Oscillatory Chemiluminescence during Peroxidation of Umbelliferone Catalyzed by Horseradish Peroxidase

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The chemiluminescence (CL) which occurred during oxidation of umbelliferone catalyzed by horseradish peroxidase (HRP) oscillated, when a high concentration of H_2O_2 was employed. The oscillatory CL response reflected periodic changes in the concentration of HRP intermediates between active states, compound **I** and compound **II**, and an inactive state of compound **III**. When the reaction was initiated, the CL was generated by the action of compound **I** and compound **II**. Then, the concentration of compound **III** was gradually increased, because the formation of compound **III** from compound **II** and H_2O_2, the rate constant of which was very little, could not be ignored in a high H_2O_2 condition; thus the CL was decreased by degrees. Compound **III** was then decomposed into the active HRP intermediates and the CL was re-increased. The repetition of those processes causes the oscillation. A kinetic study on the mechanism of the oscillatory CL was performed with trial rate equations.

Peroxidase (EC 1,11,1,7; POD) is known to catalyze two oxidation reactions. One is aerobic oxidation using O_2, and the other is peroxidation using H_2O_2. The mechanisms of the two oxidation reactions have been enthusiastically studied.[1,2] One of the unique properties of POD is to occur together with an oscillatory reaction.[3—6] Oscillations in the oxidation state of POD are observed spectrometrically during the aerobic oxidation of a hydrogen donor such as NADH[3] with continuous O_2 aeration. Interestingly, the oscillatory reactions have been hitherto reported only in the aerobic oxidation.[1—6] In the peroxidation, an oscillation seems likely, since the oxidation states of POD found in the peroxidation cycle are similar to those in the aerobic oxidation. Usually, a reaction which controls concentration of an intermediate is indispensable in an oscillatory reaction. In the POD-catalyzed aerobic oxidation of NADH, O_2 controls the concentration of NAD·, an active intermediate produced in a POD cycle. Such a reaction must be necessary for the appearance of oscillation in the peroxidation catalyzed by POD.

We have studied a chemiluminescence (CL) reaction during peroxidation of fluorescein catalyzed by horseradish peroxidase (HRP).[7—9] We have found that 3-morpholino-1-propanesulfonic acid (MOPS) could react with a fluorescein radical which was produced in a HRP cycle, and acted as an energy transferor in the CL system.[10] In our subsequent study on the CL reaction of umbelliferone, whose structure is similar to fluorescein, we found an oscillatory chemiluminescence (OSC-CL) reaction occurred at higher H_2O_2 concentration only in the MOPS buffer solution. In the present work, we investigated the conditions for the appearance of the OSC-CL and the reaction mechanism.

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.). Computer simulation was performed with a personal computer (PC-286VG, EPSON Co., Ltd.)

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.

An H_2O_2 solution was made daily by dilution of 31% H_2O_2 solution with a 0.10 M (1 M=1 mol dm^{-3}) MOPS solution adjusted at prescribed pH.

Procedure. A 0.50 cm^3 portion of a CL reagent solution, containing HRP and fluorescein or umbelliferone, was placed 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 the voltage corresponding to photon numbers radiated each time.

The absorption spectra of HRP was measured by mixing a 1.0 cm^3 portion of a CL reagent solution and a 1.0 cm^3 portion of an H_2O_2 solution in a 1-cm quartz cell. The absorption spectra were measured 70 s after the initiation of the reaction.

In the measurement of the absorbance–time course of compound **III**, a 1.0 cm^3 portion of a CL reagent solution and a 1.0 cm^3 portion of an H_2O_2 solution were mixed in a 1-cm quartz cell. Then the absorbance at 417 nm (the maximum absorption wavelength of compound **III**) was monitored from the initiation of the reaction.

All the reagent concentrations shown in the figures were final concentrations.

