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The Luciferin Binding Site Residues C/T311 (S314) Influence the Bioluminescence Color of Beetle Luciferases through Main-Chain Interaction with Oxyluciferin Phenolate

V. R. Viviani,*[,†,‡] D. T. Amaral,[†,‡] D. R. Neves,[†] A. Simões,[†] and F. G. C. Arnoldi[§]

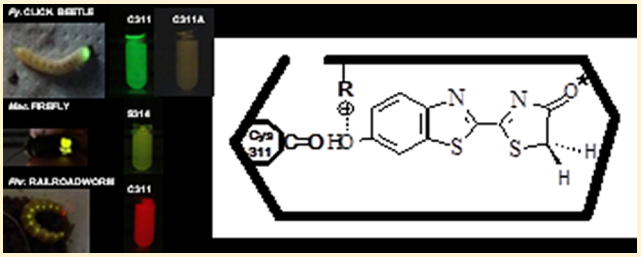
[†]Department of Physics, Chemistry and Mathematics, Graduate Program of Biotechnology and Environmental Monitoring, Federal University of São Carlos (UFSCAR), Rodovia João Leme dos Santos, km 110, Itinga, Sorocaba, SP, Brazil

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

[§]Ribeirão Preto School of Medicine, São Paulo University, Ribeirão Preto, São Paulo, Brazil

Supporting Information

ABSTRACT: Beetle luciferases emit different bioluminescence colors from green to red; however, no clear relationship between the identity of the luciferin binding site residues and bioluminescence colors was found in different luciferases, and it is unclear whether critical interactions affecting emission spectra occur on the thiazolyl or on the benzothiazolyl sides of the luciferin binding site. Through homology modeling and site-directed mutagenesis using our multicolor set of beetle luciferases (*Pyrearinus termitilluminans* larval click beetle, Pte, λ_{max} = 534 nm; *Phrixothrix hirtus* railroad worm red emitting, PxRE, λ_{max} = 623 nm; and *Macrolampis* sp2 firefly, Mac, λ_{max} = 564 nm), we show that the residues C/T311 (S314) play an important role in bioluminescence color determination. Modeling studies indicate that the main-chain carbonyls of these residues are close to both oxyluciferin phenolate and AMP, whereas the side chains pack against second-shell residues. The C311(S314)A mutation considerably red shifts the spectra of the green-yellow-emitting luciferases (Pte λ_{max} = 534 to 590 nm; Mac λ_{max} = 564 to 583/613 nm) and affects the K_M values for luciferin and ATP, but not the spectrum of the red-emitting luciferase. On the other hand, whereas the exchange between C/T311 (S314) caused smaller effects on the emission spectra of green-yellow-emitting luciferases, the C311T substitution (naturally found in green-emitting railroad worm luciferases) resulted in the largest reported blue shift in *P. hirtus* red-emitting luciferase (λ_{max} = 623 to 606 nm). Altogether, these results indicate that the stability of residues C/T311 (S314) and the size of the cavity around oxyluciferin phenolate affect bioluminescence colors and suggest, for the first time, the occurrence of a critical interaction between main-chain carbonyls of position 311 (314) residues and oxyluciferin phenolate.



Beetle luciferases emit a wide range of bioluminescence colors, from green to red.[1,2] They were originally grouped into pH-sensitive (fireflies) and pH-insensitive (click beetles and railroad worms) classes, according to the sensitivity of their bioluminescence spectra to factors such as pH, temperature, and other denaturing conditions.[3] However, recent studies with click beetle luciferases show that at higher pH values, click beetle luciferases may also undergo a slight red shift.[4,5] During the past decades, beetle luciferases gained a wide variety of applications as bioanalytical reagents, as reporter genes for investigating gene expression, and as cell and tissue markers.[6,7]

Several beetle luciferases have been cloned,[8−18] most of them from fireflies. The three-dimensional structure has been determined for the North American firefly luciferase *Photinus pyralis* (Ppy) in the absence of substrates[19] and more recently for the Japanese *Luciola cruciata* (Lcr) firefly luciferase in the presence of either the luciferyl-adenylate analogue DLSA or oxyluciferin and AMP,[20] showing closed and open active site conformations with the former analogue and the latter products, respectively. Luciferin binding site residues in firefly luciferases were identified in the three-dimensional structures,[19,20] through modeling studies,[21,22] and via site-directed mutagenesis.[21−24]

