BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Bioluminescence of Beetle Luciferases with 6′-Amino-D-luciferin Analogues Reveals Excited Keto-oxyluciferin as the Emitter and Phenolate/Luciferin Binding Site Interactions Modulate Bioluminescence Colors

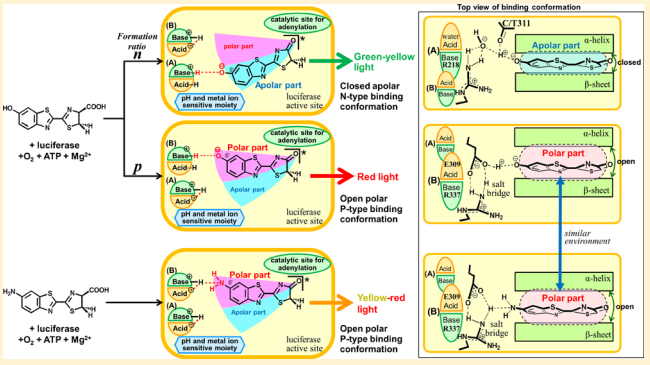
Vadim R. Viviani,*[,†,‡] Deimison Rodrigues Neves,[†] Danilo Trabuco Amaral,[†,‡] Rogilene A. Prado,[†] Takuto Matsuhashi,[§] and Takashi Hirano[§]

[†]Department of Physics, Chemistry and Mathematics, Graduate Program of Biotechnology and Environmental Monitoring, Federal University of São Carlos (UFSCAR), RodoviaJoãoLeme dos Santos, km 110, Itinga, Sorocaba, SP, Brazil

[‡]Graduate Program of Evolutive Genetics and Molecular Biology, Federal University of São Carlos (UFSCAR), São Carlos, SP, Brazil

[§]Department of Engineering Science, Graduate School of Informatics and Engineering, The University of Electro-Communications, Chofu, Tokyo 182-8585, Japan

ABSTRACT: Beetle luciferases produce different bioluminescence colors from green to red using the same D-luciferin substrate. Despite many studies of the mechanisms and structural determinants of bioluminescence colors with firefly luciferases, the identity of the emitters and the specific active site interactions responsible for bioluminescence color modulation remain elusive. To address these questions, we analyzed the bioluminescence spectra with 6′-amino-D-luciferin (aminoluciferin) and its 5,5-dimethyl analogue using a set of recombinant beetle luciferases that naturally elicit different colors and different pH sensitivities (pH-sensitive, *Amydetes vivianii* λ_{max} = 538 nm, *Macrolampis* sp$_2$ λ_{max} = 564 nm; pH-insensitive, *Phrixotrix hirtus* λ_{max} = 623 nm, *Phrixotrix vivianii* λ_{max} = 546 nm, and *Pyrearinus termitilluminans* λ_{max} = 534 nm), a luciferase-like enzyme (Tenebrionidae, *Zophobas morio* λ_{max} = 613 nm), and mutants of C311 (S314). The green-yellow-emitting luciferases display red-shifted bioluminescence spectra with aminoluciferin in relation to those with D-luciferin, whereas the red-emitting luciferases displayed blue-shifted spectra. Bioluminescence spectra with 5,5-dimethylaminoluciferin, in which enolization is blocked, were almost identical to those of aminoluciferin. Fluorescence probing using 2-(4-toluidino)naphthalene-6-sulfonate and inference with aminoluciferin confirm that the luciferin binding site of the red-shifted luciferases is more polar than in the case of the green-yellow-emitting luciferases. Altogether, the results show that the keto form of excited oxyluciferin is the emitter in beetle bioluminescence and that bioluminescence colors are essentially modulated by interactions of the 6′-hydroxy group of oxyluciferin and basic moieties under the influence of the microenvironment polarity of the active site: a strong interaction between a base moiety and oxyluciferin phenol in a hydrophobic microenvironment promotes green-yellow emission, whereas a more polar environment weakens such interaction promoting red shifts. In pH-sensitive luciferases, a pH-mediated switch from a closed hydrophobic conformation to a more open polar conformation promotes the typical red shift.

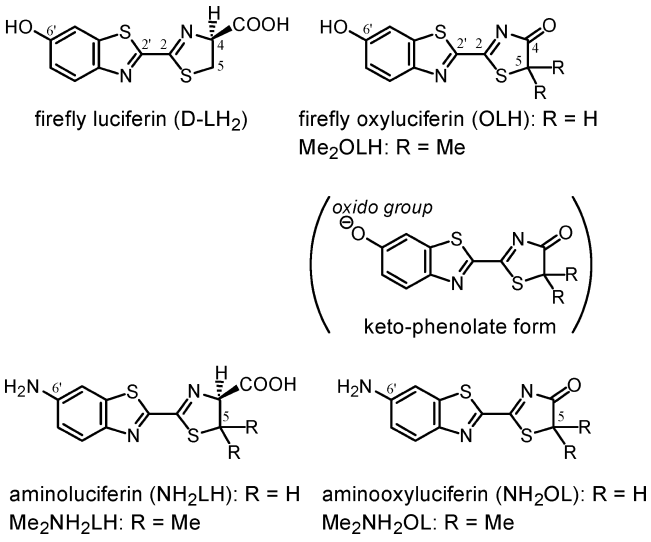


Beetle luciferases naturally emit a wide range of bioluminescence colors from green (534 nm) to red (638 nm) using the same substrates, D-luciferin [D-LH$_2$ (Scheme 1)], ATP, and molecular oxygen.[1] Because D-LH$_2$ is the common substrate for the luciferases of different luminescent beetles, bioluminescence colors are determined by modulation of the properties of the emitter, oxyluciferin [OLH (Scheme 1)], in the active sites of these luciferases. Luciferases are additionally classified as pH-sensitive or pH-insensitive, according to their bioluminescence spectral sensitivity to factors such as pH, added metal ions, temperature, and other denaturing conditions.[2,3] During the past decades, beetle luciferases were extensively applied as bioanalytical reagents and as reporter genes to investigate gene expression and cell and tissue markers.[4,5] Because they may emit different bioluminescence colors, beetle luciferases are currently used as versatile multicolor reporter systems.[6]

Considering that the emission spectrum is determined by the structure of the emitter and its surrounding luciferase environment, studies have focused on one side on the structure and function of luciferase enzymes[1,7,8] and on the other side on

Received: February 6, 2014
Revised: July 7, 2014
Published: July 15, 2014

Scheme 1. Structures of Firefly Luciferin (D-LH_2), Firefly Oxyluciferin (OLH), and Their Analogues and the Keto-Phenolate Form of OLH



the identification and photophysical properties of the putative emitters based on theoretical and spectroscopic properties of D-LH_2, OLH, and their analogues.[9−13]

To explain the nature of the emitters of different bioluminescence colors, White et al. originally proposed that the structural change of the excited state of oxyluciferin phenolate anion (Scheme 1) by tautomerization between keto red-emitting and enol/enolate yellow-green-emitting forms, under the influence of a basic residue abstracting a C5 proton, would determine red and green bioluminescence in firefly luciferases, respectively.[9,14] In addition, the importance of a nonspecific solvent effect on the emitter in bioluminescence color determination has been long proposed by many researchers.[11,12] Later, McCapra et al.[15] proposed that rotation of the C2−C2′ single bond between the thiazole and benzothiazole rings of the excited keto-phenolate form of OLH could determine a continuum of bioluminescence spectra in beetle luciferases. Theoretical studies with TDDFT calculations reported by Orlova et al.[16] suggested that the planar s-*trans* structures of the excited states of OLH are the most likely emitters in beetle bioluminescence, and the twisted structures with C2−C2′ bond rotation between the thiazole and benzothiazole rings are unlikely. Branchini et al.[17] showed definitive experimental evidence that a single structure, the keto-phenolate form of OLH, can emit both red and green bioluminescence and that tautomerization is not required for green-red emission. In this case, the color determination mechanism would be explained by deprotonation of the phenolic 6′-hydroxy group as well as π-electronic conjugation of the keto-phenolate form of the excited oxyluciferin providing a variety of colors. While a recent spectroscopic study of wild-type oxyluciferin reported by Naumov et al.[13] still supports the tautomerization hypothesis based on the results that show the enol-phenolate and enolate-phenolate forms are the yellow-green and yellow-orange emitters, Branchini's hypothesis was confirmed by other experiments. Spectroscopic studies using 5,5-dimethyloxyluciferin [Me_2OLH (Scheme 1)] reported by Hirano et al. support this conclusion that the keto-phenolate form of OLH has the potential to efficiently emit light from green to red depending on the polarity of molecular environments and the interaction between the phenolate 6′-oxido group and a countercation.[12] Thus, most of the current experimental and theoretical studies support the involvement of the keto-phenolate form of OLH as a single emitter, the emission properties being modulated by nonspecific polarity, orientation polarizability, and interactions with specific basic amino acid residues in the luciferase active sites.[12,18]

Several beetle luciferases have been cloned in the past two decades, most of them from fireflies that emit green-yellow light (538−580 nm) and are pH-sensitive.[19−24] The three-dimensional structure of the luciferase of the North American firefly *Photinus pyralis* has been determined in the absence of substrates[25] and that of the luciferase of the Japanese firefly *Luciola cruciata* in the presence of the luciferyl-adenylate analogue DLSA {5′-*O*-[*N*-(dehydroluciferyl)sulfamoyl]-adenosine} or with OLH and AMP,[26] showing a closed active site conformation with DLSA and an open conformation with AMP and OLH. Although these structures provide valuable information about the identity of the active site, they probably fail to identify critical interactions during the reacting and emitting steps. Luciferin binding site residues in firefly luciferases also have been identified in the three-dimensional structures by modeling studies,[27,28] and their function has been probed by site-directed mutagenesis.[29]

Our group has cloned several new beetle luciferases from click beetles,[30,31] railroad worms,[32,33] and fireflies,[34−36] which naturally elicit different bioluminescence colors from the extreme green (534 nm) to red (623 nm) and different pH sensitivities, and investigated their structure and function.

