The structural origin of the color differences in the bioluminescence of firefly luciferase

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Abstract Six chimeric mutants between *Hotaria parvula* (λmax = 568 nm) and *Pyrocoelia miyako* (λmax = 550 nm) luciferases were reconstructed to determine the structural origin of the color differences in firefly luciferase. Based on light-emitting color, five chimeric luciferases could be divided into two groups: the three green-emitting mutants, classified as *P. miyako* luciferase, and the two yellow-emitting mutants, classified as *H. parvula* luciferase. Their common fragments between Val-209 and Ala-318 within each group contain the active site for the color differences.

Key words: Luciferase; Firefly; Light emitting; Color difference; Active site

1. Introduction

Luciferases are the enzymes that catalyze the light-emitting reactions in bioluminescence organisms. Firefly luciferase catalyzes the oxidation of luciferin in the presence of ATP, Mg^{2+}, and molecular oxygen, yielding as products light, oxyluciferin, CO_2 and AMP [1,2]. The bioluminescence reaction of firefly luciferases in vitro emits light ranging from green (λmax547 nm) to red (λmax = 604 nm) [3–5], although the firefly luciferin is a substrate common to all luminous beetles, including the true firefly. Thus, the color differences seen in fireflies are due to the structural differences in luciferases. Seven clones of true firefly luciferases have been cloned and analyzed. The clones of fireflies were derived from *Luciola cruciata* (Lc) [6], *Luciola lateralis* (Ll) [7], *Hotaria parvula* (Hp) [8], and *Pyrocoelia miyako* (Pm) [8] from Japan, *Photinus pyralis* (Pp) from America [9], *Luciola mingrelica* (Lm) from Russia [10], and *Lampyris noctiluca* (Ln) from England [11]. These luciferases consisted of 548 amino acid residues, except for the 550 amino acid residues of Pp luciferase. The four luciferase isozymes from the click beetle *P. plagiophthalamus* (Cb), which were characterized by different luminescence colors, were cloned and shown to consist of 543 amino acid residues [12]. Multiple alignment of the amino acid sequences of Pm with those of Pp, Lc, Ll, Hp, Lm, and Cb (green-light isozyme) luciferases yielded sequence homologies of 82.1%, 66.2%, 66.6%, 64.4%, 63.3%, and 46.5%, respectively [8]. They contain a highly conserved region of amino acid sequence for catalyzing a luminous reaction. These luciferases should also have an amino acid region that determines the color of the firefly's luminescence.

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction.

The light-emitting colors were also changed from yellow to red by various manipulations [13,14] or random mutations of luciferases [15]. Given these results, three possibilities for the chemical origin of the color differences have been proposed. The first explanation is based on the different ionic structure of the excited oxyluciferin; the first excited monoanion state deprotonates to form a dianion which emits yellow light [16,17]. A second possibility involves polarity of the oxyluciferin binding site [16]. The third explanation points to a conformation of oxyluciferin so rigid that the first-excited singlet state suffers a structural restriction of luciferases that changes the energy of electronic state transitions [18]. A comparison of native structures on four Cb luciferases indicated that a 25 amino acid region (223–247) was responsible for the color differences [19]. Also, random mutagenesis of Lc luciferase resulted in color differences induced by single substitutions at Val-239, Ser-286, Gly-326, His-433, and Pro-452, respectively [15]. However, the color changes effected by the luciferase mutants identified above only caused a shift to longer wavelengths in the spectrum.

To clarify the active site for the color differences of luciferase, we are making the chimeric enzymes between Hp and Pm luciferases. The emission spectrum of the recombinant Pm is a shift to a value 18 nm shorter than that of the recombinant Hp luciferase. The emission spectra of the chimeric luciferases will reveal the important region for determining the color differences of firefly luciferase.

2. Material and methods

2.1. Enzymes and chemicals

Restriction enzymes were obtained from Takara Shuzo (Kyoto, Japan), and *Taq* polymerase, Isopropyl β-D-thiogalactopyranoside (IPTG), firefly D-luciferin-Na, and ATP-Na were provided by Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the highest grade commercially available.

