An Alternative Mechanism of Bioluminescence Color Determination in Firefly Luciferase†

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ABSTRACT: Beetle luciferases (including those of the firefly) use the same luciferin substrate to naturally display light ranging in color from green (λ_{max} ~530 nm) to red (λ_{max} ~635 nm). In a recent communication, we reported (Branchini, B. R., Murtiashaw, M. H., Magyar, R. A., Portier, N. C., Ruggiero, M. C., and Stroh, J. G. (2002) *J. Am. Chem. Soc. 124*, 2112−2113) that the synthetic adenylate of firefly luciferin analogue D-5,5-dimethylluciferin was transformed into the emitter 5,5-dimethyloxyluciferin in bioluminescence reactions catalyzed by luciferases from *Photinus pyralis* and the click beetle *Pyrophorus plagiophthalamus*. 5,5-Dimethyloxyluciferin is constrained to exist in the keto form and fluoresces mainly in the red. However, bioluminescence spectra revealed that green light emission was produced by the firefly enzyme, and red light was observed with the click beetle protein. These results, augmented with steady-state kinetic studies, were taken as experimental support for mechanisms of firefly bioluminescence color that require only a single keto form of oxyluciferin. We report here the results of mutagenesis studies designed to determine the basis of the observed differences in bioluminescence color with the analogue adenylate. Mutants of *P. pyralis* luciferase putative active site residues Gly246 and Phe250, as well as corresponding click beetle residues Ala243 and Ser247 were constructed and characterized using bioluminescence emission spectroscopy and steady state kinetics with adenylate substrates. Based on an analysis of these and recently reported (Branchini, B. R., Southworth, T. L., Murtiashaw, M. H., Boije, H., and Fleet, S. E. (2003) *Biochemistry 42*, 10429−10436) data, we have developed an alternative mechanism of bioluminescence color. The basis of the mechanism is that luciferase modulates emission color by controlling the resonance-based charge delocalization of the anionic keto form of the oxyluciferin excited state.

The familiar flashing lights produced by fireflies on warm summer evenings are striking examples of bioluminescence, the conversion of chemical energy into light by a living organism. Through basic research mainly focused on the North American firefly *Photinus pyralis* (*1*−*4*), there exists a very good understanding of the chemical transformations leading to light emission. The applications of firefly bioluminescence that include in vivo luminescence monitoring (*5*, *6*) constitute an impressive list of medical and pharmaceutical methods, many of which use the firefly luciferase gene as a reporter of gene expression and regulation (*7*−*9*).

The luciferase enzyme (Luc)[1] functions as a monooxygenase, although it does so without the apparent involvement of a metal or cofactor. The multistep oxidation of firefly luciferin (D-LH_2) results in the production of a complex of Luc, AMP, and electronically excited oxyluciferin (eq 1−2) (*1*, *10*). The excited state product is likely formed from a dioxetanone intermediate via an intramolecular chemically initiated electron-exchange luminescence mechanism (*11*). Relaxation of excited-state oxyluciferin to the corresponding ground state is accompanied by the emission of light (eq 3) with great efficiency (*12*).

$$\text{Luc} + \text{D-LH}_2 + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{Luc} \cdot \text{LH}_2\text{-AMP} + \text{PPi} \quad (1)$$

$$\text{Luc} \cdot \text{LH}_2\text{-AMP} + \text{O}_2 \rightarrow \text{Luc} \cdot \text{AMP} \cdot \text{oxyluciferin}^* + \text{CO}_2 \quad (2)$$

$$\text{Luc} \cdot \text{AMP} \cdot \text{Oxyluciferin}^* \rightarrow \text{Luc} + \text{Oxyluciferin} + \text{AMP} + h\upsilon \quad (3)$$

All beetle luciferases[2] (*13*−*18*) including Luc are members of the "acyl-adenylate/thioester-forming" superfamily of enzymes (*19*−*21*) that also includes a variety of acyl/CoA ligases; the acyl-adenylate-forming domains of enzyme complexes involved in the nonribosomal synthesis of peptides and polyketides; and several other types of enzymes. The luciferase-catalyzed formation of enzyme-bound D-LH_2-AMP (eq 1) is an example of the common chemistry. Most of the superfamily enzymes generate thioester (e.g., of CoA) intermediates or products from the initially formed corre-

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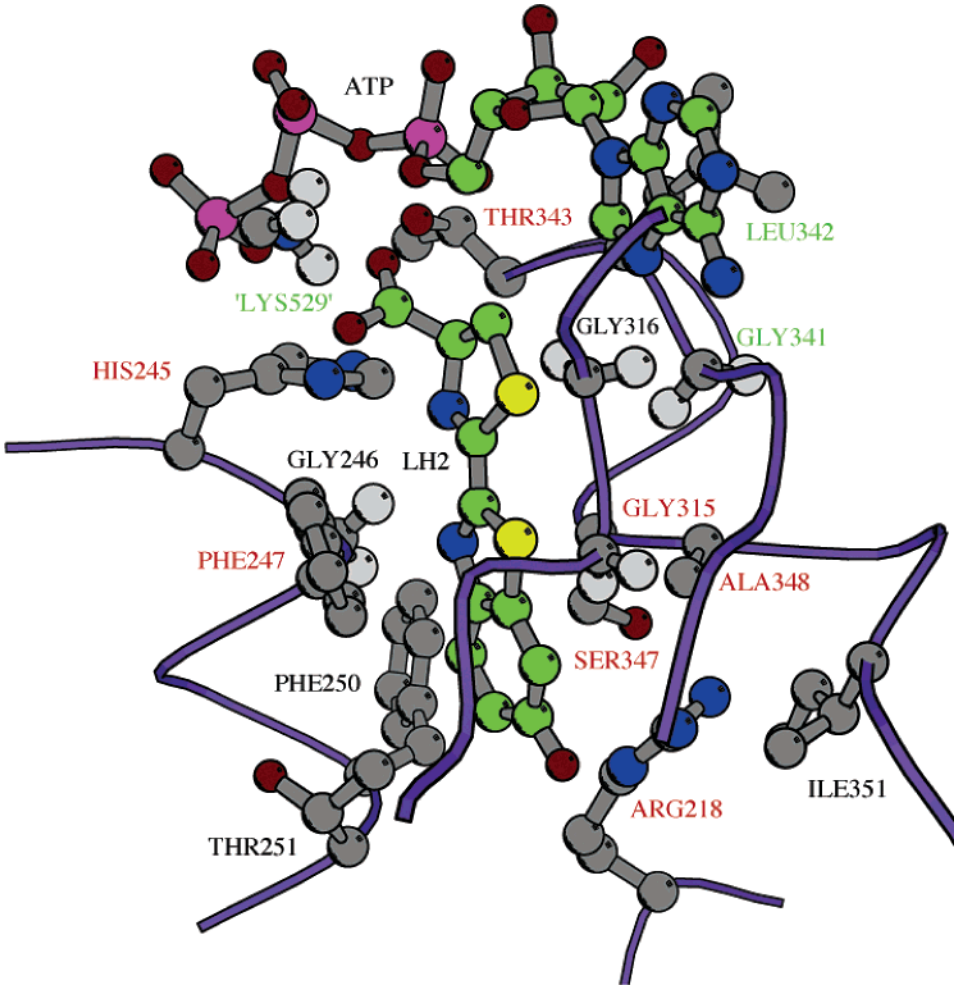


