論文の内容の要旨

論文題目　Quantitative and *in situ* spectroscopic study on color change in firefly bioluminescence

(ホタル生物発光色変化の定量およびその場分光による研究)

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Bioluminescence of firefly is a photon emission process accompanying oxidation of the substrate luciferin in the enzyme luciferase (Luc) with cofactors ATP and magnesium ion. Its color can be shifted from the natural yellow-green to red in *in vitro* reaction by decrease in the pH value of the buffer solutions, addition of transition metal ions, or mutagenesis to the luciferase.

The color-change mechanism of firefly bioluminescence was explained as equilibrium or competition between two species or states of the light emitter oxyluciferin (OL). However, Ando et al. (Ando et al. *Nat. photonics* **2**(1), 44-47, 2007) found that the mere intensity change in a green peak determined the spectral shape and peak position by studying the pH-sensitive quantitative spectra recently. The first objective of this dissertation is to investigate the influence of the micro-environmental change on the green, orange, and red emission components, and thus to further verify the feasibility of Ando and coworkers' interpretation. I measured the quantitative spectra of firefly bioluminescence under circumstances modified by addition of transition metal ions or mutagenic Lucs. Then I carried out curve fitting with Gaussian-type functions to quantitatively analyze how the reaction environment affect each emission component. Meanwhile, the spectroscopic properties of free OL in various solvents with different acidity have been clarified recently, but those of OL in Luc environment (OL-Luc complex) still remain to be studied. The second objective is to understand the influence of Luc environment on the emission of OL. I studied the spectroscopic properties of OL in the active pocket of pH-sensitive and pH-insensitive Lucs and revealed the real form it takes in Luc.

I used a homemade setup (Figure 1) for total-photon-flux spectrum measurement to quantitatively study the color change of firefly bioluminescence following the method of Ando and coworkers' (Ando et al. *Photochem. Photobiol.* **83**(5), 1205-10, 2007). I calibrated the light-collection efficiency of the sample cell, the transmissivity of the slit, and the absolute sensitivity of the system to convert relative spectrum to absolute one scaled by photon number. I constructed setups for *in situ* absorption monitoring and

fluorescence spectrum measurement to characterize the stability of OL in Luc formed in bioluminescent reaction and to study its spectroscopic properties. The setups and materials used in the measurement are stated in Chapter 2.

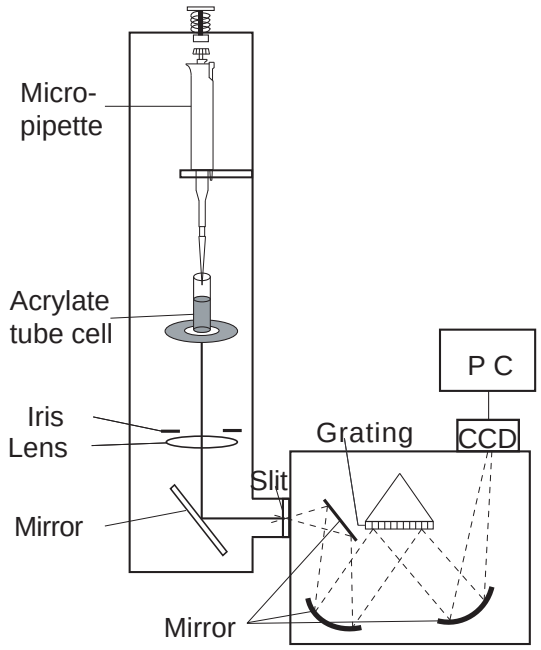

Figure 1: Scheme of the setup for quantitative spectrum measurement.