Results and Discussion

CL Reaction of Fluorescein and Umbelliferone at Several H_2O_2 Concentrations. Typical CL response curves of fluorescein and umbelliferone were observed at several levels of the H_2O_2 concentrations and at pH 6.60. The results are shown in Fig. 1. The CL

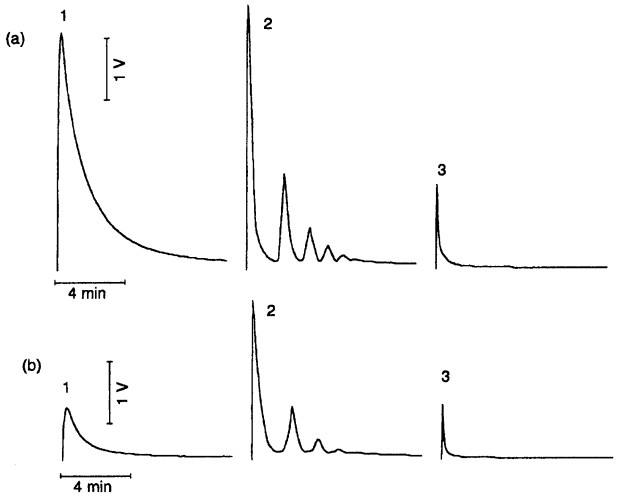

Fig. 1. Effect of H_2O_2 concentration on the CL response of fluorescein and umbelliferone. (a) fluorescein, (b) umbelliferone, [Dye]=2.2×10^{-4} M, [HRP]=1.0×10^{-6} M, [MOPS]=0.10 M, pH 6.60 [H_2O_2]=0.01 M (curve 1), 0.05 M (curve 2), 0.50 M (curve 3).

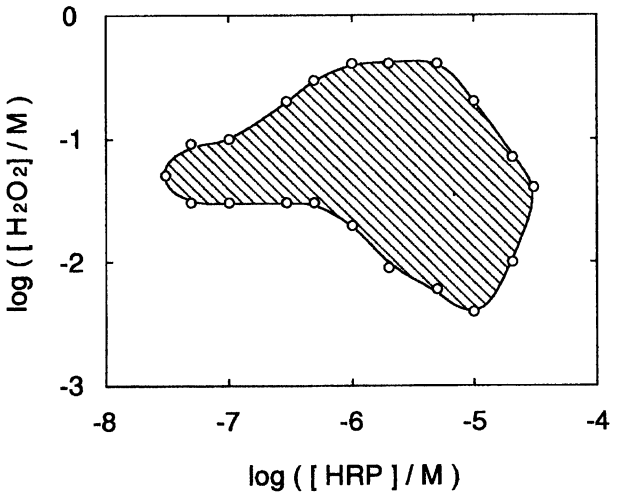

Fig. 2. Plots of [H_2O_2] vs. [HRP] for the appearance of the OSC-CL. [umbelliferone]=1.0×10^{-4} M, [MOPS]=0.10 M, pH 7.0. Within the hatched region, the OSC-CL was observed.

occurs instantaneously after mixing a CL reagent solution and the H_2O_2 solution. When a 1.0×10^{-2} M H_2O_2 solution was employed, the CL intensity reached a maximum value within a few seconds, and then decreased. On the other hand, at a 0.05 M H_2O_2 concentration, OSC-CL were observed with both dyes. The CL intensity was once decreased after a maximum value; however, it re-increased 2 min after the initiation of the reaction. The rise and fall in the CL intensity ceased within several cycles. At a 0.50 M H_2O_2 concentration, the CL peaks except the first one disappeared so that the OSC-CL did not emerge at all.

In the experiments described below, we employed umbelliferone. Umbelliferone is almost colorless, thus permitting us to measure the absorption spectra of HRP in several oxidation states without interference from umbelliferone.

Conditions for Appearance of OSC-CL. Figure 2 illustrates the concentration range of H_2O_2 for the occurrence of the OSC-CL of umbelliferone at several HRP concentrations. The OSC-CL was observed only within the hatched region. The H_2O_2 concentration range for the emergence of the OSC-CL was dependent on HRP concentration.

Effects of reaction conditions on the OSC-CL were then examined in fixed concentrations of HRP (5.0×10^{-7} M) and H_2O_2 (5.0×10^{-2} M). Effect of a buffer component on the OSC-CL was tested by using phosphate and citrate buffer solution. In both cases, no OSC-CL was observed. Only a single peak was observed in the CL response curves. Next, influence of MOPS concentration was investigated in the range from 5.0×10^{-3} to 0.25 M. The OSC-CL was observed over the MOPS concentration range from 0.010 to 0.15 M.

Variation in the CL response with pH was examined in the 6.2—8.2 pH range. The time interval between adjacent CL peaks (period) and the intensity of the OSC-CL were affected by the pH. The period was increased with an increase in the pH. The CL intensity was maximum at pH 7.0. Thus, in the experiments shown below, the pH of the H_2O_2 solution was adjusted at pH 7.0.