2.2. Construction of chimeric luciferases

The cDNA fragments coding for Pm and Hp luciferases which were digested by *NcoI*/*Hin*dIII were inserted into the *NcoI*/*Hin*dIII site of the high-expression vector pTrc-99A under the control of the trc promoter and lac repressor [8]. Two plasmids of firefly luciferases, pT-PmL(ABCD) and pT-HpL(abcd), can be divided into four regions according to their common restriction enzyme sites (Fig. 1). The chimeric luciferases were constructed by recombining the four regions in new arrangements. The six chimeric luciferases, HP(AB/cd), HP(A/bcd), HP(A/b/CD), HP(Ab1/B2/CD), HP(ab/CD), and HP(ab1/B2/cd), were obtained as follows (Fig. 2); the plasmid of pT-HP(AB/cd) was formed by ligating the *SacI*/*Hin*dIII-digested Pm(AB) fragment of pT-PmL(ABCD) into the *SacI*/*Hin*dIII-digested pT-Hp(cd) plasmid of pT-HpL(abcd), whereas pT-HP(AB/cd) was formed by ligating the *SacI*/*Hin*dIII-digested Pm(AB) fragment into the *SacI*/*Hin*dIII-digested pT-H(cd) plasmid. The plasmid of pT-HP(A/bcd) was constructed between the *Eco*RI-digested pT-P(A) and the *Eco*RI-digested H(bcd) fragment. The pT-HP(A/b/CD) was located

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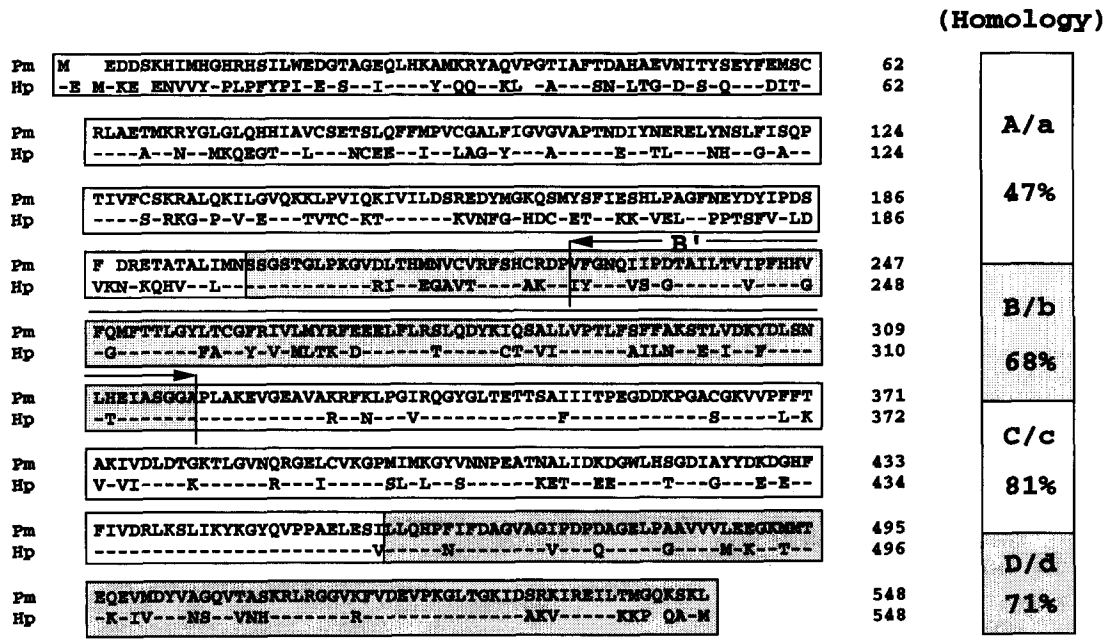

Fig. 1. Comparison of the amino acid sequences of the *P. miyako* and *H. parvula* luciferases. Four boxes show the distinct regions by common restriction enzyme sites.

between the *Nco*I/*Sac*I-digested HP(A/b) of pT-HP(A/bcd) and the *Nco*I/*Sac*I-digested pT-P(CD). The *Bam*HI site-induced Pm(B2) DNA fragment was amplified by using the polymerase chain reaction (PCR) in which the specific base substitution was introduced as a mismatch between two primers, and digested by *Bam*HI/*Sac*I. The pT-HP(ab1/B2/cd) was a combination between Pm(B2) and the *Bam*HI/*Sac*I-digested pT-HpL(ab1/cd), whereas the pT-HP(Ab1/B2/CD) was between Pm(B2) and the *Bam*HI/*Sac*I-digested pT-H(A/b1/CD).