FIGURE 1: Diagram showing the residues within 5 Å of D-LH_2 at the putative luciferase substrate binding site suggested (*28*) by molecular modeling of Luc with D-LH_2 and ATP and Mg^{2+} ion (not shown). The model was created starting with the Luc X-ray structure 1LCI (*23*), and methylammonium ion (labeled Lys529) was used to represent possible interactions of the Lys529 side-chain. Traces through the α-carbons of regions Val217-Phe219, His244-Thr252, Ile312-Pro318, and Arg337-Pro353 are shown as purple coils. This diagram was generated using the program MOLSCRIPT (*48*). The Ppy WT residues mutated in this study are labeled in black. Mutations of other residues (*32*) caused ≥ 30 nm red-shifted emission (red labels) or had little effect on bioluminescence color (green labels).

sponding acyl-adenylates. These reactions are similar to the formation of the CoA thioester of dehydroluciferin from the corresponding adenylate; a Luc-catalyzed reaction suggested (*22*) to account for the stimulatory effect of CoA on luciferase activity.

The X-ray structures of Luc (*23*, *24*) and three other acyl-adenylate forming enzymes (*25*−*27*) share a common molecular architecture consisting of a large N-terminal domain (residues 1−436) and a small C-terminal domain (residues 440−550). In the Luc crystal structure without bound substrates, the domains are separated in an "open" conformation. In contrast, both the phenylalanine-activating subunit of gramicidin synthetase 1 (PheA) in a complex with phenylalanine, Mg ion and AMP (*25*) and DhbE, the 2′-3′-dihydroxybenzoate adenylation domain of *Bacillus subtilis*, with bound adenylate DHB-AMP (*26*), the C-terminal domain was rotated 94° and was 5 Å closer to the N-terminal domain than it was in the Luc structure (*23*, *24*). In the structure of acetyl-CoA synthetase (Acs) complexed with adenosine-5′-propyl phosphate and CoA (*27*), the C-terminal domain is rotated ~140° relative to the conformation of PheA and DhbE. Possibly, the formation and oxidation of D-LH_2-AMP would require rotation of the C-terminal domain of Luc into one or more conformations similar to those revealed by the structures of PheA, DhbE, and Acs.

Starting with the structures of Luc (*23*) and PheA (*25*), we used molecular modeling techniques to produce a potential model (Figure 1) of the Luc active site containing substrates D-LH_2 and Mg-ATP (*28*). This working model has been useful in the planning and interpretation of results of site directed mutagenesis-based structure−function studies, including several related to the determination of biolumi-

[1] Abbreviations: Acs, acetyl-CoA synthetase from *Salmonella enterica*; CBA, 50 mM Tris-HCl, pH 7.0 containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.8 M ammonium sulfate and 2% glycerol; DHB, 2′-3′-dihydroxybenzoate; DhbE, the 2′-3′-dihydroxybenzoate adenylation domain for bacillibactin synthesis in *Bacillus subtilis*; D-$DiMeLH_2$, 5,5-dimethylluciferin; D-$DiMeLH_2$-AMP, 5,5-dimethyl-luciferyl-*O*-adenosine monophosphate; GST, glutathione-S-transferase; D-LH_2, D-firefly luciferin; D-LH_2-AMP, luciferyl-O-adenosine monophosphate; Luc, *Photinus pyralis* luciferase (E. C. 1.13.12.7); PheA, the phenylalanine-activating subunit of gramicidin synthetase 1; PplGR WT and PplOR WT, recombinant *Pyrophorus plagiophthalamus* green and orange emitting luciferase isozymes; Ppy WT, recombinant *Photinus pyralis* luciferase; and TICT, twisted intramolecular charge transfer.

[2] The deduced amino acid sequence of *Phengodes sp* luciferase was provided by Keith V. and Monika Wood, personal communication.

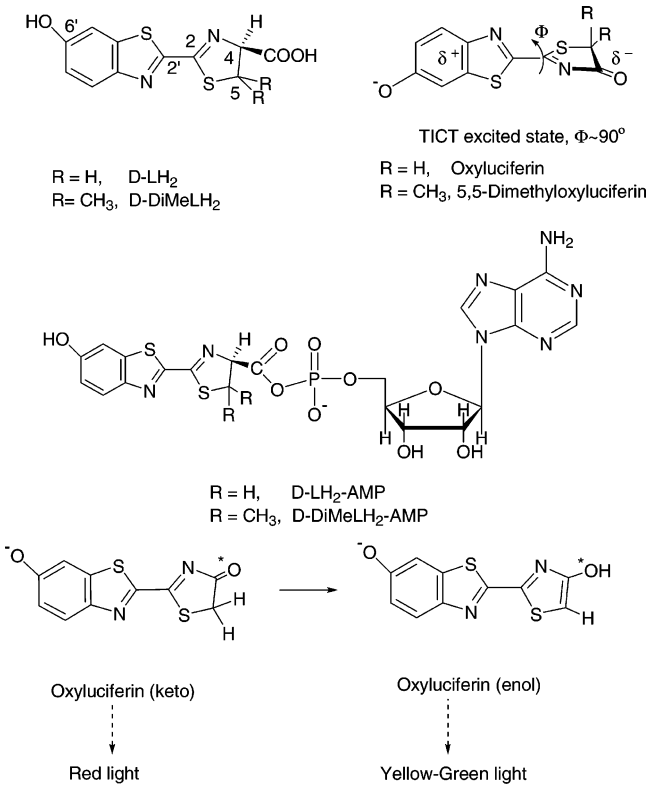


FIGURE 2: Chemical structures of D-LH_2, D-$DiMeLH_2$, and the corresponding adenylates and oxyluciferins.

nescence color (*29*−*32*). The relationship between luciferase structure and light emission color continues to be a particularly intriguing aspect of firefly bioluminescence. In nature, beetle luciferases, which presumably all utilize the same D-LH_2 substrate (Figure 2), display various colors of light from green (λ_{max} ~530 nm) to red (λ_{max} ~635 nm) (*33*−*35*). The proposed mechanisms for bioluminescence color have been reviewed (*2*, *36*, *37*).