Three basic mechanisms were proposed to explain bioluminescence color determination by the luciferase active site:[25] (I) nonspecific solvent effects,[25,27] (II) specific interactions of active site residues with excited oxyluciferin,[28] and (III) the geometry of the active site affecting the rotation of thiazinic rings of excited oxyluciferin.[29] Recent theoretical and experimental studies support both nonspecific solvent and orientation polarizability effects and specific base effects.[25,30,31] Although the identity of the emitters in firefly luciferase bioluminescence had not yet been determined, recent studies agree that the keto form of excited oxyluciferin must be the

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most likely basic structure, and that the polarization state of the 6′-phenol group may influence conjugation of oxyluciferin providing a variety of colors.[26,31] However, it is still unclear whether specific interactions affecting bioluminescence colors occur mainly on the thiazolyl or on the benzothiazolyl sides of the luciferin binding site. Furthermore, no clear relationship between the physicochemical properties of the luciferin binding site residues and bioluminescence colors in different luciferases has been established.

Recently, our group has cloned several new beetle luciferases with distinct bioluminescence properties[5,32−36] from click beetles, railroad worms, and fireflies and comparatively investigated their properties. Among them, *Phrixothrix* spp. railroad worm luciferases display green and red bioluminescence;[33] the larval click beetle *Pyrearinus termitilluminans* luciferase displays the most blue-shifted color among beetle luciferases and is pH-insensitive,[32] and *Macrolampis* firefly luciferase displays a broad pH-sensitive spectrum in the yellow-green region.[35] Some of these luciferases are gaining increasing application as multicolor reporter assays in mammalian cells. The luciferases from *Phrixothrix* railroad worms and *Py. termitilluminans* were purified and characterized.[4,37] Some residues affecting bioluminescence colors were identified by site-directed mutagenesis.[38−42] Among the luciferin binding site residues, the invariant R215 was the one whose mutation most dramatically affected the bioluminescence spectrum of *Phrixothrix vivianii* green-emitting luciferases,[38] whereas other residues important for bioluminescence colors were found in the loop of residues 223−235, mainly F/Y224, G228, and T/N226,[41,42] and interacting regions. Modeling studies with *Phrixothrix*, *Pyrearinus termitilluminans*, and *Macrolampis* luciferases[4,37] showed that the variable position represented by residues C311, T311, and S314, respectively, is located close to the luciferin phenolate and could be an important candidate in bioluminescence color determination. Therefore, we comparatively investigated the effect of this position in different beetle luciferases through site-directed mutagenesis, showing that residues at this position indeed play a critical role in bioluminescence color determination.

MATERIALS AND METHODS

Plasmids and Beetle Luciferase cDNAs. All beetle luciferase cDNAs were previously cloned in our laboratories.[32,33,35] The cDNAs for *Py. termitilluminans* (Pte) click beetle, *Phrixothrix hirtus* red-emitting luciferases, and *Zophobas* mealworm protoluciferase were subcloned into the pCold vector (Takara), and the cDNA of *Macrolampis* luciferase was subcloned into the pPro vector and expressed in *Escherichia coli* BL21-DE3 cells.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using a Stratagene mutagenesis kit (catalog no. 200518). The plasmids containing the luciferase cDNAs were amplified using *Pfu* turbo polymerase and two complementary primers containing the desired mutation, using a thermal cycler (one cycle at 95 °C, 25 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 7 min). After amplification, mutated plasmids containing staggered nicks were generated. The products were treated with *Dpn*I in order to digest nonmutated parental plasmids and used directly to transform *E. coli* XL1-Blue cells. The following primers and their respective reverse complements were used: **Pte C311A**, GGC GGA GCT GTG CGC CGG AGC CGC TCC; **Pte C311S**, GCG GAG CTG TGC AGT GGA GCC GCT CC; **PxRe C311T**, CAG AGA TCG CCT CTG GCG GCT CCC C; and **PxRE C311A**, CAG AGA TCG CCG CTG GCG GCT CCC C; and **Mac S314A**, CAC GAA ATT GCT GCC TGG TGG TGG CGC GCC: **Mac S314C**, CGAAATTGCTTGTGGTGGCGCGC; and **Zop T306C**, CGTTGTTTACTGTGTGGCGGTACTCC.