Residues affecting bioluminescence colors were identified by site-directed mutagenesis and can be classified into two main groups: luciferin binding site residues likely interacting with oxyluciferin[29,37] and residues located outside the active site indirectly affecting the conformation of the active site.[37−44] Almost all the luciferin binding site residues are invariant or conserved, and although mutations of some these residues were shown to influence bioluminescence colors in beetle luciferases, they were not proven to be natural determinants of bioluminescence colors. Among them, the invariant R218 was found to be critical for green bioluminescence in railroad worm and firefly luciferases.[29,39] More recently, the main chain carbonyls of residues Cys and Thr at position 311 of click beetles and railroad worm luciferases and Ser314 of firefly luciferases (314 of *P. pyralis* firefly luciferase) were shown to be critically located around the oxyluciferin phenolate for an interaction that may affect bioluminescence colors.[18] Other residues important for bioluminescence colors, however, were found spread in other parts outside of the active site of the luciferase structure. Among them, the loop of residues 223−235, mainly the ^{227}F(Y)GN(T)^{229} motif,[43,44] and the loop of residues 351−360,[35,8] which interact with invariant E309 (E311) and R337, were suggested to help to keep the benzothiazolyl side of the luciferin binding site in a closed conformation favorable for green light emission.[35,44] These results led us to the hypothesis that bioluminescence colors are determined by the active site conformation and compactness that indirectly modulate the polarity and specific interactions around the emitter: a closed apolar conformation would be responsible for green light emission, and an open and/or more polar conformation would result in red light emission.[18,44]

An effective approach to understanding the mechanism of bioluminescence color determination in beetle luciferases is to use luciferin analogues. One of the important luciferin

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

analogues is 6′-amino-D-luciferin [NH_2LH (Scheme 1)], which has an amino group instead of a hydroxy group at position C6′ of D-LH_2. White et al. synthesized NH_2LH for the first time and investigated its spectroscopic properties with firefly and click beetle luciferases, showing that the bioluminescence spectrum of firefly luciferase is in the orange-red region, independent of the pH, and that the bioluminescence spectra with *Pyrophorus plagiophthalamus* click beetle dorsal and abdominal lanterns luciferases were in the same region as that of D-LH_2.[45] More recently, we revisited the spectroscopic properties with NH_2LH and showed that polarity around the 6′-oxido group of OLH is essential for modulating bioluminescence colors and suggested that firefly luciferase may lead to conformations displaying different polarities around the emitter at different pH values.[46] In addition, the authors suggested that 6′-aminooxyluciferin [NH_2OL (Scheme 1)] could be a good fluorescent probe for assessing luciferin binding site polarity around the phenolate group. Therefore, a study of the bioluminescence properties of various luciferases with NH_2OL will provide valuable information about our hypothesis for the bioluminescence color determination mechanism.

Considering the controversial matter of the identity of the light emitter and whether the critical interactions affecting bioluminescence colors in beetle are located on the benzothiazole side or on the thiazolone side of the luciferin binding site, we decided to investigate the bioluminescence spectra and kinetics of recombinant beetle luciferases displaying different bioluminescence colors together with their 311 (314) mutants and a luciferase-like enzyme using NH_2LH and 5,5-dimethylaminoluciferin (Me_2NH_2LH), in which the resulting 5,5-dimethylaminooxyluciferin (Me_2NH_2OL) is constrained to the keto form (Scheme 1).

MATERIALS AND METHODS

Plasmids and Beetle Luciferase cDNAs. All beetle luciferase cDNAs were previously cloned in our laboratories.[30,31,34−36] The cDNAs for *Pyrearinus termitilluminans* click beetle luciferase, *Phrixotrix hirtus* red-emitting luciferases, and *Zophobas morio* mealworm luciferase-like enzyme were subcloned into the pCold vector (Takara). The cDNA of *Macrolampis* sp_2 luciferase and *Phrixotrix viviani* green-emitting luciferase were subcloned in pPro and pCAN vectors (Invitrogen), respectively, and the luciferase from *Amydetes vivianii* (a new species previously mistakenly identified as *Amydetes fanestratus*[36]) was cloned into the pSport vector (Invitrogen) and expressed in *Escherichia coli* BL21-DE3 cells.

Luciferase Expression and Purification. For luciferase expression, transformed *E. coli* BL21-DE3 cells were grown in 500−1000 mL of LB medium supplemented with ampicillin at 37 °C until the OD_{600} reached 0.4 and then induced at 18 °C with 0.4 mM isopropyl β-D-1-thiogalactopyranoside overnight. Cells were harvested by centrifugation at 2500*g* for 15 min at 4 °C and resuspended in extraction buffer consisting of 0.10 M sodium phosphate buffer, 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Roche) (pH 7.0), lysed with a French press or via ultrasonication, and centrifuged at 15000*g* for 15 min at 4 °C. The N-terminal histidine-tagged *Py. termitilluminans*, *Phrixotrix* spp. and *Zophobas* recombinant luciferases and their mutants were further purified by agarose-nickel affinity chromatography followed by dialysis and anion-exchange chromatography. Samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Western blotting using primary polyclonal antibodies raised against *Py. termitilluminans* click beetle and *Ph. hirtus* railroad worm luciferases and the anti-rabbit secondary antibody and detected using the ECL Western blotting detection kit (GE Healthcare) and an ATTO (Tokyo, Japan) LightCapture II CCD camera.

Aminoluciferin Synthesis. NH_2LH and Me_2NH_2LH were synthesized using previously reported procedures.[46−48]

Measurement of Luciferase Activity. Luciferase bioluminescence intensities and kinetics were measured using AB2200 (ATTO) and TD3000III luminometers as previously reported.[36] The assays were performed by mixing 5−10 μL of a 40 mM ATP/80 mM $MgSO_4$ mixture with a solution consisting of 10 μL of luciferase (0.5−1 mg/mL) and 80−85 μL of 0.5 mM luciferin in 0.10 M Tris-HCl (pH 8.0) at 22 °C. All assays were measured in triplicate.

Kinetic Measurements and K_M Determination. The effect of pH on the activity was assayed in 0.10 M phosphate (pH 6.0−8.0) or 0.10 M Tris-HCl (pH 8) buffer. The K_M assays for NH_2LH and Me_2NH_2LH were performed by mixing 5 μL of 40 mM ATP and 80 mM $MgSO_4$ in a solution containing 10 μL of luciferase (0.5−1 mg mL^{-1}), 75 μL of 0.10 M Tris-HCl (pH 8.0), and aminoluciferins at final concentrations between 0.01 and 1 mM. The K_M values were calculated using Lineweaver−Burk plots taking the peak of intensity (I_0) as a measure of V_0. The experimental errors in K_M estimates were within 10%.

Fluorescence Spectra. Fluorescence spectra were recorded in a Hitachi F4500 spectrofluorometer. Scans were run at a speed of 2400 nm/min. The spectra were automatically corrected for the spectral sensitivity of the equipment.

2,6-TNS. For 2-(4-toluidino)naphthalene-6-sulfonate (2,6-TNS) fluorescence, purified luciferases at concentrations between 50 and 100 μg/mL were mixed with 1 μM 2,6-TNS in filtered 0.10 M phosphate buffer at pH 8.0 and 6.0. A standard curve of energy maxima as a function of solvent polarity index was constructed according to the method of Viviani et al.,[39] using the following solvents: formamide (7.3), methanol (6.6), dimethyl sulfoxide (DMSO) (6.5), ethanol (5.2), 2-propanol (4.3), ethyl acetate (4.3), and ethyl ether (2.9). The fluorescence spectra for 2,6-TNS were obtained with excitation at 320 nm and scanning at 350−600 nm (excitation slit of 2.5 nm and emission slit of 10.0 nm). The fluorescence spectra of buffers and solvents were used as blanks.

Protein Fluorescence. The overall protein fluorescence arising from tryptophans and tyrosines was measured for the purified luciferases at concentrations between 50 and 100 μg/mL in 0.10 M prefiltered phosphate buffer (pH 8.0 and 6.0). Samples were excited at 280 nm and scanned from 300 to 600 nm.

Bioluminescence Spectra. Bioluminescence spectra were recorded with the same equipment (F4500) described above with the excitation shutter closed. For the *in vitro* bioluminescence, 50 μL of a purified luciferase was mixed with 450 μL of an assay solution [0.5 mM luciferin or aminoluciferins, 2 mM ATP, and 4 mM $MgSO_4$ in 0.10 M Tris-HCl (pH 8.0)]. The effect of pH on bioluminescence spectra was analyzed in 0.10 M phosphate buffer (pH 8.0 and 6.0) and 0.10 M Tris-HCl (pH 8.0). Each spectrum is the result of at least three independent measurements.

Bioinformatics: Homology Modeling. Homology-based models of *Py. termitilluminans*, *Ph. hirtus*, and *Macrolampis* sp_2 luciferases were previously constructed using as templates the closed conformation of the three-dimensional structure of *L.*

Table 1. Spectral and Kinetic Bioluminescence Properties of Beetle Luciferases with Firefly Luciferin (D-LH_2), 6′-Aminoluciferin (NH_2LH), and 5,5-Dimethylaminoluciferin (Me_2NH_2LH)

	λ_{max} [half-bandwidth] (nm)			relative activity		K_M (μM)		
luciferase	D-LH_2	NH_2LH	Me_2NH_2LH	NH_2LH/D-LH2	Me_2NH_2LH/D-LH2	D-LH_2	NH_2LH	Me_2NH_2LH
pH-sensitive Lampyridae								
Macrolampis (pH 6.0)	606 [77]	594 [73]	595 [55]	0.15	0.1	40		
Macrolampis (pH 8.0)	563 [99]	594 [70]	598 [54]	0.045	0.045	20	1	
P. pyralis (pH 6.0)	611 [85]	598 [71]	594 [77]	0.44	0.36			
P. pyralis (pH 8.0)	560 [74]	603 [94]	599 [98]	0.6	0.5	5	1	
A. vivianii (pH 6.0)	551 [98]	582 [68]	585 [68]	0.083	0.104			
A. vivianii (pH 8.0)	538 [70]	584 [72]	585 [83]	0.6	0.47	4	1	
pH-insensitive Elateridae								
Py. termitilluminans	538 [75]	560 [84]	559 [77]	0.31	0.077	80	1	2
C311A	588 [87]	579 [63]	579 [63]	0.033	0.044	150		
Phengodidae								
Ph. vivianii (green)	548 [70]	580 [66]	584 [70]	0.17	0.33	64		
RE220GR (yellow)	578 [67]	584 [68]	585 [66]	0.23	0.27	140		
Ph. hirtus (red)	623 [55]	607 [73]	605 [71]	0.48	0.30	7	2	3
C311T	606 [90]	589 [56]	598 [63]	0.7	0.8	7		
C311A	627 [52]	607 [69]	608 [68]	0.9	0.85	4		
T345I	629 [50]	604 [69]	612 [77]	0.26	0.37	79		
Tenebrionidae								
Zophobas luciferase-like	611 [92]	572 [76]	577 [77]	0.05	0.1	500	3	