In a recent communication (*38*), we reported the preparation of D-$DiMeLH_2$-AMP (Figure 2). The synthetic adenylate was transformed into 5,5-dimethyloxyluciferin, the putative emitter in bioluminescence reactions catalyzed by the firefly and click beetle luciferases. Bioluminescence from D-$DiMeLH_2$-AMP and PplGR WT was red (λ_{max} 624 nm); unexpectedly, green emission (λ_{max} 560 nm) was observed with Ppy WT (Figure 3). Since the methyl groups of 5,5-dimethyloxyluciferin prevent tautomerization, our results demonstrated that a single emitter in the keto form (Figure 2) could account for the range of bioluminescence color observed in nature. Our findings did not support the original mechanism of bioluminescence color determination advanced by White and co-workers (*4*) in which red light is ascribed to the keto form of excited-state oxyluciferin and green light to the corresponding enol form of the emitter (Figure 2). We report here the results of related mutagenesis studies and we propose an alternative mechanism of bioluminescence color.

MATERIALS AND METHODS

Materials. The following items were obtained from the indicated sources: Mg-ATP (bacterial) (Sigma); D-penicillamine and 2-cyano-6-methoxybenzothiazole (Aldrich Chemical Co.); D-LH_2 (Biosynth AG); restriction endonucleases (New England Biolabs); and mutagenic oligonucleotides (Invitrogen). 2-Cyano-6-hydroxybenzothiazole was prepared from 2-cyano-6-methoxybenzothiazole as described previously (*39*), except that the crude product was purified by flash chromatography on silica gel (eluant: hexanes−acetone, 70:30). D-$DiMeLH_2$-AMP (*38*) and D-LH_2-AMP (*31*) were prepared, purified, and stored as previously described. Ppy WT, Ppy G246A, Ppy F250S, and PplGR WT luciferases in pGex-6P-2 plasmids (*28*, *32*, *38*) were expressed in *E. coli* strain BL 21 at 22 °C and purified by the method previously reported (*38*).

General Methods. Light measurements were made in 8-× 50-mm polypropylene tubes (Evergreen Scientific, Los Angeles, CA) placed in the sample compartment of either an Aminco Chem Glow II or a Turner TD-20e luminometer. Data were acquired from the analogue output of the luminometer with a Strawberry Tree Inc. (STI) A/D converter (sampling rate 0.05−0.1 s) and stored to a Macintosh computer. Initial flash height- or integration-based light measurements were quantified with customized versions of the STI Workbench software. All measurements were corrected for the spectral response of the Hamamatsu 931B photomultiplier tubes. NMR spectra were obtained with a Varian UNITY INOVA 500-MHz spectrometer. Mass spectral analyses of the proteins were performed by tandem HPLC-electrospray ionization mass spectrometry using a PerkinElmer Series 200 HPLC system and a Sciex ABI150A mass spectrometer. The calculated molecular masses (Da) of the luciferases are: Ppy WT, 61 157; Ppy G246A, 61 171; Ppy F250S, 61 097; PplGR WT, 61 103; PplGR A243G, 61 089; and PplGR S247F, 61 163. The determined mass values were all within the allowable experimental error of 0.01% of the calculated values. The mutations of all luciferase genes were verified by DNA sequencing performed at the W. M. Keck Biotechnology Laboratory at Yale University.

Preparation of D-*5,5-Dimethylluciferin (*D-*DiMeLH$_2$)*. This procedure is an improved version of our previously published method (*38*). Under a nitrogen atmosphere, a solution of D-penicillamine was prepared by adding the amino acid (168 mg, 1.13 mmol) to 2.0 mL of deoxygenated (nitrogen bubbling for 15 min) water (adjusted to pH 8.0 with 0.3N NaOH). The penicillamine solution was added to a solution of 2-cyano-6-hydroxybenzothiazole (198 mg, 1.12 mmol) dissolved in deoxygenated (nitrogen bubbling for 15 min) methanol (5.5 mL), and the reaction mixture was stirred under nitrogen for 15 min. The pH of the mixture was readjusted to 8.0 (litmus) and stirred for an additional 90 min. Water (20 mL) was added, the pH was adjusted to 8.5 (litmus), and the solution was extracted twice with ether (20 mL) and then twice with ethyl acetate (20 mL). The aqueous layer was cooled (ice bath) and acidified to pH 1 (litmus) by dropwise addition of 5% HCl. The pale yellow precipitate that formed was collected by vacuum filtration, washed with cold water, and dried in vacuo providing 325 mg of pure product (1.05 mmol, 94% yield) with mp 190.9−191.1 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.14 (br s, 1H, COOH), 10.20 (br s, 1H, OH), 7.94 (d, J = 8.8 Hz, 1H, H_4'), 7.45 (d, J = 2.6 Hz, 1H, H_7'), 7.06 (dd, J = 8.8, 2.6 Hz, 1H, H_5'), 4.97 (s, 1H, H_4), 1.73 (s, 3H), 1.45 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 169.56(COOH), 163.56(C-2), 157.33-(C-2′ or C-6′), 157.29(C-6′ or C-2′), 146.20(C-3a′), 137.14-(C-7a′), 124.82(C-4′), 117.09(C-5′), 106.80(C-7′), 85.43(C-