Luciferase Expression and Purification. For luciferase expression, transformed *E. coli* BL21-DE3 cells were grown in 500−1000 mL of LB medium at 37 °C up to an OD_{600} of 0.4 and then induced at 18 °C with 0.4 mM IPTG overnight. Cells were harvested by centrifugation at 2500*g* for 15 min 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 (pH 7.0) (Roche), lysed by ultrasonication or a French press, and centrifuged at 15000*g* for 15 min at 4 °C. The N-terminally histidine-tagged *Py. termitilluminans*, *Phrixothrix* recombinant luciferases, and *Zophobas* luciferase-like enzyme were further purified by agarose-nickel affinity chromatography followed by dialysis. Samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Western blotting using primary polyclonal antibodies raised against *Py. termitilluminans* and *P. hirtus* railroad worm luciferases and an anti-rabbit secondary antibody employing the ECL Western Blotting detection kit (GE Healthcare).

Measurement of Luciferase Activity. Luciferase bioluminescence intensities were measured using TD3000III and AB2200 (ATTO, Tokyo, Japan) luminometers. The assays were performed by mixing 5 μL of 40 mM ATP and 80 mM $MgSO_4$ with a solution consisting of 10 μL of luciferase and 85 μL of 0.5 mM luciferin in 0.10 M Tris-HCl (pH 8.0) at 22 °C. All assays were performed in triplicate.

Kinetic Measurements and K_M Determination. The K_M assays for luciferin were performed by mixing 5 μL of 40 mM ATP and 80 mM $MgSO_4$ in a solution containing 10 μL of luciferase, 75 μL of 0.10 M Tris-HCl (pH 8.0), and luciferin at final concentrations between 0.01 and 1 mM. The K_M assays for ATP were performed by mixing 5 μL of 80 mM $MgSO_4$ in a solution containing 10 μL of luciferase, 75 μL of 0.10 M Tris-HCl (pH 8.0), and ATP at final concentrations in the range of 0.02−2 mM. Both assays were performed in triplicate. The K_M values were calculated using Lineweaver−Burk plots taking the peak of intensity (I_0) as a measure of V_0. The effect of pH on the activity was assayed in 0.10 M citrate (pH 5−6.0), phosphate (pH 6−8.0), Tris-HCl (pH 7.5−8.5), and CAPS (pH 8.5−10.0) buffers.

Bioluminescence Spectra. Bioluminescence spectra were recorded in a Hitachi F4500 spectrofluorometer. Scans were run between 450 and 700 nm at a speed of 2400 nm/min after the ATP had been mixed with the assay solution for 30 s, when there is a negligible decay of emission. The spectra were automatically corrected for the spectral sensitivity of the equipment. For the in vitro bioluminescence, 50 μL of crude extracts was mixed with 450 μL of the assay solution [0.5 mM luciferin, 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).

Bioinformatics and Homology Modeling. Homology-based models of *Py. termitilluminans*, *P. hirtus*, *P. viviani*. and *Macrolampis* sp2 luciferases and their respective mutants were constructed using as a template the three-dimensional structure of *L. cruciata* luciferase in the closed conformation in the presence of DLSA (Protein Data Bank entry 2D1S).[19] Modeler

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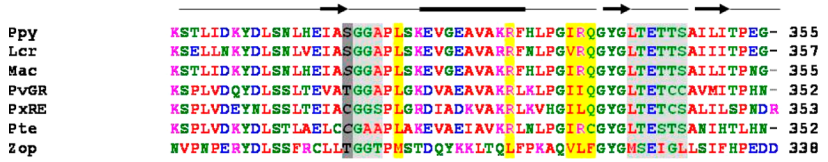

Figure 1. Multisequence alignement of the luciferin binding site module ^{314}SGGA^{317} and interacting residues in different beetle luciferases: luciferin binding site motifs highlighted in gray, residues C/T311 (S314 in *Macrolampis* firefly luciferase) under investigation highlighted in dark gray, residues interacting with luciferin binding site residues highlighted in yellow, hydrophobic residues colored red, polar residues colored green, positively charged residues colored pink and negatively charged residues colored blue.