2.3. Growth of bacteria and preparation of the luciferase solution

Transformed *E. coli* BL21 was grown at 30°C in 100 ml of LB medium, containing 50 μg/ml of ampicillin. After overnight culture, the medium was transferred to 1 l of fresh LB medium containing 50 μg/ml of ampicillin and 0.5 mM of IPTG and incubated with shaking for 24 h at 18°C [8]. After centrifuging the culture medium at 6000×*g* for 20 min at 4°C, the *E. coli* pellet was resuspended in lysis buffer (100 mM potassium phosphate, pH 7.8; 2 mM EDTA; 1 mg/ml of lysozyme), incubated on ice for 15 min and then placed in a freezer at −80°C. The frozen pellet was thawed at 25°C and centrifuged at 12000×*g* rpm for 20 min at 4°C, and the supernatant was fractionated with ammonium sulfate. The 30–70% fraction was suspended in 10 ml of 20 mM Tris-HCl/2 mM EDTA, pH 7.8 and dialyzed over-

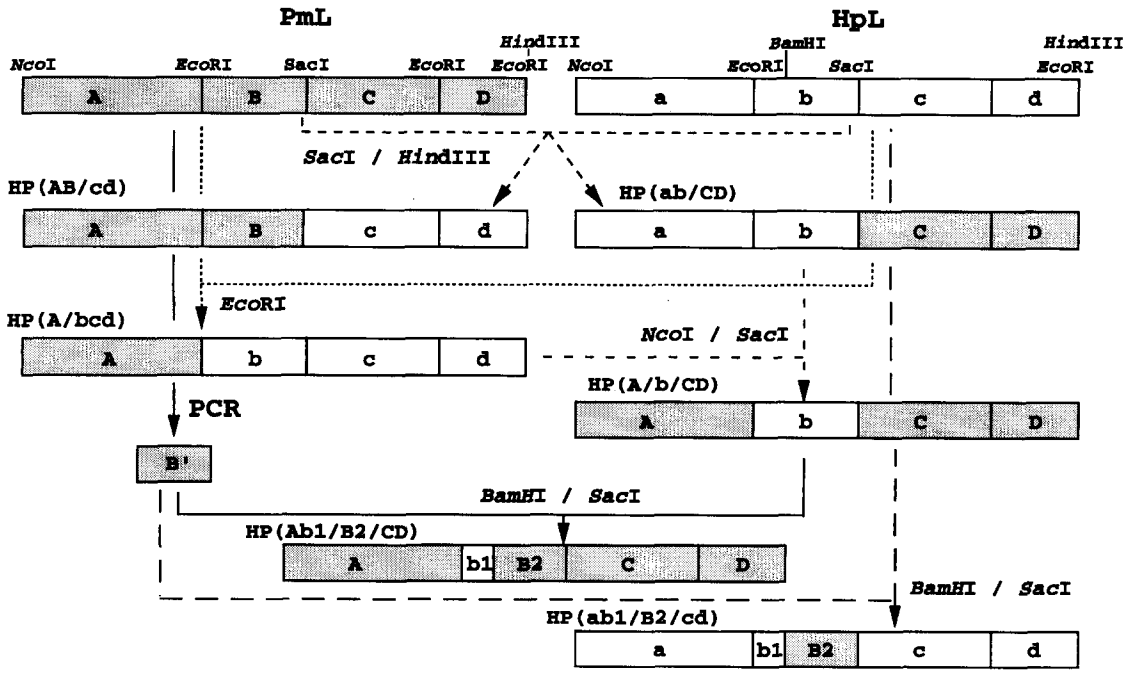

Fig. 2. The construction of the six chimeric luciferases.

Table 1
The wave length maxima of the bioluminescence of the wild type and chimeric luciferases

Luciferase	Wave length maxima (nm)
Wild type	
Pm(ABCD)	550
Hp(abcd)	568
Chimeric type	
HP(AB/cd)	553
HP(ab/CD)	568
HP(A/b/CD)	565
HP(A/bcd)	N.D.
HP(Ab1/B2/CD)	546
HP(ab1/B2/cd)	562

The spectrum was measured as described in Section 2.4.

night twice against 2 l of the same buffer. The luciferase solution from cell lysate was the sample for the measurement of bioluminescence spectrum.