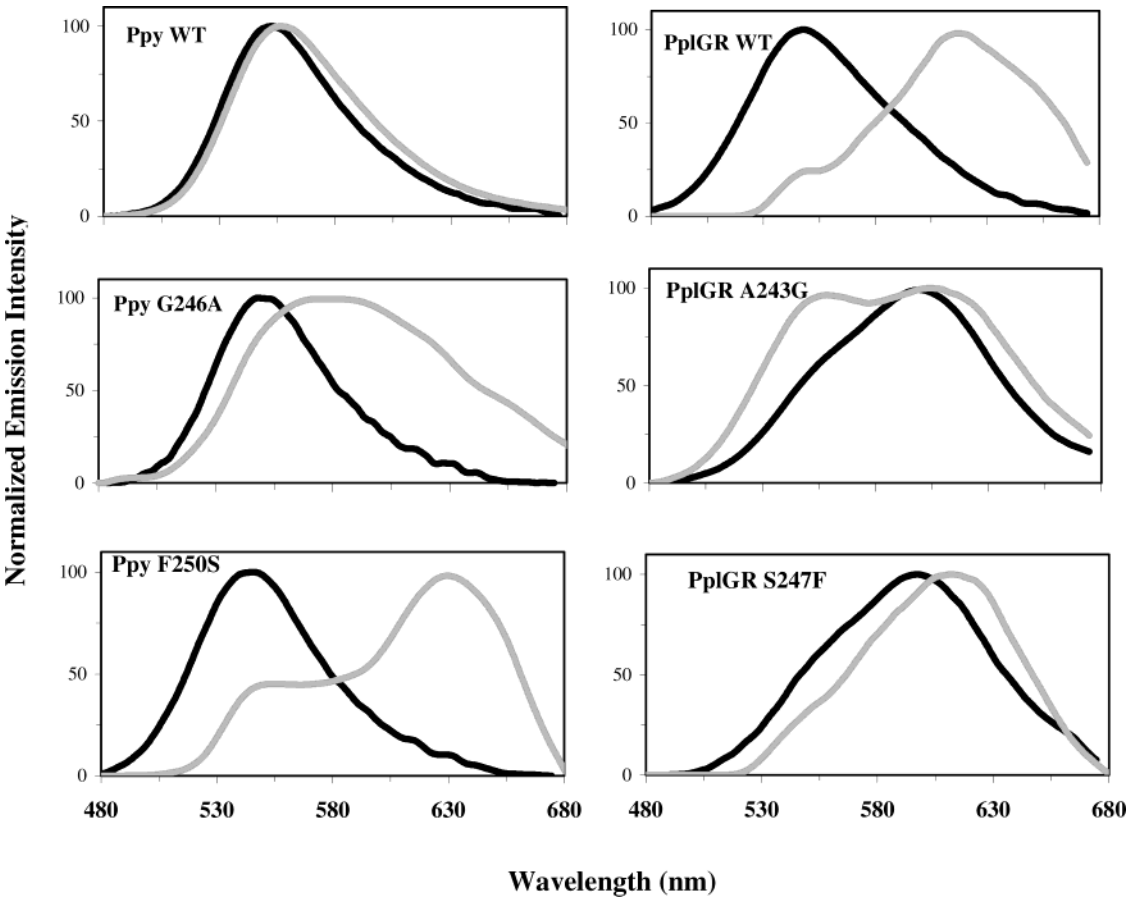


FIGURE 3: Bioluminescence emission spectra of the luciferases. Spectra were recorded with the indicated luciferases at pH 8.6 with D-LH_2-AMP (black line) and with D-DiMeLH_2-AMP (grey line), as described under Materials and Methods.

4), 60.34(C-5), 28.52(CH_3), 25.72(CH_3). IR (KBr) 2972 (w), 1728, 1569, 1481, 1224, 1068, 866 cm^{-1}. Optical rotation: $[\alpha]^{22}_D$ −33.60° (c = 0.002 DMSO). UV (ethanol) 268 nm (log ϵ = 3.81), 329 nm (log ϵ = 4.24). $[(M + H)]^+$: obsd 309.1, expected 309.1.

Specific Activity Measurements with Adenylate Substrates. Specific activity values of the luciferases with D-LH_2-AMP and D-DiMeLH_2-AMP were determined by integration-based light assays at pH 8.6. Integration times were selected (5−30 min) to ensure that ∼95% of total light emitted was collected. Each assay reaction (0.51 mL total volume) was initiated by rapid injection of 10 μL of luciferase (1−10 μg) in CBA into 50 mM Tris, pH 8.6 containing saturating concentrations of either D-LH_2-AMP or D-DiMeLH_2-AMP. Total light output was measured and corrected for the spectral response of the Hamamatsu 931B photomultiplier tube. Protein concentrations were determined as previously described (*29*).

Site-Directed Mutagenesis. Site-directed mutagenesis was carried out with the Quik Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions using PplGR WT in the pGex-6P-2 vector with the following primers and their respective reverse complements: PplGR A243G, 5′-CTG CCT TTT TTC CAT **GGC** TTT GGG TTC TCT ATA AAC TTG GG-3′ [*Nco*I] and PplGR S247F, 5′CTG CCT TTT TTC CAT GCA TTT GGG TTC **TTC** ATA AAC TTG GG-3′ [*Nsi*I] (bold represents the mutated codon, brackets indicate a screening endonuclease and underline represents silent changes to create a unique screening endonuclease site).

Steady-State Kinetic Constants. Values of K_m for D-LH_2-AMP and D-DiMeLH_2-AMP for all luciferases were determined from bioluminescence activity assays in which measurements of maximal light intensities were taken as estimates of initial velocities. Into 0.4 mL of 50 mM Tris, pH 8.6 were added 0.1 mL aliquots of solutions of either D-LH_2-AMP (final concentrations 0.06−25.0 μM) in 10 mM sodium acetate, pH 4.5 or D-DiMeLH_2-AMP (final concentrations 0.05−2.2 μM) in 25 mM ammonium acetate, pH 4.5. Immediately, light reactions were initiated by injections of 10 μL of luciferase enzymes (0.5−5.0 μg in CBA). Kinetic constants were calculated using the Enzyme Kinetics Pro (SynTex) software.

Bioluminescence Emission Spectra. Bioluminescence emission spectra were obtained using a PerkinElmer LS55 luminescence spectrometer operated in the "bioluminescence" mode. Data were collected over the wavelength range 480−680 nm in a 1 mL optical glass cuvette. Gate and delay times, detector voltage, scan rate, and slit width were adjusted to optimize instrument response. Data were corrected for the spectral response of the R928 photomultiplier tube using the PerkinElmer FL WinLab software. Bioluminescence emission spectra were obtained in 50 mM Tris, pH 8.6, with D-LH_2-AMP (0.95−9.5 μM) and D-DiMeLH_2-AMP (0.75−1.9 μM) with final concentrations of luciferases ranging from 0.15 to 0.35 μM with D-LH_2-AMP and 0.3−26.0 μM with D-DiMeLH_2-AMP. With D-LH_2-AMP, the stabilizing storage reagents NaCl, ammonium sulfate, and glycerol were kept at standard concentrations of 7.2 mM, 38 mM, and 0.1%, respectively. For studies performed with D-DiMeLH_2-AMP,

Table 1: Bioluminescence Activity and Steady State Binding Constants of Luciferases with Adenylate Substrates at pH 8.6

	relative specific activity (integrated)[a]		K_m (μM)[b]		bioluminescence emission maximum (nm)[c]	
luciferase	D-LH_2-AMP	D-DiMeLH_2-AMP	D-LH_2-AMP	D-DiMeLH_2-AMP	D-LH_2-AMP	D-DiMeLH_2-AMP
Ppy WT	100	2.9	7.7	0.25	552 (60)	560 (89)
Ppy G246A	158	0.03	12.7	0.3	548 (56)	578 (105)
Ppy F250S	79	0.04	11.4	0.3	546 (62)	631, 552 (sh) (130)
PplGR WT	30	0.7	0.8	0.3	549 (75)	624 (76)
PplGR A243G	15	1.0	0.6	0.4	599 (92)	610, 557 (125)
PplGR S247F	13	0.01	4.6	0.8	597 (89)	612 (81)