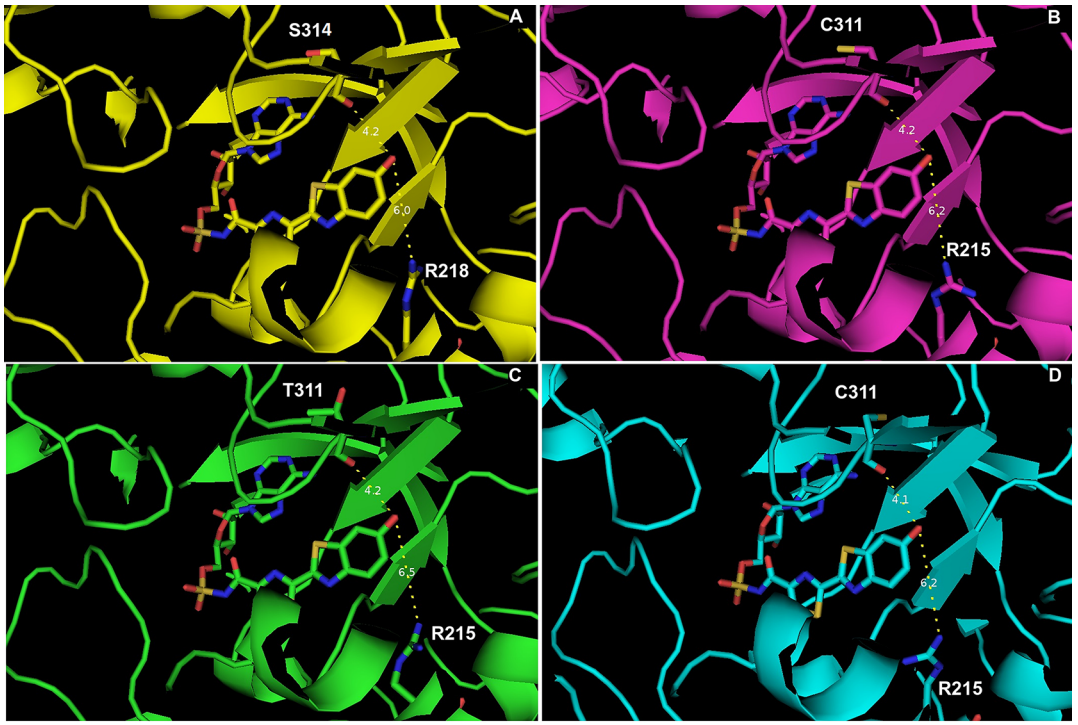

Figure 2. Homology models of the benzothiazolyl side of the luciferin binding site of beetle luciferases showing oxyluciferin phenolate and its distances from main-chain carbonyls of residues at positions 311 and 314 (corresponding to position 311 in click beetle and railroad worm luciferases and position 314 in *Macrolampis* firefly luciferase) and the guanidine group of R215 (R218 in *Macrolampis* luciferase): (A) *Macrolampis* sp2 firefly luciferase, (B) *P. hirtus* railroad worm red-emitting luciferase, (C) *P. viviani* railroad worm green-emitting luciferase, and (D) *Py. termitilluminans* larval click beetle green-emitting luciferase.

version 9.9 was used to align the sequences (using the align2d function) and to construct 200 three-dimensional models of each sequence.[43] Visualization and analyses of the best model of each luciferase were performed using PyMol version 1.4.1.[44]

RESULTS

Multialignment and Rationale behind Mutagenesis. The ^{314}S(C/T)GG(A)^{316} motif (*Ph. pyralis* luciferase) constitutes a mobile loop in the confluence of the luciferin and AMP binding sites.[21] Previous studies by Branchini et al. showed that mutations of G315 and G316 affect bioluminescence spectra and luciferin affinity, being located close to the benzothiazolic moiety of luciferin. In the same loop, position 314 of firefly luciferases displays serine, the green-emitting *P. vivianii* and *Rhagophthalmus ohbai* railroad worm luciferases display the conserved threonine, whereas *P. hirtus* red-emitting and click beetle luciferases display cysteine (Figure 1). Furthermore, the *Zophobas morio* mealworm luciferase-like enzyme also displays threonine at this position. Therefore, we performed site-directed mutagenesis, investigating the effect of Ala and of the exchange among the natural residues cysteine, threonine, and serine at this position using *Py. termitilluminans* green-emitting pH-insensitive luciferase, *P. hirtus* red-emitting pH-insensitive luciferase, and *Macrolampis* yellow-green pH-sensitive luciferase.