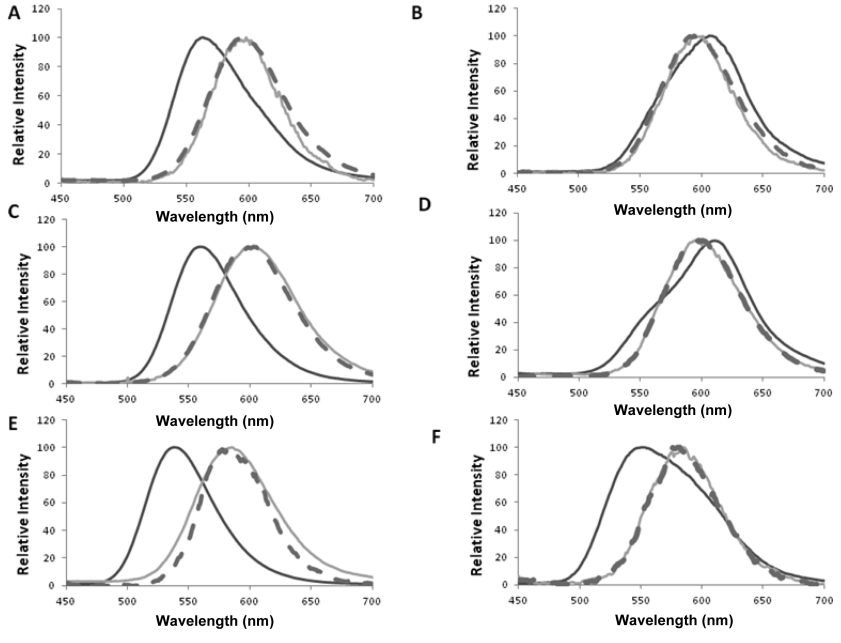


Figure 1. Bioluminescence spectra of different color-emitting pH-sensitive firefly luciferases with firefly D-luciferin and aminoluciferin analogues: (A) *Macrolampis* sp_2 at pH 8.0, (B) *Macrolampis* sp_2 at pH 6.0, (C) *P. pyralis* at pH 8.0, (D) *P. pyralis* at pH 6.0, (E) *A. vivianii* at pH 8.0, and (F) *A. vivianii* at pH 6.0. Black for D-luciferin, dashed for NH_2LH, and gray for Me_2NH_2LH.

cruciata luciferase in the presence of DLSA [Protein Data Bank (PDB) entry 2D1S] or OLH and AMP (PDB entry 2D1R).[18] Modeler version 9.9 was used to construct 200 three-dimensional models of each sequence.[49] Visualization and analyses of the best model of each luciferase were performed using PyMol version 1.4.1.[50]

RESULTS

Bioluminescence Activity and Kinetics with Aminoluciferin and 5,5-Dimethylaminoluciferin. The bioluminescence activities with NH_2LH and Me_2NH_2LH were determined as a percentage of the luminescence intensities of luciferase reactions with D-LH_2 as shown in Table 1. The relative activities with NH_2LH and Me_2NH_2LH were usually below unity (Table 1). Interestingly, however, for mutants C311A and C311T of *Phrixotrix* red-emitting luciferase, the activities were very close to those with D-LH_2. Furthermore, in almost all luciferases, the K_M values for the amino analogues were considerably lower than the K_M for D-LH_2 (Table 1), indicating a higher affinity for these analogues than for the natural bioluminescent substrate.

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

Bioluminescence Spectra with Aminoluciferins. We have measured the bioluminescence spectra of different beetle luciferases spanning the full bioluminescence spectrum of the three main families of luminescent beetles, Lampyridae, Elateridae, and Phengodidae (λ_{max} = 534−623 nm), and also the luciferase-like enzyme of *Zophobas* mealworm with NH_2LH and Me_2NH_2LH (Figures 1−3). Then, we systematically compared the bioluminescence spectra of aminoluciferins with those of D-LH_2.

Noteworthy is the fact that with Me_2NH_2LH the bioluminescence spectra were essentially similar if not identical to those with NH_2LH (Figures 1−3), providing clear evidence that the keto form of NH_2OL is responsible for all bioluminescence colors displayed by different beetle luciferases and that the interaction of the 6′-amino group with the active site environment of each luciferase determines bioluminescence colors.

Three luciferases, those of *Macrolampis* sp_2, *A. vivianii*, and *P. pyralis* fireflies, are pH-sensitive with D-LH_2 and show green-yellow emission at pH 8.0 and red-shifted emission at pH 6.0. As reported by White at al.[45] for *P. pyralis* firefly luciferase, these luciferases also show pH-insensitive bioluminescence spectra with NH_2LH. The bioluminescence emission maxima (λ_{max}) with NH_2LH were in general red-shifted in relation to the respective λ_{max} values with D-LH_2 at pH 8.0, however, to different degrees (Figure 1). The more green-shifted was seen in the bioluminescence spectrum with D-LH_2 at pH 8, and the larger the red shift was seen with NH_2LH (Figure 1) in the following order: *A. vivianii* luciferase > *P. pyralis* luciferase > *Macrolampis* luciferase (Figure 1). On the other hand, the λ_{max} values of *Macrolampis* and *P. pyralis* luciferases with NH_2LH were blue-shifted compared to the respective λ_{max} values with D-LH_2 at pH 6.0, while the λ_{max} value of *A. vivianii* luciferase with NH_2LH was still red-shifted compared to that with D-LH_2 at pH 6.0.

In the case of green-yellow-emitting pH-insensitive luciferases, *Py. termitilluminans*, *Ph. vivianii*, and *Phrixotrix* chimera RE220GR luciferases (Figures 2 and 3), *P. viviani* luciferase displayed the largest red shift of the emission maximum (32 nm) with NH_2LH in relation to that with D-LH_2. The bioluminescence spectra of *Phrixotrix* chimera RE220GR luciferase with D-LH_2 and NH_2LH were similar to each other.

In the case of the red-emitting *Ph. hirtus* luciferase and its mutants, and the luciferase-like enzyme of *Zophobas* mealworm that also emits red light, the bioluminescence spectra with NH_2LH were always blue-shifted in relation to the spectra with D-LH_2, the largest effect being observed with the luciferase-like enzyme (39 nm blue-shifted) (Figure 3).

To improve our understanding of the effect of the environment around the 6′-amino and oxido groups in the luciferins, we also measured the bioluminescence spectra of 311-residue mutants of *Py. termitilluminans* (C311) and *Ph. hirtus* (C311) luciferases. These are the mutants corresponding to residue S314 in *P. pyralis* and *Macrolampis* firefly luciferases that were shown to be located near the oxyluciferin phenolate group and to display an important effect in color modulation.[18] With NH_2LH, the orange-emitting C311A mutant (588 nm) of *Py. termitilluminans* luciferase also showed a red-shifted bioluminescence spectrum compared with that of wild-type luciferase (Figure 2). However, the bioluminescence spectrum of this orange-emitting mutant with NH_2LH was blue-shifted in relation to that with D-LH_2.

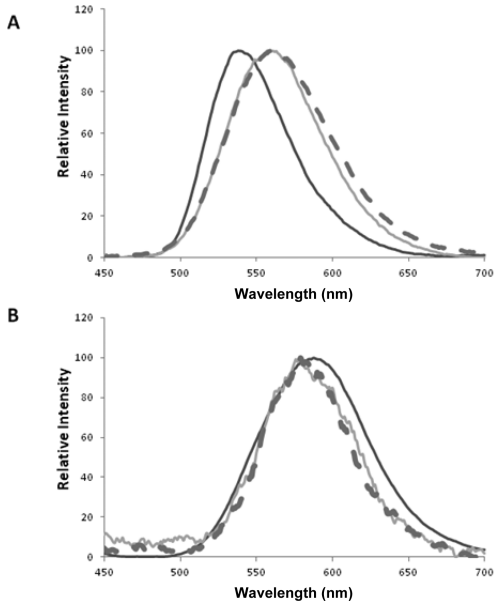


Figure 2. Bioluminescence spectra of *Py. termitilluminans* larval click beetle green-emitting luciferase with D-luciferin and aminoluciferin analogues: (A) wild type and (B) C311A mutant. Black for D-LH_2, dashed for NH_2LH, and gray for Me_2NH_2LH.

The bioluminescence spectrum of the *Ph. hirtus* C313T mutant (λ_{max} = 589 nm) with NH_2LH is very similar to that of the green-emitting *Ph. vivianii* luciferase (λ_{max} = 580 nm), which naturally displays the C311T substitution (Figure 3), indicating that this mutation indeed turns the luciferin binding site of the red-emitting luciferase more similar to that of the green-emitting one, as previously suggested.[18]

Although the bioluminescence spectra of the green-yellow-emitting luciferases with NH_2LH show a tendency to be red-shifted in relation to the spectra with D-LH_2, the magnitude of the shift of the emission maxima with this analogue is not quantitatively related with that of D-LH_2. In fact, a plot between the wavenumbers ν (in inverse centimeters) of the λ_{max} values for NH_2LH and D-LH_2 with the green-yellow luciferases together with *Macrolampis* luciferase and *P. pyralis* and *A. vivianii* firefly luciferases at pH 8.0 does not show a clear correlation (data not shown). Such a lack of correlation may reflect different interactions between the luciferase active sites and the excited states of OLH and NH_2OL.

Protein Fluorescence. Beetle luciferases have an invariant tryptophan residue at position 424 (*P. pyralis* luciferase sequence numbering), and in some luciferases more than one tryptophan, which may make an important contribution to the overall protein fluorescence (Table 2). Therefore, we measured the overall protein fluorescence as a reasonable indicator of conformational changes caused by mutations and pH changes. We verified that the change in pH from 8.0 to 6.0 did not cause any significant changes in the protein fluorescence of firefly luciferases without substrates. Similarly, the mutant luciferases of *Py. termitilluminans* and *Ph. hirtus* at position C311 also display fluorescence spectra essentially similar to those of the respective wild-type luciferases. These results could be taken as evidence of the lack of larger conformational changes upon changes in pH and C311T and -A mutations in the absence of substrates.