The CL response curves with several umbelliferone concentrations were obtained. The OSC-CL was observed in the range from 2.0×10^{-5} to 1.0×10^{-4} M umbelliferone. The CL intensity was increased with increasing umbelliferone concentration whereas the period was almost independent on the umbelliferone concentration.

Mechanism of OSC-CL Reaction. In order to investigate the mechanism of oscillation, the mechanism of umbelliferone CL was preferentially studied. Previously we have reported that MOPS functions as an energy transferor in the fluorescein CL reaction.[10] Since umbelliferone possesses similar partial structure to fluorescein and has the same ability of exhibiting the OSC-CL as fluorescein, it is reasonable to consider that the umbelliferone CL proceeds similarly to fluorescein; that is, a protonated umbelliferone (UmH) functions as the hydrogen donor for the HRP cycle, and a deprotonated umbelliferon (Um^-) functions as an energy acceptor (Eqs. 1, 2, 3, 4, 5, 6, and 7),

$$\text{native HRP} + H_2O_2 \xrightarrow{k_1} \text{compound I} \quad (1)$$

$$\text{compound I} + \text{UmH} \xrightarrow{k_2} \text{compound II} + \text{Um}\cdot \quad (2)$$

$$\text{compound II} + \text{UmH} \xrightarrow{k_3} \text{native HRP} + \text{Um}\cdot \quad (3)$$

$$\text{Um}\cdot + \text{MOPS} \xrightarrow{k_4} \text{UmH} + \text{MOPSoxd} \quad (4)$$

$$\text{MOPSoxd+dissolved } O_2 \xrightarrow{k_5} \text{excited compound (P}^*\text{)} \quad (5)$$

$$P^* + Um^- \xrightarrow{k_6} P + (Um^-)^* \quad (6)$$

$$(Um^-)^* \xrightarrow{k_7} Um^- + \text{light} \quad (7)$$

where native HRP is a native state of HRP and MOPS-oxd is an oxidized MOPS. The CL reaction can be explained by Eqs. 1, 2, 3, 4, 5, 6, and 7. Then we pursued the mechanism of the OSC-CL.

Equations 1, 2, 3, 4, 5, 6, and 7 don't include any mechanism which would depress the formation of excited products, so that the oscillation can not be explained by those equations. Therefore, HRP intermediates of other oxidation states probably participate in the OSC-CL cycle. It is well accepted that, at a high H_2O_2 concentration, compound **II** reacts with H_2O_2 to form compound **III**, a catalytically dull oxidation state: Eq. 8.[11]

$$\text{compound II} + H_2O_2 \xrightarrow{k_8} \text{compound III} + 2H^+ \quad (8)$$

In order to confirm the production of compound **III** during the OSC-CL reaction, absorption spectra of HRP was measured 70 s after the initiation of the reaction, when the CL emission was stable and minimum. The spectra obtained are shown in Fig. 3. Characteristic absorption maxima for compound **III** were observed at 417 nm in Soret band, and at 542 nm and 576 nm in Q band. These results support the formation of compound **III**.

Next, the time profile of compound **III** concentration was determined from the change in absorbance at 417 nm. In Fig. 4a, the absorbance was increased from the initiation, then reached a constant, and immediately decreased 3 min after the start; then a rise and a fall in the absorbance were observed. The CL response curve in Fig. 4b was obtained under the same condition as in Fig. 4a. The concentration of compound **III** was oscillated in a way which was synchronized with the

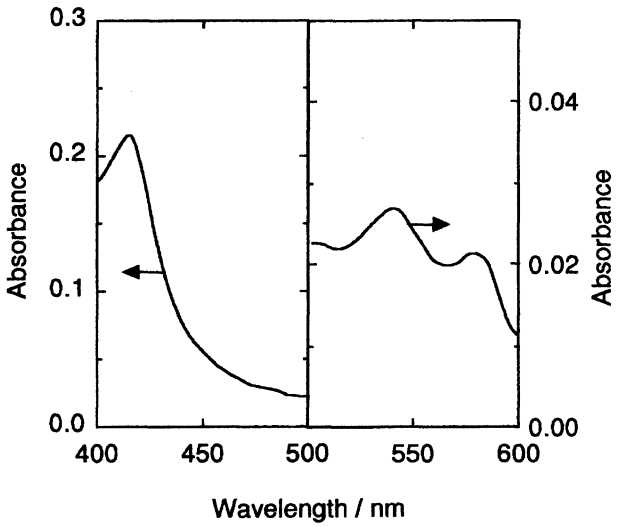

Fig. 3. Absorption spectra of HRP 70 s after the injection of H_2O_2 solution. [umbelliferone]=1.0×10^{-4} M, [H_2O_2] = 5.0×10^{-2} M, [HRP] = 2.0×10^{-6} M, [MOPS]=0.10 M, pH 7.0.