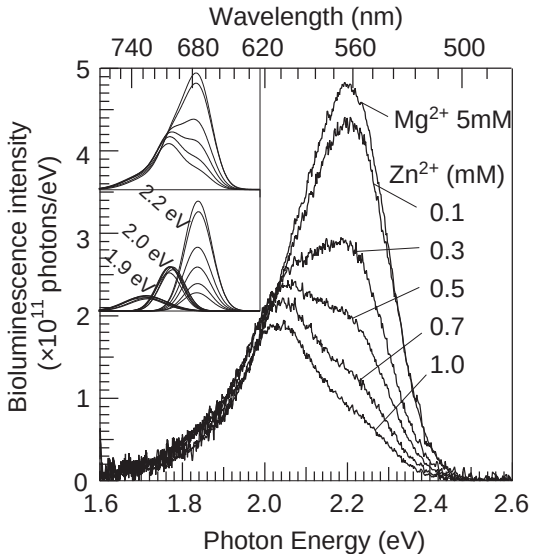

Figure 2: Quantitative spectra of bioluminescence in the presence of 5 mM of Mg^{2+} ions and Zn^{2+} ions with different final concentrations at pH 8.0. The insets show the results of Gaussian fitting and each component.

First I studied the quantitative spectra of *Photinus pyralis* (North-American firefly) bioluminescence in the presence of various transition metal ions at pH 8.0. Figure 2 shows the bioluminescence spectra in the presence of different concentrations of zinc ions. The intensity of the green peak (560 nm) was sensitive to the concentration of zinc ions, while that of the red peak (620 nm) seemed to be unchanged. I analyzed the spectra by decomposing them into three Gaussian-type functions (shown in the inset of Figure 2): green ($\sim$ 2.2 eV), orange ($\sim$ 2.0 eV), and red ($\sim$ 1.9 eV) Gaussian components. The peak energy and full-width-at-half-maximum (FWHM) value of each component are independent of the concentration of zinc ions. Only the intensity of the green component is sensitive to the concentration of zinc ion, while those of the other two are not. Similar color change was observed by adding Cd^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, or Hg^{2+} ions, while concentrated Mg^{2+}, Ca^{2+}, or Mn^{2+} ions caused no shift in spectral peak or quantum yield. According to the concentration of the metal ion required to produce identical spectral change, the sensitivity of quantum yield and spectrum to these metal ions were ordered as $Hg^{2+} > Zn^{2+}$, $Cd^{2+} > Ni^{2+}$, Co^{2+}, $Fe^{2+} \gg Mg^{2+}$, Mn^{2+}, Ca^{2+}. The order had no correlation with the electron-withdrawing strength of these metal ions. It is likely that the metal ions bind to Luc and the modified Luc affects bioluminescent emission of OL. The strong difference and the peculiar order in spectral sensitivities for the various metal ions may stem from their special binding affinity to different amino acid residues in luciferase. The experimental results mentioned above and discussions on possible bindings between the metal ion and protein are presented in Chapter 3.

To understand the color change caused by microenvironmental change in the Luc, I studied the spectra of bioluminescence catalyzed by mutagenic *Luciola cruciata* (Genji-botaru) Luc in which the amino acid residue tyrosine (Y) 257 was substituted by phenylalanine (F), alanine (A), glutamate (E),

or arginine (R) in Chapter 4. The representative spectra of Y257F and Y257E are shown in Figure 3. Curve fitting with Gaussian-type functions were employed to quantitatively analyze their bioluminescence spectra that exhibited sensitivity or insensitivity to change in the pH value of solutions. For the pH-sensitive Luc (wild type and Y257F), intensity change in the green region was observed accompanying the spectral peak shift. Their spectra were reproduced by three Gaussian components, similar to the results in Figure 2. Change in the intensities of the green Gaussian components at various pH values determined the peak positions and shapes of the total bioluminescence spectra. In addition, the maximum intensity of the green component in the spectra of Y257F was weaker than that in wild-type Luc, directly correlating with the less pH-sensitivity of the bioluminescence spectra of Y257F. In contrast, the spectra of the pH-insensitive bioluminescence were well reproduced by only two components, the orange and red ones. As an example, the spectra catalyzed by Y257E are shown in Figure 3 (b). The green component was not required in fitting the bioluminescence spectra catalyzed by Y257A/E/R, and hence resulted in no/minor change in the spectral intensities or peak positions. The FWHM and peak energies of the three Gaussian components were close for all the Lucs, although the strong intensity and blue shift of the orange component of Y257R were exceptions. The sensitivity of each component to the pH value was unchanged: the green component was pH-sensitive, and the orange and red ones were still pH-insensitive. The change in the intensity of the green component mainly determined the spectral shape and peak position. Therefore, modification to the Luc by mutagenesis influences the intensity of the green emission component, and thus regulates the pH-sensitivity of the bioluminescence. The correlation between the color change and the properties of the substituted amino acid residues are also discussed in this chapter.