[a] Specific activity measurements were made as described in Materials and Methods. The specific activity data are expressed relative to the Ppy WT value obtained with D-LH_2-AMP that is defined as 100 and is equivalent to 2.05×10^{15} photons mg^{-1} (0.213 einstein $\times 10^{-6}$ μmol^{-1}). The error associated with the specific activity measurements is estimated to be $\pm$ 10% of the value. [b] Kinetic constants were determined as described in Materials and Methods. The error associated with the K_m values falls within $\pm$ 10% of the value. [c] Bioluminescence emission spectra were measured as described in Materials and Methods. Bandwidths (nm) at 50% of emission maxima are indicated in parentheses.

stabilizing storage reagents were removed from the luciferases by exchange into 0.1 M phosphate buffer, pH 8.0, by gel chromatography using PD-10 columns (Pharmacia).

RESULTS AND DISCUSSSION

With D-LH_2-AMP (Figure 3) or D-LH_2 plus Mg-ATP (*38*) in the pH range 7.8−8.6, the wild-type luciferases from *Photinus pyralis* (Ppy WT) and *Pyrophorus plagiophthalamus* (PplGR WT) catalyze the emission of yellow-green and green light, respectively. Although these luciferases did not produce light with D-DiMeLH_2 (Figure 2), we demonstrated (*38*) that at pH 8.6, Ppy WT and PplGR WT convert preformed D-DiMeLH_2-AMP into 5,5-dimethyloxyluciferin with the concomitant production of yellow-green and red light emission, respectively (Figure 3). In an attempt to better understand the structure−function relationship of bioluminescence color, we compared residues at the putative Luc binding site (Figure 1) to amino acids at equivalent sequence positions in PplGR WT. We considered the straightforward idea that the observed differences in bioluminescence color with D-DiMeLH_2-AMP might be accounted for by the variable residues, Gly246, Phe250, Thr251, Gly316, and Ile351 (click beetle residues Ala243, Ser247, Ile248, Ala313, and His348, respectively). Accordingly, we designed, expressed, and purified mutants of Ppy WT and PplGR WT, in which each of the five amino acids was converted to the residue found in the equivalent position of the other enzyme. Unfortunately, a thorough examination of the bioluminescent properties of the luciferase proteins (data not shown) did not produce significant insights into the issues of bioluminescence color. Results representative of the noteworthy findings of the mutational studies, including the substantiation of the ability of the luciferases to produce various colors of light from a single excited-state keto emitter, are reported (Table 1 and Figure 3) and discussed here. Many of the luciferases displayed low activity with D-DiMeLH_2-AMP, so for comparison purposes, data were collected for all substrates and enzymes at pH 8.6, the optimum pH for reactions with D-DiMeLH_2-AMP.

Expression, Purification, and Characterization of the Luciferase Proteins. The wild-type and the modified luciferases listed in Table 1 were expressed as GST-fusion proteins and contained the additional N-terminal peptide GPLGS-, which remained after PreScission protease cleavage from GST. Average yields (mg) of purified proteins per 0.5 L culture were Ppy WT (12.5), Ppy G246A (9.5), Ppy F250S (12.0), PplGr WT (3.0), PplGR A243G (2.3) and PplGR S247F (1.3). The firefly enzymes remained fully active for up to 6 months at 4 °C. The click beetle luciferases were less stable, losing ~20% activity over 2 weeks; therefore, these enzymes were characterized within 2 weeks of purification.

Bioluminescence Activity and Substrate Binding. Specific activities based on the total light emitted by the luciferases were measured with saturating concentrations of D-LH_2-AMP and D-DiMeLH_2-AMP and were corrected for differences in the colors and shapes of the emission spectra. These values (Table 1) are not based on initial flash height intensities that were difficult to compare because the rise times and decay values varied widely (data not shown). Under the conditions of our measurements (Materials and Methods), the Ppy WT integration-based specific activity with D-LH_2-AMP is ~12-fold lower than that obtained with D-LH_2 and Mg-ATP at pH 7.8 (*30*). When assessed with D-LH_2-AMP, the specific activity of the Ppy mutant F250S was slightly reduced compared to Ppy WT; however, the G246A mutant displayed an ~1.6-fold increased value (Table 1). The specific activities of the PplGR WT and mutants (A243G and S247F) were reduced ~3.3- and ~7-fold, respectively, compared to Ppy WT. With the wild-type luciferases, much lower (~35- to ~140-fold) specific activities were observed with D-DiMeLH_2-AMP than with D-LH_2-AMP. With the exception of PplGR A243G, the single amino acid changes further reduced activity (Table 1).

The binding affinity of the luciferases for D-LH_2-AMP varied little except for the PplGR WT and the A243G enzymes that had ~10-fold lower K_m values for this substrate. Interestingly, the K_m values for D-DiMeLH_2-AMP did not vary significantly for Ppy WT and PplGR WT (0.25 and 0.3 μM, respectively) or the mutated luciferases (0.3−0.8 μM) (Table 1). The active site mutations reported here produced significantly greater effects on activity than on adenylate substrate binding. Possibly, this is because the luciferases adopt conformations similar to the one reported (*27*) for Acs bound to an adenylate analogue resulting in initially distant binding residues being brought into favorable contact with the adenylates.

Bioluminescence Emission Spectra. Bioluminescence emission spectra produced by the luciferases were measured with the adenylate substrates; these data are presented in Figure 3 and summarized in Table 1. With D-LH_2-AMP, minor blue-shifted emission maxima were observed with the position

246 and 250 Ppy mutants compared to Ppy WT. In contrast, ~50 nm red shifts were obtained when mutations were introduced at positions 243 and 247 of PplGR WT. With D-DiMeLH$_2$-AMP, there was great variation in the observed bioluminescence emission spectra of the luciferases (Figure 3 and Table 1). As documented in an earlier report (*38*), Ppy WT catalyzed the emission of yellow-green light (λ_{max} = 560 nm) with D-DiMeLH$_2$-AMP, while PplGR WT displayed red bioluminescence (λ_{max} = 624 nm). The emission spectrum of the Ppy WT single mutant F250S displayed substantial red emission similar to PplGR WT (Figure 3). The Ppy G246A emission was broadened and red-shifted 18 nm compared to that of Ppy WT. The emission of the click beetle enzyme S247F with D-DiMeLH$_2$-AMP was similar to that of PplGR WT (Figure 3 and Table 1). A distinctly bimodal emission was observed with PplGR A243G that was characterized by equal emission maxima in the yellow-green (λ_{max} = 557 nm) and red (λ_{max} = 610 nm). Apparently, Ala243 in the click beetle luciferases is important in determining bioluminescence color, as previously suggested by Viviani and co-workers (*40*).