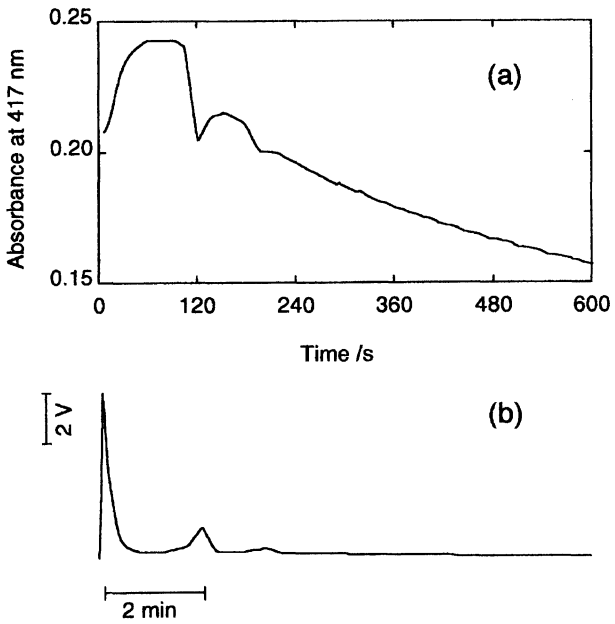

Fig. 4. Changes in absorbance and the CL response during the peroxidation of umbelliferone catalyzed by HRP. (a) absorbance, (b) CL, [umbelliferone]=1.0×10^{-4} M, [H_2O_2]=5.0×10^{-2} M, [HRP]=2.0×10^{-6} M, [MOPS]=0.10 M, pH 7.0.

CL intensity in a reversed phase. Hence, the periodic formation and decomposition of compound **III** was responsible for the periodic change in the CL intensity, which reflected the periodic production of Um·. Meanwhile, in Fig. 4a a gradual decrease in the absorbance was also observed. This may indicate the destruction of HRP at the high concentration of H_2O_2.

The decomposition of compound **III** was then investigated. Since a phenolic compound such as umbelliferone has a potentiality of reducing compound **III**,[12] the reductive decomposition of compound **III** by umbelliferone was tested. In the experiment, compound **III** was produced by mixing HRP and excess H_2O_2 in advance of the addition of umbelliferone. The results are shown in Fig. 5. Absorbance at 417 nm increased just after mixing of HRP and the H_2O_2 solution, and then reached a constant value 90 s after the initiation. This stands for the production of compound **III**. Immediately after the injection of a small amount of umbelliferone solution, absorbance at 417 nm decreased. The decrease in the absorbance was ended after 30 s from the injection of umbelliferone. Following this, the absorbance showed damped oscillation.

Since the decomposition of compound **III** was observed by squirting an umbelliferone solution, umbelliferone probably acted as a reductant of compound **III**: Eq. 9.

$$\text{compound III} + UmH + H^+ \xrightarrow{k_9} \text{compound I} + Um\cdot \quad (9)$$

On the other hand, compound **III** is known to react with compound **I**, the product of the reaction 9, to pro-

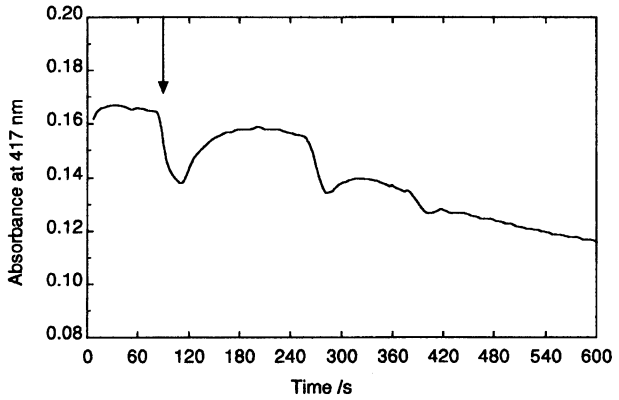

Fig. 5. Change in absorbance at 417 nm by the addition of umbelliferone into compound **III** solution. $[H_2O_2]=5.0\times10^{-2}$ M, $[HRP]=1.6\times10^{-6}$ M, [MOPS]=0.10 M, pH 7.0. At first, HRP solution and the H_2O_2 solution was mixed. Then at the arrow, a 0.050 cm^3 portion of 3.0×10^{-3} M umbelliferone was injected.