Modeling Studies. According to modeling studies, the residues at position 311 (316 in *L. cruciata* firefly, 314 in *Ph. pyralis* and *Macrolampis* sp2 fireflies, and 311 in click beetles and railroad worms) are located close to luciferin phenolate (Figure 2). In all cases, it is not the side chain of residue 311 (314) that faces oxyluciferin phenolate, but the main-chain carbonyls. Additionally, the main-chain carbonyls of these residues are also located close to the adenine amino group of AMP (within 4.8 Å). The guanidine group of the invariant residue R218 (R215 in click beetle and railroad worm luciferases) is 6−7 Å from oxyluciferin phenolate, in the opposite direction of residue 311 (314) main-chain carbonyls (Figure 2).

Figure 3 shows the residues surrounding the side chains of residue 311 (314) in the wild-type and mutant luciferases. The calculated distances between the side chains of residue 311 (314) and the closest atoms from the side chains of

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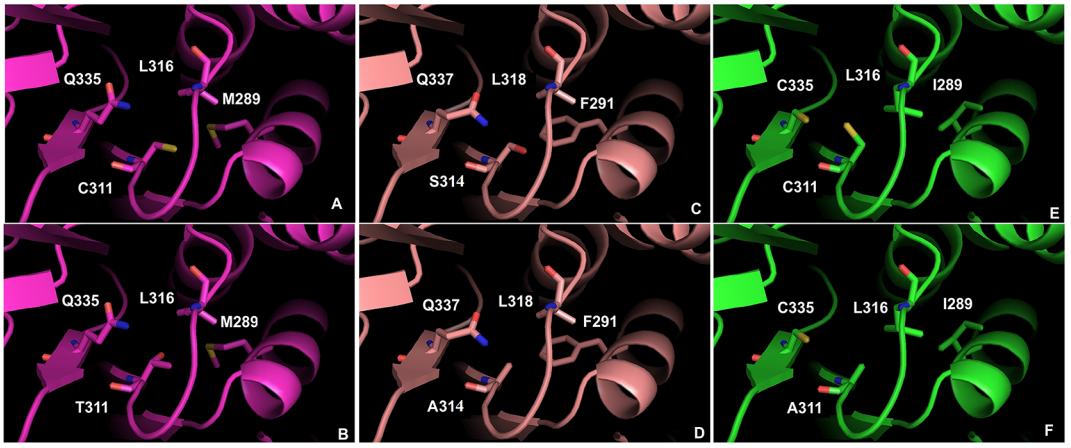

Figure 3. Homology models showing the surrounding environment of residue 311 (314) side chains in wild-type and residue 311 (314) mutant luciferases: (A) *P. hirtus* wild-type railroad worm luciferase, (B) *P. hirtus* C311T mutant luciferase, (C) *Macrolampis* sp2 wild-type firefly luciferase, (D) *Macrolampis* sp2 S314A mutant luciferase, (E) *Py. termitilluminans* wild-type larval click beetle luciferase, and (F) *Py. termitilluminans* C311A mutant luciferase.

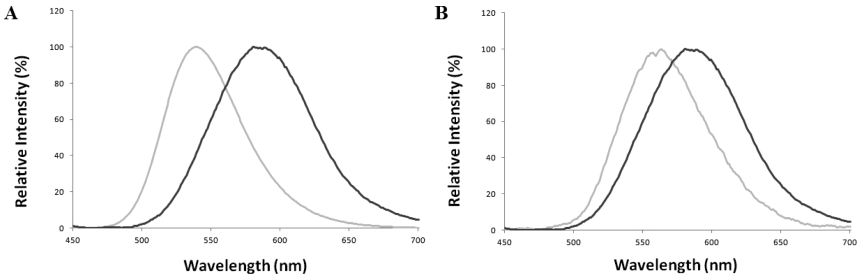

Figure 4. Bioluminescence spectra of *Py. termitilluminans* larval click beetle green-emitting luciferase and its C311A mutant: (A) wild-type luciferase (gray) and C311A mutant (black) at pH 8.0 and (B) C311A mutant at pH 8.0 (black) and pH 6.0 (gray).

surrounding residues are listed in Table S1 of the Supporting Information.