2.4. Measurement of bioluminescence emission spectrum

To 1 ml of the luciferase solution in a quartz cell, 1 ml of 1 mM firefly luciferin/25 mM glycylglycine, pH 7.8 containing 5 mM ATP and 15 mM $MgSO_4$ was injected [20], and the emission spectrum was measured with a Hitachi F-4010 Fluorescence Photometer with the excitation lamp turned off. In every experiment, the wave length maxima for luciferase mutants were calibrated as a standard for the emission spectra of Hp and Pm luciferases.

3. Result and discussion

The Hp and Pm luciferases can be separated into four regions ("Region ABCD" and "Region abcd") by their common restriction enzyme sites (Fig. 1). "Region C" from Pro-319 to Ile-458 in Pm luciferase is the most homologous part, whereas "Region A" from Met-1 to Asn-208 is less homologous than the other parts. Fig. 2 shows the reconstruction of chimeric enzymes by recombining the four regions in new arrangements. "Region B2" was amplified by the PCR method between Val-226 and Ala-318 in Pm luciferase restriction sites corresponding to *Bam*HI/*Sac*I restriction sites in Hp luciferase. Finally, we obtained six chimeric luciferases; HP(AB/cd), HP(ab/CD), HP(A/bcd), HP(A/b/CD), HP(Ab1/B2/CD) and HP(ab1/B2/cd).

Bioluminescence emission spectra of Pm and Hp luciferases measured by a Hitachi F-4010 Fluorescence Photometer were the same as those obtained using a Hamamatsu PMA-10 Photon Counting Multichannel Analyzer [8]. Also, the spectra of the *E. coli* lysates of Pm and Hp luciferases were the same as those of the purified Pm and Hp luciferases (data not shown). Table 1 shows the wavelength maxima for the lysates of Pm, Hp, and six chimeric luciferases. The emission peak of HP(AB/cd) at 553 nm was almost the same as that of Pm luciferase at 550 nm, whereas HP(ab/CD) at 568 nm was the same as Hp luciferase at 568 nm. These results suggested "Region AB" and "Region ab" determined the emitting colors of Pm and Hp luciferases from 550 nm to 570 nm. Furthermore, the spectrum of HP(A/b/CD) showed an emission peak at 565 nm that was similar to those of HP(ab/CD) and Hp luciferase, indicating that the "Region b" fragment (208–318), may be more important for the emission of yellow color than "Region a". On the other hand, the wavelength maxima of HP(Ab1/B2/CD) (λmax = 546 nm) and HP(ab1/B2/cd) (λmax = 562 nm) exhibited a shift to shorter wavelengths from the pre-modified luciferases, HP(A/b/CD) and Hp luciferase, respectively. These results indicated that the fragment (226–318) of "Region B2" was also the important region for the color differences, since the spectra of these luciferases containing "Region B2" showed a shift to shorter wavelengths, i.e. a green-shift. These results suggested that the fragment of "Region B2" and "Region b" could contain the active site for the color differences in firefly luciferase. However, the slight difference for the spectra of HP(ab1/B2/cd) and HP(Ab1/B2/CD) indicated that another region, "Region A" or "Region B1", but not "Region B2" may influence on the green-shift.

The active sites for the color differences have been supposed to be at a fragment (223–247) on Cb luciferase [19] and at some single amino acid residues, Val-239, Ser-286, Gly-326, His-433, and Pro-452 on Lc luciferase [15]. In this study, it is distinctly the fragment (208–318) containing "Region B2" and "Region b" that appears to be involved in the color determination from 550 nm to 570 nm on bioluminescence reaction. This result supports the assumption of an active fragment (223–247) on Cb luciferase. However, this region can not always determine the emission color because the emission spectra of HP(Ab1/B2/CD), HP(ab1/B2/cd), and Pm luciferase were not identified completely. Recently, computer analysis for the primary structure of Lm, Lc, Ll, Pp, and Cb luciferases showed that the active sites of the color differences may be located in fragments (223–247) and (352–358) according to the Cb luciferase sequence [21]. It is impossible for a specific amino acid residue alone to contribute to color determination. In conclusion, one of the active sites for the color determination between green and yellow must be located in the fragment (208–318) of Pm and Hp luciferase.

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