duce native HRP and compound **II**.[12]

$$\text{compound III} + \text{compound I} \xrightarrow{k_{10}} \text{native HRP} + \text{compound II} + O_2 \quad (10)$$

In the present system, native HRP was quite rapidly converted into compound **I** (Eq. 1), because a high concentration of H_2O_2 was present and the value of rate constant of Eq. 1 is great (ca. 10^7 $M^{-1}s^{-1}$). Therefore, once compound **III** is decomposed in reaction 9, compound **III** is further decomposed autocatalytically because native HRP is converted into compound **I**, the reactant of Eq. 10.

The mechanism for the reproduction of compound **III** was next considered. When the rate of compound **III** formation in Eq. 8 exceeded the rate of compound **III** decomposition in Eqs. 9 and 10, the production of compound **III** recommenced. In the two decomposition processes, the rate of Eq. 10 was predominant since no effect of the concentration of umbelliferone was observed on the period of the oscillation. Thus we can assume the inhibitory reaction for the autocatalytic reaction 10; that is, compound **I** reacts with MOPSoxd, produced from the reaction of MOPS with Um·, to form compound **II** and an oxidized product: (Eq. 11).

$$\text{compound I} + \text{MOPSoxd} \xrightarrow{k_{11}} \text{compound II} + \text{product} \quad (11)$$

The production of MOPSoxd became lower with increasing compound **III** concentration. Simultaneously, the consumption of MOPSoxd increased so that the reaction of Eq. 11 became slow. Accordingly, the decomposition of compound **III** began when the rate of Eq. 10 surpassed that of Eq. 11. As the MOPSoxd was an intermediate of the CL emission on one hand, the CL intensity was anticipated to decrease while the MOPSoxd was consumed in reaction 11. This is consistent with the fact that the CL intensity decreases with increasing compound **III** concentration.

Consequently, in this peroxidation system, the oscillation reflected the periodic change in compound **III** concentration, just as it did in the aerobic oxidation. However, the elementary reaction for the formation of compound **III** is different for the two oxidation reactions. In the peroxidation, compound **III** is produced from the reaction of compound **II** with H_2O_2, whereas in the aerobic oxidation compound **III** is formed from the reaction between native HRP and superoxide anion radical. The alternative of the peroxidation or the aerobic oxidation, may be due to the nature of the hydrogen donor, which is converted to a key intermediate radical in the HRP cycle.

Analysis of Reactions by Computer Simulation. In order to investigate the validity of the trial mechanism of OSC-CL, kinetic analysis was carried out by using a computer. For the computer simulation, some assumptions were made as shown below.

(i) The concentration of HRP in native state was neglected, because the rate of the reaction 1 is rapid.

(ii) The rate of reaction 3 was neglected, because the value of this rate constant was expected to be little.

(iii) The quenching reaction of Um· was taken into account (Eq. 12).

$$\text{Um·}(+\text{Um·} \text{ or } O_2) \xrightarrow{k_{12}} \text{oxidized Um} \quad (12)$$

(iv) The destruction of HRP was omitted.

(v) The concentrations of MOPS and H_2O_2 were assumed to be constant.

From assumption (i), the concentration of compound **II** can be represented by using the total HRP concentration ($[HRP]_{total}$), compound **I** concentration and compound **III** concentration; that is, [compound **II**]= $[HRP]_{total}$ − [compound **I**] − [compound **III**]. Meanwhile, reactions 6 and 7 are not concerned with the change in the oxidation state of HRP, thus precluding their use for the simulation.