Fluorescence Spectra of 2,6-TNS in Luciferases. We also measured the fluorescence spectra of 2,6-TNS included in

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

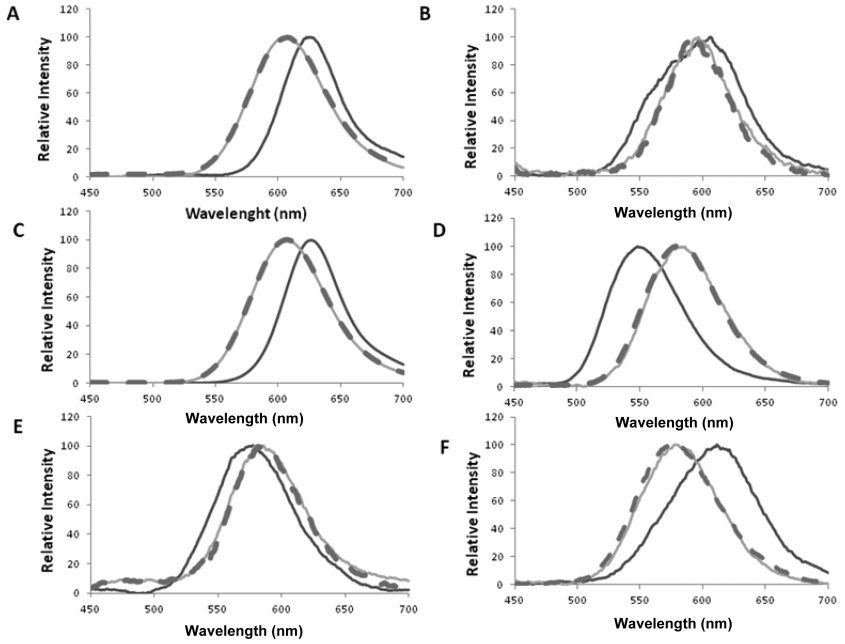


Figure 3. Bioluminescence spectra of *Phrixotrix* railroad worm with firefly D-luciferin and aminoluciferin analogues: (A) *Ph. hirtus* red-emitting luciferase, (B) *Ph. hirtus* C311T mutant, (C) *Ph. hirtus* C311A mutant, (D) *Ph. vivianii* green-emitting luciferase, (E) *Phrixotrix* yellow-emitting chimeric luciferase, and (F) *Z. morio* luciferase-like enzyme. Black for D-LH_2, dashed for NH_2LH, and gray for Me_2NH_2LH.

Table 2. Summary of Protein and 2,6-TNS Fluorescence Maxima with Beetle Luciferases and Mutants

		λ_{max} (nm)			
		protein FL		2,6-TNS	
luciferase	tryptophans	pH 8	pH 6	pH 8	pH 6
pH-sensitive (Lampyridae)					
P. pyralis	W440	337	333	428	429
pH-insensitive					
Elateridae					
Py. termitilluminans	W77, W400, W424	336	338	428	429
C313A	W77, W400, W424	336	339	436	434
Phengodidae					
Ph. vivianii	W424	340		422	
Ph. hirtus	W424	334	333	438	
RE T345I	W424	335		428	
RE C313T	W424	339		431	
luciferase-like enzyme					
Z. morio	W227, W432	347	345	440	440
I327S	W227, W432	348		441	

luciferases (Table 2), which is considered a classical fluorescence probe for the polarity of protein active sites and is a competitive inhibitor with regard to firefly luciferin. Fluorescence spectra of 2,6-TNS in the *Zophobas* luciferase-like enzyme and *Ph. hirtus* red-emitting luciferase gave more red-shifted emission maxima, whereas in the green-emitting *Ph. vivianii*, *Py. termitilluminans*, and *P. pyralis* luciferases, it gave more blue-shifted emission maxima. These results suggest that the *Zophobas* luciferase-like enzyme and *Ph. hirtus* red-emitting luciferase have more polar active sites and the green-yellow-emitting *Ph. vivianii*, *Pyrearinus*, and *P. pyralis* luciferases have more hydrophobic active sites (Table 2). In the case of the pH-sensitive *P. pyralis* luciferase, there were not considerable changes in the fluorescence spectrum of 2,6-TNS at pH 8 and 6, despite the bioluminescence spectrum with D-LH_2 undergoing a dramatic red shift, suggesting that there are not considerable changes in polarity with this probe at different pH values.

Upon comparison of the critical mutants at position 311 (314) of *Py. termitilluminans* and *Ph. hirtus* luciferases, whose main chains carbonyls were shown to be close to phenolate, we found that the fluorescence spectra of 2,6-TNS followed a trend similar to that of the bioluminescence spectra: red-shifted in the case of the *Py. termitilluminans* C311A mutant in relation to that of its wild type and blue-shifted in the case of the *Ph. hirtus* C311T mutant in relation to that of its wild type. Because the magnitude of the shifts of the fluorescence maxima is smaller than that of the respective bioluminescence ones, it is difficult to obtain clear correlations between the wavenumbers of the bioluminescence emission maxima with D-LH_2 (and NH_2LH) and those of the fluorescence maxima of 2,6-TNS.

DISCUSSION

To understand the identity of the emitter(s) of beetle bioluminescence and the nature of its interactions in the active sites of luciferases emitting different bioluminescence colors, we investigated the bioluminescent properties of recombinant beetle luciferases eliciting distinct bioluminescence colors, their 311 (314 in fireflies) amino acid residue mutants and a luciferase-like enzyme with NH_2LH and Me_2NH_2LH. NH_2LH is an attractive substrate for mechanistic studies, because its 6′-amino group is an electron-donating group similar to the 6′-oxido (O^-) group of luciferin phenolate but has basic character (pK_b of ∼9; pK_a of the conjugate acid of ∼4.6) instead of acidic character (pK_a of ∼8.6) of the 6′-hydroxy (OH) group of D-LH_2. Our previous study of the bioluminescence properties of *P. pyralis* luciferase with NH_2LH and Me_2NH_2LH led us to suggest that the emitter of the bioluminescence with NH_2LH is

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

the excited state of the keto form of NH_2OL.[46] The fluorescence properties of Me_2NH_2OL indicated that the excited state of the keto form of NH_2OL can efficiently emit various colors of light from yellow-green to orange depending on the solvent polarity,[46] being a sensitive probe for the polarity in the luciferin phenolate binding pocket of the luciferase active site.

The major finding of this work is that the bioluminescence spectra of the 12 pH-sensitive and pH-insensitive luciferases tested with Me_2NH_2LH almost overlap with those of NH_2LH, clearly showing that the structure of the excited state of the emitter, NH_2OL generated from NH_2LH, is in its keto form as previously reported for *P. pyralis* luciferase.[46] These results provide compelling evidence that the keto form of the excited oxyluciferin phenolate could be the actual emitter for bioluminescence of different luciferases with wild-type D-LH_2, because it is unlikely that the keto−enol tautomerism is specifically involved in the bioluminescence with D-LH_2. Therefore, the differences in the λ_{max} values of the luciferases with D-LH_2 and NH_2LH are determined mainly by interactions of 6-oxido and NH_2 groups in the luciferase active sites modulating the π-electronic properties of the keto form.

Next, we evaluated the oxyluciferin phenolate binding pocket environment of different bioluminescence color-emitting luciferases, by comparing bioluminescence spectra of amino-luciferin analogues with those of D-luciferin and correlating them with the polarity indexes. The λ_{max} values for Me_2NH_2LH bioluminescence were evaluated with the correlation between the fluorescence data of Me_2NH_2OL in various organic solvents and Rechardt's solvent polarity parameter $E_T(30)$ (in kilocalories per mole) (Figure 4).[46] Similarly, the correlation between the fluorescence data of the phenolate anion of Me_2OLH generated with tributylamine and the $E_T(30)$ parameter was also available (Figure 5).[12]

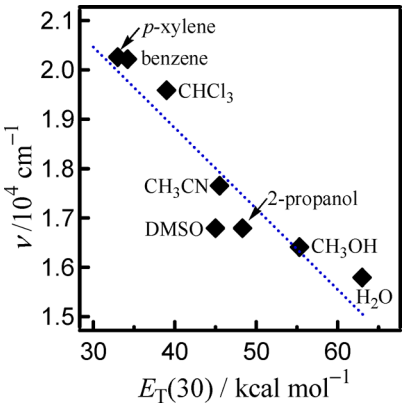


Figure 4. Plot of wavenumbers ν of fluorescence emission maxima of Me_2NH_2OL vs solvent parameter $E_T(30)$.

The luciferases were classified into three subgroups: (1) pH-sensitive, (2) pH-insensitive green-yellow-emitting (534−578 nm), and (3) pH-insensitive orange/red-shifted and red-emitting (>580 nm).

pH-Sensitive Luciferases (group 1). We first evaluated the bioluminescence of the pH-sensitive *Macrolampis* and *A. vivianii* firefly luciferases. Similar to *P. pyralis* luciferase, *Macrolampis* sp_2 and *A. vivianii* luciferases did not show pH-dependent character of bioluminescence with NH_2LH as reported by White et al.,[45] providing evidence that pH

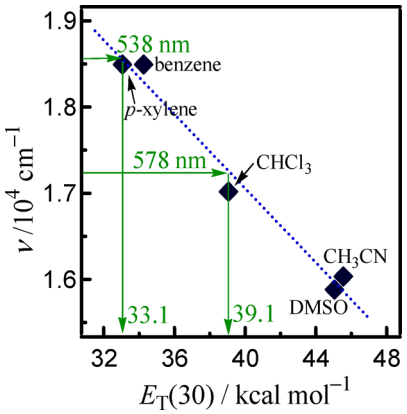


Figure 5. Plot of wavenumbers ν of fluorescence emission maxima of the phenolate anion of Me_2OLH generated with tributylamine vs solvent parameter $E_T(30)$.

sensitivity depends on the presence of the 6′-hydroxy group of OLH and is related to an acid−base interaction between the 6′-hydroxy group of OLH and a basic moiety of the luciferase active site.

It was suggested that pH sensitivity in firefly luciferases arises from a conformational switch between a closed apolar to an open polar conformation.[26,35,44] Our previous study of *P. pyralis* luciferase bioluminescence with D-LH_2 and NH_2LH led us to propose that the luciferin binding site indeed displays two different oxyluciferin phenolate binding modes that may arise from distinct conformations, apolar N and polar P types for OLH, while NH_2OL is able to bind only to a P-type conformation.[46] These binding conformations are illustrated in Figure 6. The results shown here with the pH-sensitive luciferases of *Macrolampis* and *A. vivianii* fireflies support this hypothesis.

