M), 0.37 (5×10^{-6} ~ 10^{-4} M) for Na_2CO_3 and 0.71 (10^{-7} ~ 10^{-6} M), 0.20 (5×10^{-6} ~ 10^{-4} M) for the CL without the enhancer. The enhanced factor varied from 8.5 ~ 14.0 for GdnHCl and 1.8 ~ 3.0 for Na_2CO_3 in the entire concentration range of luminol.

Rates of the formation of the intermediate and its reaction with luminol

The assessment of the effect of the enhancer on the rate of each step involved in the CL mechanism is valuable in understanding the CL-enhancement. The formation of intermediate of Mn(III)-MP8 can be monitored by the stopped-flow technique using the photodiode array detection. Fig. 8 shows a typical stopped-flow time-resolved absorption spectra for the reaction of 2.4 μM Mn(III)-MP8 and 9.6 μM H_2O_2 in 5.0 mM phosphate buffer at pH 12.0. Upon addition of H_2O_2, a gradual disappearance of the bands at 368 and 464 nm for Mn(III)-MP8 and the concomitant appearance of a new band at 404 nm with sharp isosbestic points at 389 and 418 nm were observed. The results indicate the formation of a unique intermediate, whose spectral features resemble those of O=Mn(IV)-MP8.[17] Similar spectral changes were observed in the entire range of pH 9 ~ 13 except that the intermediate was produced at a much smaller rate and in much less an amount at low pH than at high pH.

Since the maximum CL enhancement occured at pH 10, the following kinetic studies were carried out at this pH. The rate for the formation of intermediate can be determined by following the change in absorbance at 404 nm as demonstrated in Fig. 9a for the CL reactions of 2.4 μM Mn(III)-MP8 and 2.4 μM H_2O_2 in 5.0 mM phosphate buffer in the absence or presence of GdnHCl (3 M) or Na_2CO_3 (0.5 M). The presence of enhancers, especially GdnHCl, significantly accelerated the rate of formation of intermediate. Moreover, the presence of GdnHCl greatly increased the amount of intermediate produced. A decrease in absorbance was observed in the late stage of the reaction (e.g., beyond ~ 10 s for GdnHCl). It was attributed to the regeneration of the original Mn(III)-MP8, as supported by a concomitant increase in absorbance at 368 nm in the same time period (data not shown). This process is much slower than the reaction of intermediate with luminol and is not expected to have a significant effect on the CL reaction. One- or two-exponential fitting of the curve allowed the calculation of the initial rate of step 1 (R_1). The relative values of R_1 obtained in the presence and absence of an enhancer (denoted as R_{1r}) are given in Table 1. The values of

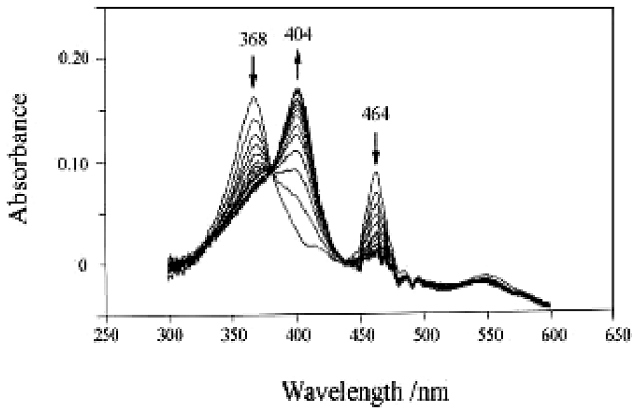

Fig. 8. Stopped-flow time-resolved absorption spectra for the oxidation of 2.4 μM Mn(III)-MP8 with 9.6 μM H_2O_2 in 5 mM phosphate buffer at pH 12.0. The time interval per trace is 0.2 s. The absorbance increased at 404 nm and decreased at 368 and 464 nm as indicated by the arrows.