Speculative Mechanism of Bioluminescence Color Determination. The results reported here and previously (*38*) provide experimental support for mechanisms of bioluminescence color that require only a single keto form of oxyluciferin. One such mechanism proposed by McCapra and co-workers (*37*, *41*, *42*), contends that color modulation is associated with conformations of the keto form of oxyluciferin related by rotation about the C2−C2′ bond (Figure 2). Red emission is attributed to the minimum energy conformation of an unusual TICT excited state (Figure 2), in which the aromatic rings of oxyluciferin are rotated 90° about the C2−C2′ bond. Yellow-green light emission would result from a higher energy approximately coplanar excited state conformer. Further, luciferase-emitter interactions would maintain specific conformations of the excited state, thereby influencing emission color. The McCapra proposal is certainly sustained by our findings; however, it is not without shortcomings. The results of recently reported (*43*) time dependent density functional theory-based calculations are particularly problematic because they suggest that the involvement of the TICT excited state is less likely than that predicted by the semiempirical methods used by McCapra (*41*, *42*).

We have previously shown that mutations in Luc at stringently conserved residues Arg218, His245, Gly315, Thr343, and Ala348 have produced significantly (> 40 nm) red-shifted bioluminescence (*29*, *31*, *32*). Additionally, mutation of Luc residue Asp422 produced similarly red-shifted emission (unpublished results). No absolute and straightforward correlation could be made between any of the shared characteristics of these residues (e.g., location in structural elements, conservation among luciferase primary sequences, or kinetic parameters) and ability to alter bioluminescence color. In general, however, bioluminescence color is altered by many of the changes (*32*) in residues at or near the putative substrate/emitter binding site (Figure 1). The new bioluminescence results with D-DiMeLH$_2$-AMP substantiate our earlier findings (*38*) on the role of the keto form of oxyluciferin and strengthen the argument (*40*) for the importance of PplGR residue Ala243.

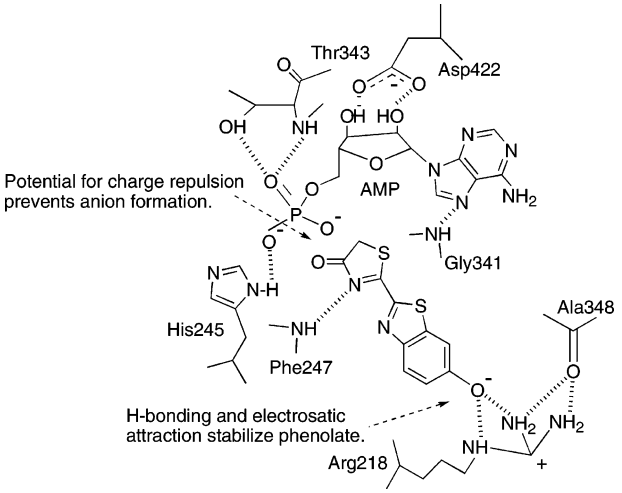
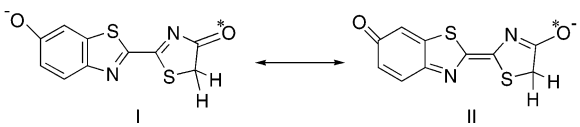


FIGURE 4: Schematic representation of the speculative mechanism for bioluminescence color determination based on the degree of delocalization of the keto anion of excited-state oxyluciferin.

The basis of the mechanism for beetle bioluminescence color determination we propose here is that luciferase modulates emission color by controlling the resonance-based charge delocalization of the anionic keto form (I) of the oxyluciferin excited state (eq 4). Resonance stabilization of the anion I (eq 4) would extend the π-electron skeleton, producing a resonance hybrid of the excited state of lower energy than if the oxyluciferin anion were constrained as mainly species I. A resonance stabilized excited state more nearly resembling species II (eq 4) would be the red emitter. Limitations on delocalization imposed by protein structure would produce higher energy excited states more closely resembling species I that would produce green emission spectra. In many cases, emission spectra contain two peaks, as is the case for Luc with the natural substrates at ~pH 7 and as has been observed with several luciferases containing mutations (*32*, *36*, *44*), including those in Figure 3. In these instances, the emission spectra would be described by resonance hybrids comprised of structures resembling species I and II in the approximate proportions that are found in the respective emission spectra. Our proposal is quite similar in its resonance-based rationale to the keto−enol mechanism of White, with the recognition that species I alone has the same degree of stabilization by conjugation as does the enol form of oxyluciferin (Figure 2) that was previously identified (*4*) as the green light emitter. Moreover, phenolate anion stabilization and a concomitant reduction in charge delocalization have been shown by Tonge and co-workers (*45*) to account for solvent-induced blue-shifts in absorption maxima of model green fluorescent protein chromophores.



(4)

I II

Our view of how the beetle luciferase structures produce green bioluminescence by minimizing charge delocalization and thus favoring species I is schematically presented in Figure 4. The key Luc residues Arg218, His245, Phe247, Thr343, Ala348, and Asp422 are absolutely conserved among the luciferases and have major roles. Species I is favored by

fixing the charge on the phenolate ion through favorable H-bonding and electrostatic interactions with the side chain group of Arg218, which is partially held in position by H-bonds to Ala348. Phe247 fixes the positions of the D-LH_2 aromatic rings mainly through π-stacking interactions (Figure 1), a concept supported by recent mutagenesis studies (*32*). A key role is attributed to the phosphate ion of AMP, which is shown in a position to create a destabilizing electrostatic interaction, thus limiting delocalization to species I. In turn, the side chains of His245, Thr343, and Asp422 are essential for proper positioning of AMP. Possibly, the differences in the emission spectra of the wild-type and Gly246/Ala243 mutant luciferases with D-$DiMeLH_2$-AMP (Figure 3) reflect the disruption of these key interactions with AMP (Figure 4) caused, in part, by unfavorable steric interactions between the methyl groups of 5,5-dimethyloxyluciferin and Ala243.