The difference in the λ_{max} values with D-LH_2 and NH_2LH must be considered with the substituent effects of the oxido and amino groups of oxyluciferin phenolate and NH_2OL, respectively. Because the electron donating ability strength of the amino group (Hammett constant σ_p of NH_2 = −0.66) is lower than that of the oxido group (σ_p of O^- = −0.81),[51] the fluorescence emission maximum of NH_2OL is blue-shifted in relation to that of oxyluciferin phenolate in environments with the same polarity. Therefore, the bioluminescence spectrum of a beetle luciferase with NH_2LH is expected to be blue-shifted compared to that with D-LH_2, assuming that both are found in the same binding site microenvironment. As in the case of *P. pyralis* luciferase, the λ_{max} values of *Macrolampis* sp_2 luciferase with NH_2LH are blue- and red-shifted compared to those with D-LH_2 at pH 6.0 and 8.0, respectively, indicating that the *Macrolampis* luciferase active site also displays both N- and P-type binding conformations with OLH. However, in the case of *A. vivianii* green-emitting luciferase with NH_2LH at pH 6, the bioluminescence spectrum is considerably red-shifted compared to that with D-LH_2. Because the pH-dependent character of *A. vivianii* luciferase is weaker than those of *P. pyralis* and *Macrolampis* luciferases, the bioluminescence spectrum of *A. vivianii* luciferase with D-LH_2 at pH 6.0 still displays a broad shape with a predominance of green emission over red emission,[36] which is indicative of a more stable N-type binding conformation despite the acidic pH. Thus, we estimated the λ_{max} value (~590 nm) of red emission by decomposition of the green and red emission spectra using the spectrum at pH 8.0.

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

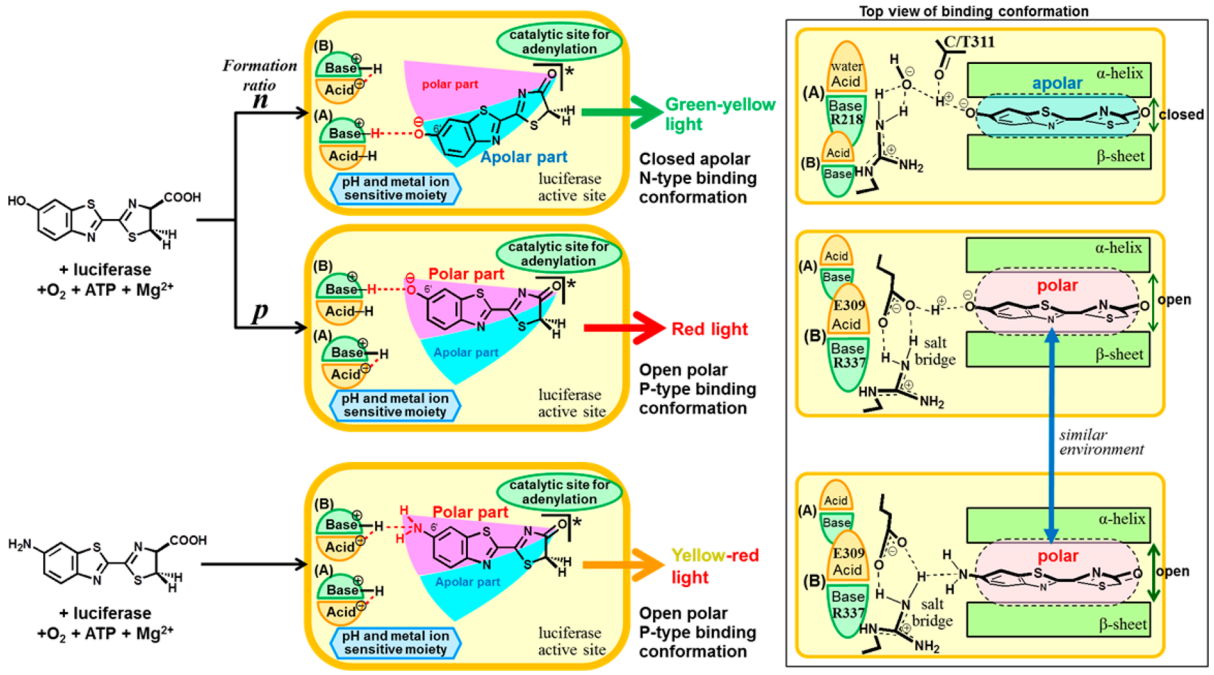


Figure 6. Scheme summarizing the active site environment experienced by the excited states of oxyluciferin phenolate and aminooxyluciferin during bioluminescence with closed apolar N-type and open polar P-type conformations, including acid−base interactions.

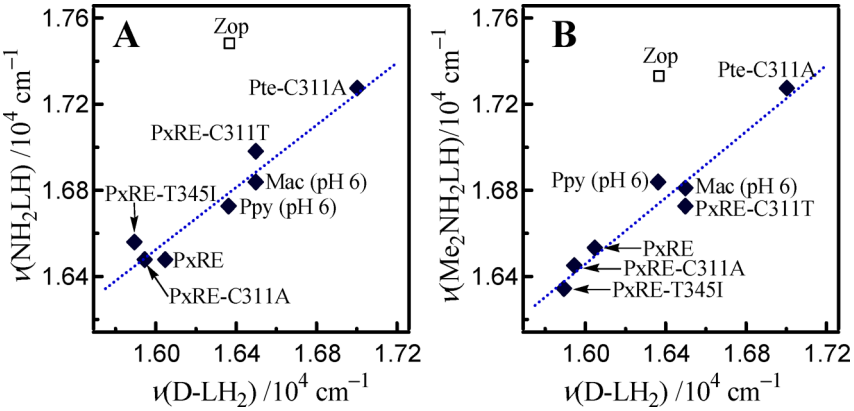


Figure 7. Plots of wavenumbers ν of the λ_{max} values of bioluminescence of the red-shifted and red-emitting luciferases together with *Macrolampis* luciferase (pH 6.0), *P. pyralis* luciferase (pH 6.0), and *Zophobas* luciferase-like enzyme using NH_2LH (A) and its 5,5-dimethyl analogue Me_2NH_2LH (B) vs the ν values of the λ_{max} values with D-LH_2. Abbreviations: Mac, *Macrolampis* luciferase; Ppy, *P. pyralis* luciferase; PxRE, *Ph. hirtus* luciferase; PxRE-C311T, *Ph. hirtus* C311T mutant; PxRE-C311A, *Ph. hirtus* C311A mutant; PxRE-T345I, *Ph. hirtus* T345I mutant; Pte-C311A, *Py. termitilluminans* C311A mutant; Zop, *Z. morio* luciferase-like enzyme.

As seen for other firefly luciferases, the λ_{max} of *A. vivianii* luciferase with NH_2LH (582 nm) is blue-shifted compared to the maximum of ∼590 nm with D-LH_2 at acidic pH, indicating that the red-emitting conformation of *A. vivianii* luciferase with OLH is also polar. However, the peaks at 590 and 582 nm with D-LH_2 and NH_2LH, respectively, are slightly blue-shifted in relation to the corresponding peaks observed for *Macrolampis* and *P. pyralis* luciferases at pH 6.0 (∼610 and ∼595 nm, respectively), indicating that the active site environment for the red-emitting conformation of *A. vivianii* luciferase is slightly less polar than the other two luciferases.

Green-Yellow-Emitting pH-Insensitive Luciferases (group 2). In the case of the green-yellow-emitting *Py. termitilluminans* and *Ph. vivianii* luciferases, the λ_{max} values with NH_2LH at pH 8.0 are always red-shifted compared to those with D-LH_2, like the cases of pH-sensitive firefly luciferases at pH 8.0. The λ_{max} values of these green-yellow-emitting luciferases with D-LH_2 are in the range from 538 nm (*Py. termitilluminans* luciferase) to 578 nm (*Phrixotrix* RE220GR yellow chimera). The polarity of the active sites of the most blue-shifted luciferases of *Py. termitilluminans* (538 nm) and *A. vivianii* at pH 8.0 (538 nm), predicted from the fluorescence spectra of the phenolate anion of Me_2OLH (Figure 5), is similar to that of solvents like *p*-xylene [$E_T(30)$ = 33.1], whereas the active site of *Phrixotrix* yellow-emitting chimera RE220GR (578 nm) displays a polarity similar to that of chloroform [$E_T(30)$ = 39.1]. In contrast, the λ_{max} values with NH_2LH and Me_2NH_2LH for the green-yellow-emitting luciferases of *Py. termitilluminans*, *Ph. vivianii*, and the *Phrixotrix* RE220GR chimera together with *Macrolampis*, *P. pyralis*, and *A. vivianii* firefly luciferases at pH 8.0 are in the ranges of 560−603 and 559−599 nm, respectively. A linear correlation of the

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

fluorescence maximum wavenumbers ν (in inverse centimeters) of Me_2NH_2OL versus the $E_T(30)$ [$\nu = -164E_T(30) + 25400$ (Figure 4)] indicates that the active sites of green-yellow-emitting luciferases have polarities for Me_2NH_2OL similar to the level of solvation of different solvents: acetonitrile [$E_T(30) = 45.6$] for *Py. termitilluminans* luciferase (λ_{max} = 559 nm, yellow-green), 1-propanol [$E_T(30) = 50.7$] for *Ph. vivianii* green-emitting luciferase (λ_{max} = 584 nm) and the *Phrixotrix* yellow-emitting chimera (λ_{max} = 585 nm), and an 8:2 ethanol/water mixture [$E_T(30) = 53.7$] for *P. pyralis* luciferase (pH 8.0) (λ_{max} = 599 nm, orange). Therefore, the active sites of the green-yellow-emitting pH-insensitive luciferases and the pH-sensitive luciferases at pH 8.0 provide apolar environments in the closed N-type binding conformation for the excited state of the keto-oxyluciferin phenolate, and more polar environments for NH_2OL and Me_2NH_2OL with a P-type binding conformation (Figure 6).

pH-Insensitive Orange- and Red-Emitting Luciferases (group 3). In the case of the orange-emitting *Py. termitilluminans* C311A mutant and the true red-emitting wild-type *Ph. hirtus* luciferase and its mutants (C311T, C311A, and T345I), the λ_{max} values with NH_2LH were always blue-shifted compared to those with D-LH_2. These differences in the λ_{max} values are consistent with the prediction based on the electron donating property of the substituents. Thus, both OLH and NH_2OL may experience similar polar environments in the case of these red-shifted luciferases. To confirm whether OLH and NH_2OL are found in a similar environment in these orange- and red-emitting luciferases, correlations between the wavenumbers ν (in inverse centimeters) of the λ_{max} values for NH_2LH (and Me_2NH_2LH) and D-LH_2 for these luciferases together with the red-emitting forms of *Macrolampis* and *P. pyralis* firefly luciferases at pH 6.0 were estimated (Figure 7). The linear correlations of the ν values for NH_2LH and Me_2NH_2LH versus those for D-LH_2 indeed indicate that OLH, NH_2OL, and Me_2NH_2OL are located in a similar environment in the active site of these orange- and red-emitting luciferases corresponding to a polar P-type binding conformation (Figure 6). The correlation between the fluorescence ν values of Me_2NH_2OL and the $E_T(30)$ parameters (Figure 4) shows that the λ_{max} values of the *Py. termitilluminans* C311A orange-emitting mutant (579 nm) and the *Ph. hirtus* T345I red mutant (612 nm) display polarities corresponding to $E_T(30)$ values of 50 and 55, respectively, which are similar to those of 1-butanol [$E_T(30) = 49.7$] and methanol [$E_T(30) = 55.4$].