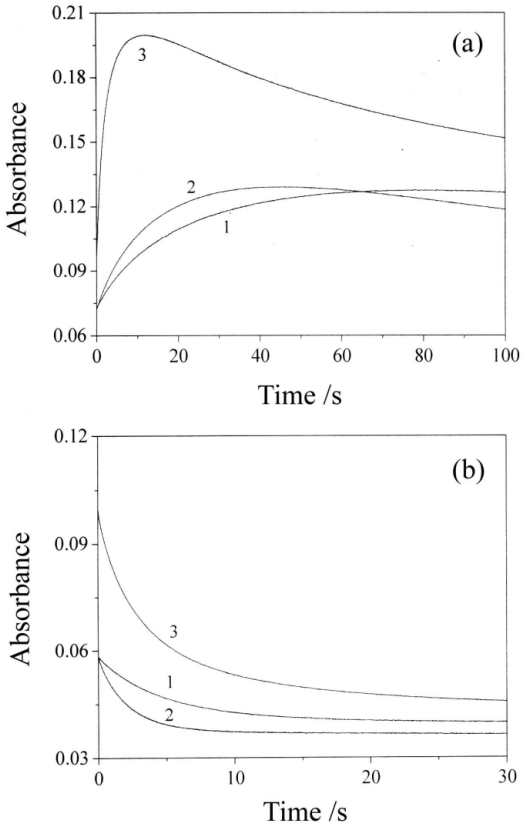

Fig. 9. Stopped-flow time courses for the changes in absorbance at 404 nm for (a) the oxidation of 2.4 μM Mn(III)-MP8 by 2.4 μM H_2O_2 and (b) the reaction of the intermediate with 50 μM luminol in the presence of none (trace 1), 0.5 M Na_2CO_3 (trace 2) and 3.0 M GdnHCl (trace 3) at pH 10.0. The PMT voltage was set at 220 V.

R_{1r} were 16.19 ± 0.28 and 1.74 ± 0.04 for GdnHCl and Na_2CO_3, respectively. The guanidinium ion may interact with MP-bound hydroperoxide through electrostatic force and hydrogen bonding. These interactions may decrease the activation energy, accelerating the formation of intermediate. Similar arguments may also apply to the bicarbonate ion. However, the stabilization of the transition state seems to be much smaller for bicarbonate than for a guanidinium ion.

Upon addition of luminol (25 μM, final concentration) to O=Mn(IV)-MP8, the time-resolved absorption spectra showed an opposite change in absorbance (i.e., decrease at 404 nm and increase at 368 and 464 nm) as compared to the spectra in Fig. 8 (data not shown). This is an indication of the occurrence of step 2 in the CL mechanism. The rate for this step can be determined by monitoring the disappearance of the absorbance at 404 nm as demonstrated in Fig. 9b for the CL reactions carried out in the presence and absence of enhancers. Single exponential fitting of the stopped-flow traces allowed the calculation of the initial rate for step 2 (R_2). The relative values of R_2 obtained in the presence and absence of an enhancer (denoted as R_{2r}) are also given in Table 1. The values of R_{2r} were 2.73 ± 0.05 and 1.94 ± 0.04 for GdnHCl and Na_2CO_3, respectively. Thus moderate acceleration of step 2 was observed by the presence of the enhancers. The combined effect of R_{1r} and R_{2r} (given in Table 1 as $R_{1r} \times R_{2r}$) revealed that the presence of 3 M GdnHCl or 0.5 M Na_2CO_3 accelerated the CL cycle by 44.2 ± 1.1 and 3.38 ± 0.10, respectively.