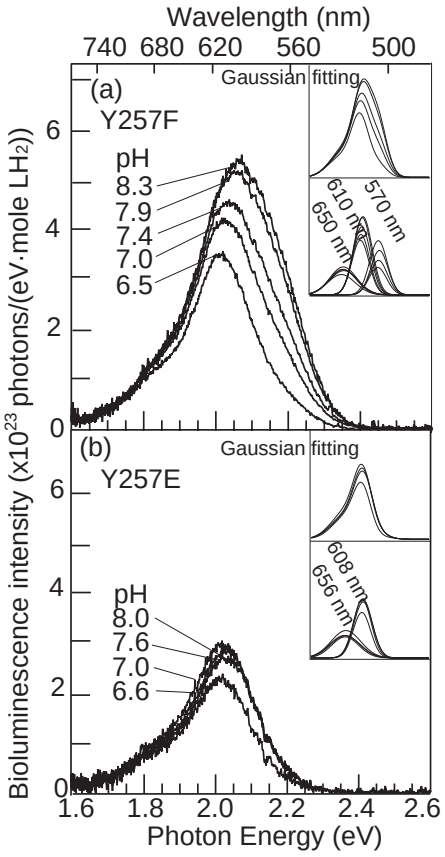

Figure 3: Quantitative spectra of bioluminescence catalyzed by (a) Y257F and (b) Y257E mutants of Genji-botaru luciferase at various pH values. The insets show the results of Gaussian fitting and each component.

Chapter 5 aimed at characterizing the properties of the light emitter OL in Luc environment and thus revealing the real forms of OL in the active pocket of the enzyme. I demonstrated the stability of OL in Luc formed in bioluminescent reaction via monitoring the *in situ* absorption spectra. OL in complex with Luc was stable for about an hour under aerobic basic conditions, and the stable period was longer in acid solution. This enabled the studies on the *in situ* spectroscopic properties of OL.

For OL in complex with pH-sensitive *Photinus pyralis* Luc (Figure 4), a peak with $\lambda^{\mathrm{abs}} = 380$ nm is dominant in the absorption spectra. The trade-off in its intensity with that of a weak shoulder $\lambda^{\mathrm{abs}} \sim 430$ nm reveals equilibrium between two species of OL. Excitation at 380 nm generated a blue ($\lambda^{\mathrm{fl}} = 450$ nm) and green ($\lambda^{\mathrm{fl}} = 560$ nm) fluorescence bands with comparable intensities (Figure 4 (a)). Excitation at 430 nm generated a single green fluorescence band with peaks at 560 nm (Figure 4 (b)). Very weak red fluorescence ~ 610 nm was observed with excitation at 510 nm, although no corresponding absorption

peaks was detected.