In the case of the red-emitting *Zophobas* luciferase-like enzyme, the λ_{max} values with NH_2LH and Me_2NH_2LH are also blue-shifted compared to that with D-LH_2, while the points of the ν values deviate from the linear lines of Figure 7. This result indicates that the excited states of oxyluciferin phenolate and NH_2OL or Me_2NH_2OL are also found in a polar environment in the enzyme active site, but there is a difference in the position of OLH and NH_2OL or Me_2NH_2OL in the active site: polar environments for the excited state of oxyluciferin phenolate and less polar environments for the excited states of NH_2OL and Me_2NH_2OL. This behavior is consistent with previous homology modeling studies that predicted an environment for the luciferase-like enzyme luciferin binding site less polar than that with the *Phrixotrix* red-emitting enzyme.[52]

The active site polarities predicted from the λ_{max} values are consistent with those probed with 2,6-TNS and 1,5-ANS, which show nonpolar environments for the green-emitting and yellow-green-emitting luciferases of *Py. termitilluminans*, *P. viviani*, and *P. pyralis* and more polar environments for the red-shifted luciferases and mutants (Table 2). The results with these probes also indicate that mutations C311A in *Pyrearinus* luciferase and C311T in *Ph. hirtus* red-emitting luciferase, and their main chain carbonyls that are located close to oxyluciferin phenolate,[18] directly influence the polarity of the phenolate binding pocket, reinforcing a critical role for bioluminescence colors.

The λ_{max} values of D-LH_2 with pH-insensitive red-emitting luciferases and pH-sensitive luciferases at pH 6.0 (<629 nm) are still considerably blue-shifted in comparison with the chemiluminescence maximum of luciferyl-adenylate in an aqueous environment (~640 nm),[53] indicating that the luciferin binding site environment in the P-type binding conformation is still relatively anhydrous and less polar than an aqueous solution. In fact, the active site polarities of these red-emitting luciferases were predicted to be in the range of alcohol solvation.

The variation in the λ_{max} values of bioluminescence for different luciferases and pH-sensitive luciferases at different pH values with D-LH_2 can be explained by the three following mechanisms: (1) a change in polarity around the emitter in the active site that may have a solvatochromic effect on the emission maximum, (2) an acid−base interaction between a basic moiety in the active site and the 6′-hydroxy group of OLH, which may determine major green−red shifts, and (3) the compactness and rigidity of the active site cavity, which may affect the spectral bandwidth. As in the case of D-LH_2, a specific acid−base interaction of the amino group of NH_2LH and Me_2NH_2LH in the luciferase active site should be considered for color modulation.

The K_M values indicated that both NH_2LH and Me_2NH_2LH are better bioluminescence substrates for beetle luciferases than D-LH_2. Their higher affinity may reflect a better stabilization of the amino group in the luciferin binding pocket, due to its smaller size and hydrogen bond acceptor (basic) character. In particular, the amino group can make stronger hydrogen bonding interactions with hydrogen bond donor groups stabilizing NH_2LH and Me_2NH_2LH in the luciferin binding sites. This in turn may explain why a single P-type binding mode is stabilized with amino analogues.

The relationship of the bioluminescence color with D-LH_2 (Figure 6) and the ratio of light emission from the excited oxyluciferin phenolate in the N- and P-type binding conformations is summarized in Table 3. Because the quantum yield of green-yellow emission is higher than that of red emission for the *P. pyralis* luciferase bioluminescence, red emission remains hidden by the predominant green-yellow emission component.[54] Thus, the excited state of oxyluciferin phenolate could be generated in both a closed apolar N-type and/or open polar P-type binding modes, whose distribution (n:p) is characteristic of each luciferase. In the case of pH-sensitive luciferases [(1) in Table 3], the closed apolar N-type conformation predominates at pH 8.0, with an n:p ratio above the unity. At this pH, a strong interaction between a basic moiety at (A) and the 6′-hydroxy group of the excited oxyluciferin is promoted and stabilized in a less polar environment of the active site, setting the excited oxyluciferin π system at a higher energy level, resulting in green-yellow emission. At pH 6, the closed N-type conformation is disrupted and the red light emission arising from an open polar P-type binding conformation becomes dominant. In this case, the 6′-

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

Table 3. Ratio of N-Type and P-Type Conformations in Beetle Luciferases

(1) pH-dependent luciferases	*Macrolampis* sp_2 luciferase	*n*:*p*
	P. pyralis luciferase	$n > p \neq 0$
	A. vivianii luciferase	
(2) pH-independent green-yellow luciferases	*Py. termitilluminans* luciferase	*n*:*p*
	Ph. vivianii green luciferase	$n \gg p$
	Phrixotrix quimera RE220GR	
(3) pH-independent orange/red luciferases	*Py. termitilluminans* C311A mutant	*n*:*p*
	Ph. hirtus luciferase	$n \ll p$
	Ph. hirtus C311T mutant	
	Ph. hirtus C311A mutant	
	Ph. hirtus T345I mutant	

hydroxy group of the excited oxyluciferin could be displaced to a more polar environment, making a new weaker interaction with the position (B) setting the oxyluciferin π system at a lower energetic level, resulting in red light emission.

The structural motifs comprising the loops of residues 223−235 and 352−361 and interacting residues S284 and E311−R337 are candidates for the pH-sensitive moiety, although the exact identity of residues mediating this effect remains to be elucidated.[44] At acidic pH values, protonation of residues of this network may disrupt stabilizing interactions leading to a conformational switch to the P-type conformation. The pH-sensitive moieties are also sensitive to metal ions such as Zn^{2+} and temperature.[2]

In contrast, the 6′-amino group of NH_2OL cannot interact with the basic moieties at (A) and (B) in the active sites of the pH-sensitive luciferases (Figure 6). In the absence of the substrate, the basic moieties at (A) and (B) could make an acid−base interaction with neighboring acidic moieties in the luciferase active site. Because the binding affinity of NH_2LH is higher than that of D-LH_2, the acidic moieties may play an important role in binding NH_2LH. In particular, the acidic moiety at (B) could selectively make an acid−base interaction with the 6′-amino group of NH_2LH, fixing the excited NH_2OL in a polar P-type binding mode of the active site. In the P-type binding conformation, the polarity of the active site varies depending on the luciferase, affecting the stability of the excited state of NH_2OL, which emits light in the range from yellow to orange.

In the case of pH-insensitive luciferases, only a single conformation is displayed with D-LH_2 depending on the luciferase: a closed apolar N-type for green-yellow-emitting luciferases or a polar P type for orange- and red-emitting luciferases [(2) and (3) in Table 3]. In the active sites of the green-yellow-emitting luciferases of *Py. termitilluminans*, *Ph. vivianii* green, and the *Phrixotrix* RE220GR chimera, a strong acid−base interaction between the 6′-hydroxy group of the excited oxyluciferin and the basic moiety at (A) could be stabilized ($n \gg p$) by a more rigid apolar environment, fixing the excited oxyluciferin phenolate anion in an N-type binding mode in a manner similar to that of pH-sensitive luciferases at pH 8. The polarity of the luciferase active sites for the excited oxyluciferin phenolate is similar to that between *p*-xylene and chloroform. In these luciferases, the pH-sensitive moiety is not functional and the closed N-type binding conformations are not affected by pH. In contrast, the acidic moiety at (B) could still make an acid−base interaction with the 6′-amino group of NH_2LH, giving only the excited state of NH_2OL in the P-type binding conformation (Figure 6). In this case, excited NH_2OL emits yellow-orange light, indicating that the polarity of the active sites is somewhere between those of acetonitrile and 1-propanol [$E_T(30)$ = 46−51].

The orange-emitting *Py. termitilluminans* C311A mutant and the true red-emitting *Ph. hirtus* luciferase and its C311T, C311A, and T345I mutants are categorized in group (3) in Table 3. In these luciferases, the excited states of oxyluciferin phenolate and NH_2OL experience only a polar P-type binding environment (Figure 6) with a polarity similar to different alcohol solvations, emitting orange-red light. The pH-independent character of these luciferases with D-LH_2 ($n \ll p$) would result from the absence of a pH-sensitive moiety that could stabilize a more closed apolar N-type conformation. In the active site of the red-emitting *Zophobas* luciferase-like enzyme, the excited states of oxyluciferin phenolate and NH_2OL are also found in the P-type binding conformation, but their positions could be slightly different.