The acceleration of steps 1 and 2 accounts for only 26.7% (44.2/165.3) and 6.0% (3.38/56.3) of the observed CL enhancement caused by GdnHCl and Na_2CO_3, respectively. The acceleration of steps 3 and 4 in the CL mechanism and the increase in CL quantum yield by the presence of GdnHCl or Na_2CO_3 may increase the overall CL efficiency, leading to further CL enhancement. The CL enhancement caused by Na_2CO_3 is too large to be explained fully by the above arguments. There must exist some alternate route for its CL. To better understand the mechanism of CL emission and enhancement, it is necessary to identify the chemiluminescent species, for which CL emission spectrum is helpful. The CL emission spectrum can be obtained by using a continuous flow manifold in conjunction with a fluorimeter. By adjusting the flow rate, a steady-state CL signal was achieved for the CL reaction of 2.4 μM Mn(III)-MP8, 2.4 μM H_2O_2, 50 μM luminol, in 5 mM phosphate buffer (pH 12.0) in the presence of 3 M GdnHCl as shown in Fig. 10a. With the establishment of the steady-state level of signal, the CL emission spectrum can then be determined by a fluorimeter as illustrated in Fig. 10b. The emission spectrum exhibits a maximum at 425 nm, which is the characteristic emission for the excited 3-aminophthalate.[19] No other emission bands were observed. Therefore the excited 3-aminophthalate is responsible for the emission in this CL system.

The carbonate or bicarbonate may react with O=Mn(IV)-MP8 to form a $^{\bullet}CO_3^-$ radical. The $^{\bullet}CO_3^-$ radical is known to react very efficiently with luminol to form a luminol radical with a rate constant of 9×10^8 M^{-1} s^{-1}:[20]

$$^{\bullet}CO_3^- + LH^- \rightarrow CO_3^{2-} + {}^{\bullet}L^- + H^+ \quad (5)$$

The dissolved carbon dioxide in solution can react with hydroperoxide to give peroxycarbonate (HCO_4^-), which also yields $^{\bullet}CO_3^-$ upon decomposition.[21] The increased production of $^{\bullet}L^-$ by the presence of carbonate radical tends to further enhance the CL signal significantly.

CONCLUSION

The CL intensity of the Mn(III)-MP8-luminol-H_2O_2 system is enhanced dramatically by the presence of guanidine hydrochloride or Na_2CO_3, especially at pH 10.0. The acceler-

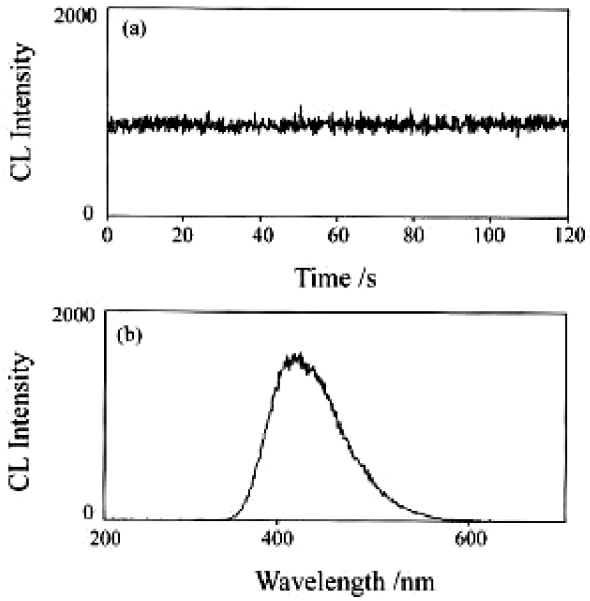

Fig. 10. (a) The time scan of the CL intensity (λ_{em} = 425 nm) and (b) the CL emission spectrum (scan rate = 240 nm/min) for the reaction of 2.5 μM Mn(III)-MP8, 2.5 μM H_2O_2, 50 μM luminol, 3.0 M GdnHCl in 5 mM phosphate buffer at pH 12.0. The PMT voltage was set at 700 V and the bandpass was 10 nm.

ation of the CL cycle by the enhancers plays an important role in the CL enhancement. The CL intensity increases by several orders of magnitude over a wide concentration range of hydrogen peroxide or luminol. This CL system can be applied to detect any one of its reaction components, including CL substrate, oxidant, catalyst, enhancer or inhibitor.

ACKNOWLEDGMENT

The authors thank the National Science Council of the ROC for financial support.

Received August 23, 2002.

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