From Eqs. 2, 4, 5, 8, 9, 10, 11, and 12, simultaneous rate equations for the concentrations of compound **I** (CP **I**), compound **III** (CP **III**), UmH, Um·, and MOPSoxd were constructed as follows:

$$\frac{d[\text{CP I}]}{dt} = -k_2[\text{CP I}][\text{UmH}] + k_9[\text{CP III}][\text{UmH}] + k_{10}[\text{CP I}][\text{CP III}] - k_{11}[\text{CP I}][\text{MOPSoxd}]$$

$$\frac{d[\text{CP III}]}{dt} = k_8([HRP]_{total} - [\text{CP I}] - [\text{CP III}])[H_2O_2] - k_9[\text{CP III}][\text{UmH}] - k_{10}[\text{CP I}][\text{CP III}]$$

$$\frac{d[\text{UmH}]}{dt} = -k_2[\text{CP I}][\text{UmH}] - k_9[\text{CP III}][\text{UmH}] + k_4[\text{Um·}][\text{MOPS}]$$

$$\frac{d[\text{Um·}]}{dt} = k_2[\text{CP I}][\text{UmH}] + k_9[\text{CP III}][\text{UmH}] - k_4[\text{Um·}][\text{MOPS}] - k_{12}[\text{Um·}]$$

$$\frac{d[\text{MOPSoxd}]}{dt} = k_4[\text{Um·}][\text{MOPS}] - k_{11}[\text{CP I}][\text{MOPSoxd}] - k_5[\text{MOPSoxd}]$$

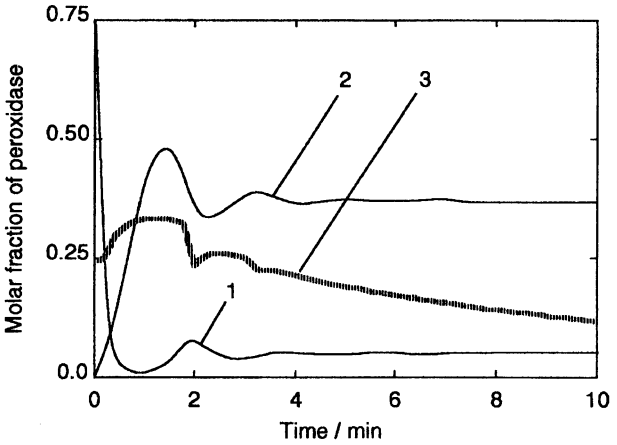

Fig. 6. Simulation of compound **I** and compound **III** concentrations during the OSC-CL reaction. curve 1: compound **I**, curve 2: compound **III**, curve 3: experimental results shown in Fig. 4a. Simulated constants: $k_2=3$, $k_4=0.05$, $k_5=0.5$, $k_8=0.00055$, $k_9=0.00001$, $k_{10}=400$, $k_{11}=5$, $k_{12}=0.5$, $[\mathrm{HRP}]_{\mathrm{total}}=0.002$, $[\mathrm{MOPS}]=100$, $[\mathrm{H_2O_2}]=50$. Due to the limitation of calculable orders, the second-order rate constants were divided by 10^6, and the concentration factors were multiplied by 10^3.

The time course of each concentration was solved by using the Runge–Kutta–Gill method.[13] In the calculation, the rate constants of k_2 and k_8 were estimated from the change in absorbance during the conversion of HRP oxidation states. The values of k_4, k_5, k_9, k_{10}, k_{11}, and k_{12} were obtained by varying within a reasonable range.

Figure 6 shows an example of a simulation of concentrations of compound **I** and compound **III**. The concentration of compound **III** increased from the initiation, and reached a maximum value within 2 min, and then decreased up to 3 min from the start. Following this, the concentration increased and decreased. On the other hand, compound **I** was synchronizing with compound **III** in a reversed phase. In Fig. 6 curve 3, the change in the absorbance at 417 nm was also represented under the same conditions as the simulation. The oscillation pattern of compound **III** clearly resembles that of the absorbance. These results suggest that the proposed mechanism is reasonable.

In conclusion, HRP was proved to be concerned with an oscillatory phenomenon in peroxidation as well as in the aerobic oxidation. MOPS controlled the decomposition of compound **III**. A minute periodic change in the oxidation state of HRP was successfully monitored by the CL method, even in a strongly-colored fluorescein solution in which the dynamics of the oxidation state could hardly be observed by spectrometry. The effects of biological reductants on the OSC-CL response curve are currently examined in our laboratory for the analytical application of the OSC-CL reaction. This system will be useful for constructing a new model in a research field of nonlinear phenomena.

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