Currently, we cannot assertively identify the basic groups involved in phenol stabilization. In enzyme active sites, the basic moieties could be represented by the neutral form of imidazoles from histidines, guanidine groups from arginines, amino groups of lysines, and backbone amide carbonyls. On the other hand, the acidic moieties are represented by carboxylic acid groups of aspartic and glutamic acids, sulfhydryl groups of cysteines, phenolic hydroxy groups of tyrosines, and hydroxy groups of serines. Practically, near physiological pH, the basic moieties, with the exception of histidine imidazoles, will be in their conjugate acid forms such as ammonium and guanidinium, and the acidic moieties will be in their conjugate base forms such as carboxylates and phenolate. Of course, the microenvironment of the active site can considerably modulate the acidity of these groups. A prospect of the beetle luciferase active sites based on the three-dimensional crystal structures of *L. cruciata* and *P. pyralis* luciferases with DLSA[26,55] and modeling studies of *P. pyralis* luciferase[27,28] show that the following acid−base groups surround the oxyluciferin phenolate: guanidine of R218,[39] hydroxyl from Y257,[56] carboxylic acid of E309,[35,44] backbone amide carbonyl of T/C311 (S314),[18] and guanidine of R337.[28] Arginine residues R218 (R218) and R337 (R339) of *P. pyralis* luciferase (*L. cruciata* luciferase) are located close to the benzothiazole ring of luciferin, and their main chains may work as putative bases. A salt bridge between E309 (E311) and R337 (R339) (Figure 8) was postulated to stabilize a closed conformation of the active site that would be favorable for green light emission.[26,35] One possibility is that the basic moieties at (A) and (B) of *P. pyralis* luciferase (Figure 6) could be the guanidine groups of R218 and R337, respectively, and the corresponding acid for (A) could be a water molecule and for (B) the carboxylic acid group of E309 (E311). The main chain carbonyl oxygen of C/T311 (S314 in firefly luciferases) may assist the basic function of R218. Indeed, the planes of the acid−base moieties at (A) and (B) are almost perpendicular to each other in relation to the oxyluciferin phenolic OH group: the R218 guanidine group and the C/T311 carbonyl face the oxyluciferin phenolate at opposite sides in one plane, whereas the R337 and E309 functional groups face the oxyluciferin phenolate at a different angle. R218 has been previously reported to be important for green bioluminescence in *Ph. vivianii* railroad worm and *P. pyralis* firefly luciferase, whereas it is not essential for red bioluminescence in *Ph. hirtus* red-emitting luciferase.[53] In

dx.doi.org/10.1021/bi500160m | *Biochemistry* 2014, 53, 5208−5220

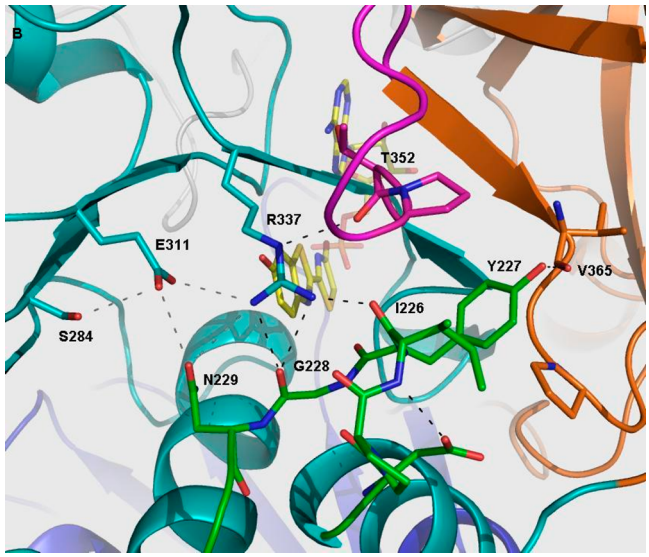


Figure 8. Model of *Macrolampis* sp_2 firefly luciferase showing the benzothiazolyl side of the luciferin binding site and the putative pH-sensing moiety, involving the loop of residues 223−235 (green) and its interactions with residues E311 (E309) and R337, which make a salt bridge that keeps a closed active site conformation and may also work as acidic and basic moieties for oxyluciferin phenolate (yellow) (according to Viviani et al., 2008[42]).

support of the importance of an arginine for green bioluminescence, it was shown that guanidine blue-shifts the spectrum of *Phrixotrix* red-emitting luciferase to orange and additionally partially rescues green emission in the orange-emitting R215S mutant of *Ph. vivianii* luciferase.[38,40] When R218 works as a basic moiety at (A) by the interaction with oxyluciferin phenolate, the salt bridge between R337 and E309 at (B) may stabilize a closed hydrophobic conformation of the active site that would be favorable for green light emission[26,35]- (Figure 6). While R218 is invariant in beetle luciferases, the active site models show that its guanidine group is located ∼6 Å from phenolate. Thus, R218 could not be easily associated with green and red bioluminescence, if one does not assume conformational changes that affect the position of the guanidinium ion in relation to the excited oxyluciferin phenolate or the mediation of a water molecule. A water molecule has indeed been proposed and shown to mediate an interaction among R218, N229, and phenolate.[40,57] On the other hand, the guanidine side chain of R337 (*P. pyralis* luciferase), which is normally involved in the active site-stabilizing interaction with E309, could be a candidate for the basic moiety at (B). In the case of disruption of the salt bridge between E309 and R337 by pH-mediated or other structural changes, the excited oxyluciferin phenolate may switch its interaction to the basic moiety (B) in a P-type polar environment arising from the active site opening, resulting in red emission. In the case of the bioluminescence of NH_2LH, E309 could work as the interacting acidic moiety (B), also promoting a switch to a P-type binding conformation of the excited NH_2OL (Figure 6), in a manner independent of pH. These acid−base interactions of the excited oxyluciferin phenolate and aminooxyluciferin with R337 and E309, respectively, weaken the salt bridge of R337 and E309 at (B), resulting in an open P-type binding conformation of the active site.

CONCLUDING REMARKS

Through the use of NH_2LH and its 5,5-dimethyl analogue Me_2NH_2LH with a set of beetle luciferases spanning the entire bioluminescence spectrum of beetle bioluminescence, we revealed compelling evidence that the emitter in beetle bioluminescence is always in the keto form and that bioluminescence colors are essentially determined by interactions of the 6′-hydroxy group of the excited OLH with basic and acidic groups under the influence of the surrounding polarity of the luciferin binding site of different luciferases. Accordingly, a closed apolar N-type binding site conformation that favors a strong interaction of excited oxyluciferin phenol with a basic group is responsible for green-yellow light emission, whereas a more polar P-type binding conformation would be responsible for orange-red light emission. The presence of basic and acidic residues is essential not only for binding D-LH_2 and aminoluciferins but also for making salt bridges that stabilize closed apolar N-type active site conformations. In pH-sensitive firefly luciferases at alkaline pH, a closed hydrophobic N-type conformation is stabilized by a pH-sensitive moiety, promoting a strong interaction between excited oxyluciferin phenol and a basic group, whereas at acidic pH, the breakdown of the N-type active site stabilizing interactions will promote a switch to a more open and polar P-type conformation, displacing oxyluciferin phenolate to another weaker base interaction, resulting in red light emission. In pH-insensitive luciferases, only a single type of conformation is found: a rigid closed apolar N-type conformation for yellow-green-emitting luciferases or a polar P-type conformation for orange-red-emitting luciferases. In these luciferases, intermediate color variations from green to yellow and from orange to red can be explained by solvatochromic effects and also cavity size variations of these two conformations.

AUTHOR INFORMATION

Corresponding Author

*Graduate Program of Biotechnology and Environmental Monitoring, Department of Physics, Chemistry and Mathematics, Federal University of São Carlos (UFSCAR), Rodovia JoãoLeme dos Santos, km 110, Itinga, Sorocaba, SP, Brazil. Telephone: 55-15-32295983. E-mail: viviani@ufscar.br.

Funding

V.R.V. is grateful fo financial support from the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP Grants 2012/04857-0 and 2011/23961-0) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). T.H. is grateful for financial support for this work from a Grant-in-Aid for Scientific Research C (22550031) from the Japan Society for the Promotion of Science, the Iketani Science and Technology Foundation, and the Izumi Science and Technology Foundation.

Notes

The authors declare no competing financial interest.

DEDICATION

D.R.N. dedicates the manuscript to the memory of Luzineti do Nascimento Neves. T.H. dedicates the manuscript to the memory of Kiichi Hirano.

REFERENCES

(1) Viviani, V. R. (2002) The origin, diversity and structure function relationships of insect luciferases. *Cell. Mol. Life Sci. 59*, 1833−1850.