According to the spectroscopic properties of free OL and NMR data, the three emitters were assigned to neutral enol-OL (blue), monoanionic phenolate-enol-OL$^-$ (green), and monoanionic phenolate-keto-OL$^-$ (red), respectively. The strong intensity of the green fluorescence with excitation at 380 nm (Figure 4 (a)) is not proportional to the weak absorbance at 430 nm, which ruled out the possible direct excitation of the emitter corresponding to $\lambda^{\mathrm{abs}} \sim 430$ nm. A possible reason is that partial excited-state proton transfer occurred to the neutral OL generated the green-emitting phenolate-enol-OL$^-$. The absorbance at 430 nm was not proportional to the intensity of corresponding green fluorescence shown in Figure 4 (b), which indicated that its fluorescence efficiency depended on pH value of the solution. The absorption and fluorescence spectra reveal that OL mainly exists as neutral enol form in the active pocket of Luc after bioluminescent emission. A minority of green-emitting phenolate-enol-OL$^-$ displays equilibrium with enol-OL, which is moderately regulated by change in pH value. The red emitter phenolate-keto-OL$^-$ is absent in the ground state, reason of which may be its transition to enol-OL that has lower free energy.

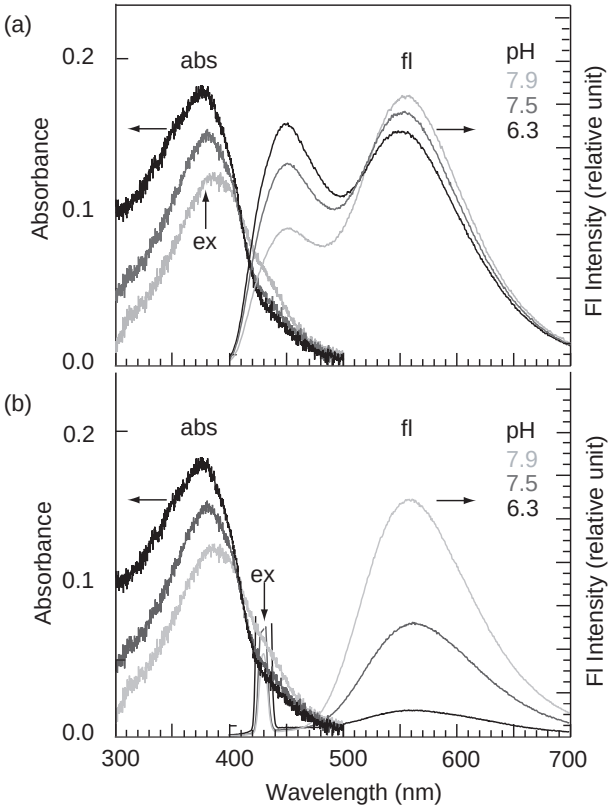

Figure 4: *In situ* absorption (left axis) and fluorescence (right axis) spectra of oxyluciferin in Genji-botaru luciferase formed in bioluminescent reaction at various pH values with excitation at (a) 380 nm and (b) 430 nm.

The spectroscopic properties of OL in a red-emitting H433Y mutant of *Luciola cruciata* Luc were studied and compared with those in the wild-type one in Chapter 6. Both the intensities and peak positions of the absorption and fluorescence spectra of OL in complex with the pH-insensitive red H433Y mutant were very similar to those of pH-sensitive wild-type Luc. The similarities indicate that the difference in the environment of wild-type and H433Y mutant Lucs does not significantly change the spectroscopic properties of OL in the ground state. They also indicate that the total concentrations of all species of final products of the green- and red-bioluminescent reaction are similar.

The quantitative spectra and analyses show that the intensity of the green bioluminescent emission component is sensitive to the changes in reaction environment and thus determines the spectral shape and peak energy, verifying the assumption of Ando and coworkers'. This suggests the necessity of explaining the intensity change in the green peak to understand the color-change mechanism of firefly bioluminescence. Studies on the *in situ* spectroscopic properties of OL clarified the real forms of OL in the Luc and enabled assignment of bioluminescent emitters. Similarities between its properties in pH-sensitive and -insensitive Luc reveal that different Luc environment causes little change in the properties of OL in the ground state, but affects the excited state generated via chemical reaction, indicating the importance of studying the intermediate state of the reaction to explain the color-change mechanism.