While there are many examples of mutations at residues shown in Figure 4 and others likely to be at or very near the D-LH_2 binding pocket that have been shown to elicit color shifts (*32*), there also are several cases, for example, His431 to Tyr in Luc (unpublished results), *L. cruciata* (*46*), and *H. parvula* (*44*), in which mutations of remote residues have produced red-shifted emission. Changes at remote residues are generally thought to exert long-range effects through the alteration of polypeptide packing that in turn could modify the structure of the Luc active site and alter the stabilization of species I. Other mutations that mainly affect Mg-ATP binding (*32*) may induce red-shifted bioluminescence by altering the position of the phosphate group of AMP (Figure 4). DeLuca (*1*, *10*) recognized the importance of the nucleotide structure in color determination based on studies with a synthetic luciferyl-adenylate analogue derived from ϵATP (1, N^6-ethenoadenosine triphosphate) that produced efficient red bioluminescence (λ_{max} = 620 nm). The ability of AMP to influence color requires that it remain bound as light is emitted. This point is supported by the structural data (*25*, *26*) for PheA and DhbE that include AMP formed by the hydrolysis of the corresponding adenylates.

The active site geometry proposed for Luc in Figure 4 may be similar in other beetle species. With only two exceptions, PplOR WT (λ_{max} = 593 nm) and the railroad worm *P. hirtus* (λ_{max} = 623 nm), there is only moderate natural variation (λ_{max} = 536 nm to 578 nm) in the emission spectra produced by the known beetle luciferases. Possibly, this variation is produced by minor differences in the degree of stabilization of phenolate ion species I (eq 4) through local or long-range effects altering the local polarity of the emitter site (*43*, *47*). The PplOR WT emission may be accounted for by a slight alteration in the interaction between the Arg218 side chain group and the oxyluciferin phenolate ion as a result of the Ser247 to Gly change that distinguishes it from the other click beetle isozymes (*13*). The natural red bioluminescence of *P. hirtus* is intriguing because this luciferase too contains all of the residues proposed here (Figure 4) to be necessary for green light emission. Apparently, through a change(s) originating in the primary structure, the railroad worm luciferase has evolved to efficiently delocalize the charge of the oxyluciferin anion at an emitter site containing residues that promote green emission. Current research in our laboratory is addressing the feasiblity of the proposed mechanism of bioluminescence color determination.

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REFERENCES

1. DeLuca, M. (1976) Firefly luciferase, *Adv. Enzymol. 44*, 37−68.
2. Wood, K. V. (1995) The chemical mechanism and evolutionary development of beetle bioluminescence, *Photochem. Photobiol. 62*, 662−673.
3. Wilson, T., and Hastings, J. W. (1998) Bioluminescence, *Annu. Rev. Cell. Dev. Biol. 14*, 197−230.
4. White, E. H., Rapaport, E., Seliger, H. H., and Hopkins, T. A. (1971) The chemi- and bioluminescence of firefly luciferin: an efficient chemical production of electronically excited states, *Bioorg. Chem. 1*, 92−122.
5. Contag, C. H., and Bachmann, M. H. (2002) Advances in in vivo bioluminescence imaging of gene expression, *Annu. Rev. Biomed. Eng. 4*, 235−260.
6. Greer, III, L. F., and Szalay, A. A. (2002) Imaging of light emission from the expression of luciferases in living cells and organisms: a review, *Luminescence 17*, 43−74.
7. Kricka, L. J. (1995) Chemiluminescence and bioluminescence, *Anal. Chem. 67*, 499R-502R.
8. Price, R. L., Squirrell, D. J., and Murphy, M. J. (1998) Luciferase and recombinant luciferase labels, *J. Clin. Ligand Assay 21*, 349−357.
9. Kricka, L. J. (2000) Application of bioluminescence and chemiluminescence in biomedical sciences, *Methods Enzymol. 305*, 333−345.
10. DeLuca, M., Leonard, N. J., Gates, B. J., and McElroy, W. D. (1973) Role of 1,N^6-ethenoadenosine triphosphate and 1,N^6-ethenoadenosine monophosphate in firefly luminescence, *Proc. Natl. Acad. Sci. U.S.A. 70*, 1664−1666.
11. Koo, J.-Y., Schmidt, S. P., and Schuster, G. B. (1978) Bioluminescence of the firefly: key steps in the formation of the electronically excited state for model systems, *Proc. Natl. Acad. Sci. U.S.A. 75*, 30−33.
12. Seliger, H. H., and McElroy, W. D. (1960) Spectral emission and quantum yield of firefly bioluminescence, *Arch. Biochem. Biophys. 88*, 136−141.
13. Wood, K. V., Lam, Y. A., Seliger, H. H., and McElroy, W. D. (1989) Complementary DNA coding click beetle luciferases can elicit bioluminescence of different colors, *Science 244*, 700−702.
14. Ye, L., Buck, L. M., Schaeffer, H. J., and Leach, F. R. (1997) Cloning and sequencing of a cDNA for firefly luciferase from *Photuris pennsylvanica*, *Biochim. Biophys. Acta 1339*, 39−52.
15. Viviani, V. R., Bechara, E. J. H., and Ohmiya, Y. (1999) Cloning, sequence analysis, and expression of active *Phrixothrix* railroad worms luciferases: relationship between bioluminescence spectra and primary structures, *Biochemistry 38*, 8271−8279.
16. Viviani, V. R., Silva, A. C. R., Perez, G. L. O., Santelli, R. V., Bechara, E. J. H., and Reinach, F. C. (1999) Cloning and molecular characterization of the cDNA for the Brazilian larval click-beetle *Pyrearinus termitilluminans* luciferase, *Photochem, Photobiol. 70*, 254−260.
17. Choi, Y. S., Lee, K. S., Bae, J. S., Lee, K. M., Kim, S. R., Kim, I., Lee, S. M., Sohn, H. D., and Jin, B. R. (2002) Molecular cloning and expression of a cDNA encoding the luciferase from the firefly, *Hotaria unmunsana*, *Comp. Biochem. Physiol. Part B 132*, 661−670.
18. Lee, K. S., Park, H. J., Bae, J. S., Goo, T. W., Kim, I., Sohn, H. D., and Jin, B. R. (2001) Molecular cloning and expression of a cDNA encoding the luciferase from the firefly, *Pyrocoelia rufa*, *J. Biotechnol. 92*, 9−19.
19. Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T., and Yamamoto, T. (1990) Structure and regulation of rat long-chain acyl-CoA synthetase, *J. Biol. Chem. 265*, 8681−8685.
20. Babbitt, P. C., Kenyon, G. L., Martin, B. M., Charest, H., Sylvestre, M., Scholten, J. D., Chang, K.-H., Liang, P.-H., and Dunaway-Mariano, D. (1992) Ancestry of the 4-chlorobenzoate dehalogenase: analysis of amino acid sequence identities among families of acyl/adenyl ligases, enoyl-CoA hydratases/isomerases, and acyl-CoA thioesterases, *Biochemistry 31*, 5594−5604.