(2) Seliger, H. H., and McElroy, W. D. (1964) The colors of firefly bioluminescence: Enzyme configuration and species specificity. *Proc. Natl. Acad. Sci. U.S.A. 52*, 75−81.
(3) Viviani, V. R., and Bechara, E. J. H. (1995) Bioluminescence of Brazilian fireflies (Coleoptera: Lampyridae): Spectral distribution and pH effect on luciferase-elicited colors. Comparison with elaterid and phengodid luciferases. *Photochem. Photobiol. 62*, 490−495.
(4) Roda, A., Pasini, P., Mirasole, M., Michenini, E., and Guardigli, M. (2004) Biotechnological application of bioluminescence and chemiluminescence. *Trends Biotechnol. 22*, 295−303.
(5) Viviani, V. R., and Ohmiya, Y. (2006) Beetle luciferases: Colorful lights on biological processes and diseases. *Photoproteins in Bioanalysis*, Chapter 3, pp 49−63, Wiley, New York.
(6) Nakajima, Y., Yamazaki, T., Nishii, S., Noguchi, T., Hoshino, H., Niwa, K., Viviani, V. R., and Ohmiya, Y. (2010) Enhanced beetle luciferase for high-resolution bioluminescence imaging. *PLoS One 5* (4), 10011.
(7) Wood, K. V. (1995) The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol. 62*, 662−673.
(8) Hosseinkhani, S. (2011) Molecular enigma of multicolor bioluminescence of firefly luciferase. *Cell. Mol. Life Sci. 68*, 1167−1182.
(9) White, E. H., Rapaport, E., Hopkins, T. A., and Seliger, H. H. (1969) Chemi- and bioluminescence of firefly luciferin. *J. Am. Chem. Soc. 91*, 2178−2180.
(10) DeLuca, M. (1969) Hydrophobic nature of the active site of firefly luciferase. *Biochemistry 8*, 160−166.
(11) Ugarova, N. N., and Brovko, L. Y. (2002) Protein structure and bioluiminescence spectra for firefly bioluminescence. *Luminescence 17*, 321−330.
(12) Hirano, T., Hasumi, Y., Ohtsuka, K., Maki, S., Niwa, H., Yamaji, M., and Hashizume, D. (2009) Spectroscopic studies of the light-color modulation mechanism of firefly (beetle) bioluminescence. *J. Am. Chem. Soc. 131*, 2385−2396.
(13) Naumov, P., Ozawa, Y., Ohkubo, K., and Fukuzumi, S. (2009) Strucuture and spectroscopy of oxyluciferin, the light emitter of the firefly bioluminescence. *J. Am. Chem. Soc. 131*, 11590−11605.
(14) 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.
(15) McCapra, F., Gilfoyle, D. J., Young, D. W., Church, N. J., and Spencer, P. (1994) The chemical origin of color differences in beetle bioluminescence. In *Bioluminescence and Chemiluminescence: Fundamental and Applied Aspects* (Campbell, A. K., Kricka, L. J., and Stanley, P. E., Eds.) pp 387−391, John Wiley & Sons, Chichester, U.K.
(16) Orlova, G., Goddard, J. D., and Brovko, L. Y. (2003) Theoretical study of the amazing firefly bioluminescence: The formation and structure of the light emitters. *J. Am. Chem. Soc. 125*, 6962−6971.
(17) Branchini, B. R., Southworth, T. L., Murtishaw, M. H., Magyar, R. A., Gonzales, S. A., Ruggiero, M. C., and Stroh, J. G. (2004) An alternative mechanism of bioluminescence color determination in firefly luciferase. *Biochemistry 43*, 7255−7262.
(18) Viviani, V. R., Amaral, D. T., Neves, D. R., Simões, A., and Arnoldi, F. G. C. (2013) The luciferin binding site residues C/T311 (S314) influence the bioluminescence color of beetle luciferases through main-chain interaction with oxyluciferin phenolate. *Biochemistry 52*, 19−27.
(19) De Wet, J. R., Wood, K. V., Helinsky, D. R., and DeLuca, M. (1985) Cloning of firefly luciferase cDNA and expression of active luciferase in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 82*, 7870−7873.
(20) Tatsumi, H., Masuda, T., Kajiyama, N., and Nakano, E. (1989) Luciferase cDNA from Japanese firefly *Luciola lateralis*. *Biochim. Biophys. Acta 1131*, 161−165.
(21) Devine, J. H., Kutuzova, G. D., Green, V. A., Ugarova, N. N., and Baldwin, T. O. (1993) Luciferase from the East European firefly *Luciola mingrelica*: Cloning and nucleotide sequence of cDNA, overexpression in *E. coli* and purification of the enzyme. *Biochim. Biophys. Acta 1173*, 121−132.
(22) Ohmiya, Y., Ohba, N., Toh, H., and Tsuji, F. I. (1995) Cloning, expression and sequence analysis of cDNA for the Japanese fireflies, *Pyrocoelia miyako* and *Hotaria parvula*. *Photochem. Photobiol. 62*, 309−313.
(23) Sala-Newby, G. B., Thomson, C. M., and Campbell, A. K. (1996) Sequence and biochemical similarities between the luciferases of the glow-worm *Lampyris noctiluca* and the firefly *Photinus pyralis*. *Biochem. J. 313*, 761−767.
(24) Li, Y., Buck, L. M., Scaeffer, H. J., and Leach, F. R. (1997) Cloning and sequencing of a cDNA for the firefly luciferase from *Photuris pennsilvanica*. *Biochim. Biophys. Acta 1339*, 39−52.
(25) 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.
(26) Nakatsu, T., Ichiyama, S., Hiratake, J., Saldanha, A., Kobayashi, N., Sakata, K., and Kato, H. (2006) Structural basis for the spectral difference in luciferase bioluminescence. *Nature 13*, 372−376.
(27) 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.
(28) Sandalova, T. P., and Ugarova, N. N. (1999) Model of the active site of firefly luciferase. *Biochemistry (Moscow) 64*, 962−967.
(29) Branchini, B. R., Southworth, T. L., Murtiashaw, M. H., Boije, H., and Fleet, S. E. (2003) A mutagenesis study of the luciferin binding site residues of firefly luciferase. *Biochemistry 42*, 10429−10436.
(30) 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. Sci. 70*, 254−260.
(31) Amaral, D. T., Prado, R. A., and Viviani, V. R. (2012) Luciferase from *Fulgeochlizus bruchi* (Coleoptera: Elateridae), a Brazilian click-beetle with a single abdominal lantern: Molecular evolution, biological function and comparison with other click-beetle luciferases. *Photochem. Photobiol. Sci. 11*, 1259−1267.
(32) Viviani, V. R., Bechara, E. J. H., and Ohmyia, Y. (1999) Cloning, sequence analysis and expression of active *Phrixothrix* railroad-worms luciferases: Relationship between bioluminescence spectra and primary structures. *Biochemistry 38*, 8271−8279.
(33) Arnoldi, F. G., da Silva Neto, A. J., and Viviani, V. R. (2010) Molecular insights on the evolution of the lateral and head lantern luciferases and bioluminescence colors in Mastinocerini railroad-worms (Coleoptera: Phengodidae). *Photochem. Photobiol. Sci. 9*, 87−92.
(34) Viviani, V. R., Arnoldi, F. G. C., Brochetto-Braga, M. R., and Ohmiya, Y. (2004) Cloning and characterization of the cDNA for the Brazilian *Cratomorphus distinctus* larval firefly luciferase: Similarities with European *Lampyris noctiluca* and Asiatic *Pyrocoelia* luciferases. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 139*, 151−156.
(35) Viviani, V. R., Oehlmeyer, T. L., Arnoldi, F. G. C., and Brochetto-Braga, M. R. (2005) A new firefly luciferase with bimodal spectrum: Identification of structural determinants of spectral pH-sensitivity in firefly luciferases. *Photochem. Photobiol. Sci. 81*, 843−848.
(36) Viviani, V. R., Amaral, D. T., Prado, R. A., and Arnoldi, F. G. C. (2011) A new blue-shifted luciferase from the Brazilian *Amydetes fanestratus* (Coleoptera: Lampyridae) firefly: Molecular evolution and structural/functional properties. *Photochem. Photobiol. Sci. 10*, 1879−1886.
(37) Kajiyama, N., and Nakano, E. (1991) Isolation and characterization of mutants of firefly luciferase which produce different colors of light. *Protein Eng. 4*, 691−693.
(38) Viviani, V. R., and Ohmiya, Y. (2000) Bioluminescence color determinants of *Phrixothrix* railroadworm luciferases: Chimeric luciferases, site-directed mutagenesis of Arg215 and guanidine effect. *Photochem. Photobiol. 72*, 267−271.

(39) Viviani, V. R., Arnoldi, F. G. C., Venkatesh, B., Neto, A. J. S., Ogawa, F. G. T., Oehlmeyer, A. T. L., and Ohmiya, Y. (2006) Active-site properties of *Phrixothrix* railroad worm green and red bioluminescence-eliciting luciferases. *J. Biochem. 140*, 467−474.
(40) Viviani, V. R., Uchida, A., Viviani, W., and Ohmiya, Y. (2002) The influence of Ala243(Gly247), Arg 215 and Thr226(Asn230) on the bioluminescence spectra and pH-sensitivity of railroad worm, click beetle and firefly luciferases. *Photochem. Photobiol. 76*, 538−544.
(41) Viviani, V. R., Silva Neto, A. J., Arnoldi, F. G., Barbosa, J. A., and Ohmiya, Y. (2007) The influence of the loop between residues 223−235 in beetle luciferases bioluminescence spectra: A solvent gate for the active site of pH-sensitive luciferases. *Photochem. Photobiol. 84*, 138−144.
(42) Viviani, V. R., Arnoldi, F. G., Silva Neto, A. J., Oehlmeyer, T. L., Bechara, E. J. H., and Ohmiya, Y. (2008) The structural origin and biological function of pH-sensitivity in firefly luciferases. *Photochem. Photobiol. Sci. 7*, 159−169.
(43) Viviani, V. R., Uchida, A., Suenaga, N., Ryufuku, M., and Ohmiya, Y. (2001) Thr226 is a key-residue for bioluminescence spectra determination in beetle luciferases. *Biochem. Biophys. Res. Commun. 280*, 1286−1291.
(44) Moradi, A., Hosseinkhani, S., Naderi-Manesh, H., Sadeghizadeh, M., and Alipour, B. A. (2009) Effect of charge distribution in a flexible loop on the bioluminescence color of firefly luciferase. *Biochemistry 48*, 575−582.
(45) White, E. H., Worther, H., Seliger, H. H., and McElroy, W. D. (1966) Amino-analogs of firefly luciferin and biological activity thereof. *J. Am. Chem. Soc. 88*, 2015−2019.
(46) Hirano, T., Nagai, H., Matsuhashi, T., Hasumi, Y., Iwano, S., Ito, K., Maki, S., Niwa, H., and Viviani, V. R. (2012) Spectroscopic studies of the color modulation mechanism of firefly bioluminescence with amino-analogs of luciferin and oxyluciferin. *Photochem. Photobiol. Sci. 11*, 1281−1284.
(47) Reddy, G. R., Thompson, W. C., and Miller, S. C. (2010) Robust light emission from cyclic alkylaminoluciferin substrates for firefly luciferase. *J. Am. Chem. Soc. 132*, 13586−13587.
(48) Takakura, H., Kojima, R., Urano, Y., Terai, T., Hanaoka, K., and Nagano, T. (2011) Aminoluciferins as functional bioluminogenic substrates of firefly luciferase. *Chem.—Asian J. 6*, 1800−1810.
(49) Sali, A., and Blundell, T. L. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol. 234*, 779−815.
(50) DeLano, W. L. (2002) *The Pymol Molecular Graphics System*, DeLano Scientific, San Carlos, CA.
(51) Hansch, C., Leo, A., and Taft, R. W. (1991) A survey of Hammett substituent constants and resonance and field parameters. *Chem. Rev. 91*, 165−195.
(52) Viviani, V. R., Scorsato, V., Prado, R. A., Pereira, J. G., Niwa, K., Ohmiya, Y., and Barbosa, J. A. (2010) The origin of luciferase activity in *Zophobas* mealworm AMP/CoA-ligase (protoluciferase): Luciferin stereoselectivity as a switch for the oxygenase activity. *Photochem. Photobiol. Sci. 9*, 1111−1119.
(53) Viviani, V. R., and Ohmiya, Y. (2006) Bovine serum albumin displays luciferase-like activity in presence of luciferyl-adenylate: Insights on the origin of protoluciferase activity and bioluminescence colors. *Luminescence 21*, 262−267.
(54) Ando, Y., Niwa, K., Yamada, N., Irie, T., Enomoto, T., Kubota, H., Ohmiya, Y., and Akiyama, H. (2008) Firefly bioluminescence quantum yield and color change by pH-sensitive green emission. *Nat. Photonics 2*, 44−47.
(55) Sundlov, J. A., Fontaine, D. M., Southworth, T. L., Branchini, B. R., and Gulick, A. M. (2012) Crystal structure of firefly luciferase in a second catalytic conformation supports a domain alternation mechanism. *Biochemistry 51*, 6493−6495.
(56) Wang, Y., Akiyama, H., Terakada, K., and Nakastu, T. (2013) Impact of site directed mutant luciferase on quantitative green and orange/red emission intensities in firefly bioluminescence. *Sci. Rep. 3*, 1−6.
(57) Kato, D., Kubo, T., Maenaka, K., Niwa, K., Ohmiya, Y., Takeo, M., and Negoro, S. (2012) Confirmation of color determination factors for Ser286 derivatives of firefly luciferase from *Luciola cruciata* (LUC-G). *J. Mol. Catal. B: Enzym. 87*, 18−23.