21. Chang, K.-H., Xiang, H., and Dunaway-Mariano, D. (1997) Acyl-adenylate motif of the acyl-adenylate/thioester-forming enzyme superfamily: a site-directed mutagenesis study with the *Pseudomonas* sp. strain CBS3 4-chlorobenzoate/CoA ligase, *Biochemistry 36*, 15650−15659.
22. Fontes, R., Ortiz, B., De Diego, A., Sillero, A., and Günther Sillero, M. A. (1998) Dehydroluciferyl-AMP is the main intermediate in the luciferin dependent synthesis of Ap4A catalyzed by firefly luciferase, *FEBS Lett. 438*, 190−194.
23. Conti, E., Franks, N. P., and Brick, P. (1996) Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes, *Structure 4*, 287−298.
24. Franks, N. P., Jenkins, A., Conti, E., Lieb, W. R., and Brick, P. (1998) Structural basis for the inhibition of firefly luciferase by a general anesthetic, *Biophys. J. 75*, 2205−2211.
25. Conti, E., Stachelhaus, T., Marahiel, M. A., and Brick, P. (1997) Structural basis for the activation of phenylalanine in the nonribosomal biosynthesis of gramicidin S, *EMBO J. 16*, 4174−4183.
26. May, J. J., Kessler, N., Marahiel, M. A., and Stubbs, M. T. (2002) Crystal structure of DhbE, an archetype for aryl acid activating domains of modular nonribosomal peptide synthetases, *Proc. Natl. Acad. Sci. U.S.A. 99*, 12120−12125.
27. Gulick, A. M., Starai, V. J., Horswill, A. R., Homick, K. M., and Escalante-Semerena, J. C. (2003) The 1.75 Å crystal structure of acetyl-CoA synthetase bound to adenosine-5′-propyl phosphate and coenzyme A, *Biochemistry 42*, 2866−2873.
28. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M., and Zimmer, M. (1998) Site-directed mutagenesis of histidine 245 in firefly luciferase: a proposed model of the active site, *Biochemistry 37*, 15311−15319.
29. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M., Helgerson, L. C., and Zimmer, M. (1999) Site-directed mutagenesis of firefly luciferase active site amino acids: a proposed model for bioluminescence color, *Biochemistry 38*, 13223−13230.
30. Branchini, B. R., Murtiashaw, M. H., Magyar, R. A., and Anderson, S. M. (2000) The role of lysine 529, a conserved residue of the acyl-adenylate-forming enzyme superfamily, in firefly luciferase, *Biochemistry 39*, 5433−5440.
31. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., and Portier, N. C. (2001) The role of active site residue arginine 218 in firefly luciferase bioluminescence, *Biochemistry 40*, 2410−2418.
32. Branchini, B. R., Southworth, T. L., Murtiashaw, M. H., Boije, H., and Fleet, S. E. (2003) A mutagenesis study of the putative luciferin binding site residues of firefly luciferase, *Biochemistry 42*, 10429−10436.
33. Seliger, H. H., and McElroy, W. D. (1964) The colors of firefly bioluminescence: enzyme configuration and species specificity, *Proc. Natl. Acad. Sci. USA 52*, 75−81.
34. McElroy, W. D., and Seliger, H. H. (1966) The colors of bioluminescence: role of enzyme and substrate structure, in *Molecular Architecture in Cell Physiology* (Hayashi, H., and Szent-Gyorgyi, I., Eds.) pp 63−79, Prentice-Hall, Englewood Cliffs, NJ.
35. Hastings, J. W. (1996) Chemistries and colors of bioluminescent reactions- a review, *Gene 173*, 5−11.
36. Viviani, V. R. (2002) The origin, diversity, and structure function relationships of insect luciferases, *Cell. Mol. Life Sci. 59*, 1833−1850.
37. McCapra, F. (2000) Chemical generation of excited states: The basis of chemiluminescence and bioluminescence, *Methods Enzymol. 305*, 3−47.
38. Branchini, B. R., Murtiashaw, M. H., Magyar, R. A., Portier, N. C., Ruggiero, M. C., and Stroh, J. G. (2002) Yellow-green and red firefly bioluminescence from 5,5-dimethyloxyluciferin, *J. Am. Chem. Soc. 124*, 2112−2113.
39. Seto, S., Ogura, K., Nishiyama, Y. (1963) A convenient synthetic method of 2-carbamoyl-6-methoxybenzothiazole, one of the intermediates for the synthesis of firefly luciferin, *Bull. Chem. Soc. Jpn. 36*, 331−333.
40. Viviani, V. R., Uchida, A., Viviani, W., and Ohmiya, Y. (2002) The influence of Ala 243(G247), R215 and T226(N230) on bioluminescence spectra and pH-sensitivity determination in railroad worm, click beetle and firefly luciferases, *Photochem. Photobiol. 76*, 538−544.
41. McCapra, F., Gilfoyle, D. J., Young, D. W., Church, N. J., and Spencer, P. (1994) The chemical origin of colour differences in beetle bioluminescence, in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects* (Campbell, A. K., Kricka, L. J., and Stanley, P. E., Eds.) pp 387−391, John Wiley and Sons, Chichester.
42. McCapra, F. (1997) Mechanisms in chemiluminescence and bioluminescence—unfinished business, in *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons* (Hastings, J. W., Kricka, L. J., and Stanley, P. E., Eds.) pp 7−15, John Wiley and Sons, Chichester.
43. Orlova, G., Goddard, J. D., and Brovko, L. Y. (2003) A theoretical study of the amazing firefly bioluminescence: The formation and structures of the light emitters, *J. Am. Chem. Soc. 125*, 6962−6971.
44. Ueda, H., Yamanouchi, H., Kitayama, A., Inoue, K., Hirano, T., Suzuki, E., Magamune, T., and Ohmiya, Y. (1997) HIS-433 as a key residue for the color difference in firefly luciferase *Hotaria parvula*, in *Bioluminescence and Chemiluminescence: Molecular Reporting with Photons* (Hastings, J. W., Kricka, L. J., and Stanley, P. E., Eds.) pp 216−219, John Wiley and Sons, Chichester.
45. Bell, A. F., He, X., Wachter, R. M., and Tonge, P. J. (2000) Probing the ground-state structure of the green fluorescent protein chromophore using Raman spectroscopy, *Biochemistry 39*, 4423−4431.
46. Kajiyama, N., and Nakano, E. (1991) Isolation and characterization of mutants of firefly luciferase which produce different colors of light, *Protein Engin. 4*, 691−693.
47. Ugarova, N. N., and Brovko, L. Y. (2001) Relationship between the structure of the protein globule and bioluminescence spectra of firefly luciferase, *Russ. Chem. Bull., Int. Ed. 50*, 1752−1761.
48. Kraulis, P. J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures, *J. Appl. Crystallogr. 24*, 946−950.

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