The surrounding environment of the position 311 (314) side chains is composed by the polar groups from the main chains and the predominantly hydrophobic side chains (M289, L316, and Q335 of *P. hirtus*; I289, L316, and C335 of *Pyrearinus*; and L318, F291, and Q337 of *Macrolampis*). In *P. hirtus* luciferase, the mutation of C311 to the more polar threonine is apparently stabilized by the polar interaction with Q335. On the other hand, both mutations S314A in *Macrolampis* sp2 and C311A in *Py. termitilluminans* apparently decrease the stability of the polar interactions with Q337 and C335, respectively.

Additionally, there are indications that the residue 311 (314) side chains are close to the main-chain carbonyls of residue 284 (V284 of *P. hirtus*, V284 of *P. viviani*, and L287 of *Macrolampis* sp2) (Figure S1 of the Supporting Information). In *Py. termitilluminans* luciferase, the C311 side chain is shifted to the opposite direction close to the C335 side chain (3.2 Å), indicating the possibility of a unique stabilizing disulfide bond, which may explain the more blue-shifted color emitted by this luciferase.

Pyrearinus termitilluminans Luciferase. In *Py. termitilluminans* luciferase, the C311A mutation resulted in a 56 nm red shift, which is the largest red shift ever reported for a single mutation in a click beetle luciferase (Figure 4A). Furthermore, at pH 6, the spectrum undergoes a 33 nm blue shift (Figure 4B), turning this luciferase pH-sensitive, although in the opposite direction of firefly luciferases, which display a red shift at acidic pH. The mutation also decreased the luminescence activity. The K_M for luciferin was considerably increased, the K_M for ATP slightly decreased and the optimal pH shifted from 8.0 to 8.5 (Table 1). Overall, these changes indicate that the C311A mutation affects the active site, possibly weakening the main-chain interaction with luciferin and strengthening the interaction with ATP.

Considering that some compounds can be used as chemical rescuing agents simulating lost functional groups of mutated amino acids, we tested the effect of 2-mercaptoethanol and dithiothreitol as putative sulfhydryl chemical rescuing agents of the lost cysteine in the bioluminescence spectrum of the C311A mutant. However, none of them had any effect on the spectrum, making unlikely a direct interaction between the cysteine sulfhydryl group and oxyluciferin phenolate in bioluminescence color.

Phrixotrix Railroad Worm Luciferases. Phrixotrix red-emitting luciferase also displays cysteine at position 311. As expected, the C311A mutation in this red-emitting luciferase had no effect on the spectrum (Figure 5). In this case, however, the K_M for luciferin decreased and that of ATP was slightly affected. The C311T mutation, which is naturally found in *P. vivianii* green-emitting luciferase, resulted in a 17 nm blue shift and a large broadening of the spectrum, being the largest blue shift ever reported for a single mutation in this red-emitting luciferase (Figure 5). Although the K_M for luciferin was not affected in this case, the K_M for ATP considerably decreased, suggesting that this mutation may affect ATP binding (Table S1 of the Supporting Information). In *P. vivianii* luciferase, the reverse T311C substitution was previously shown to display an

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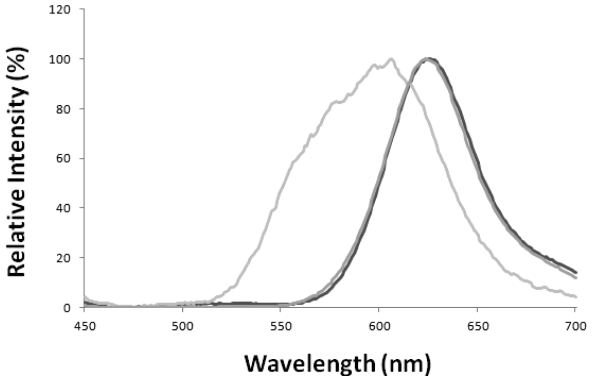

Figure 5. Bioluminescence spectra of *P. hirtus* railroad worm red-emitting luciferase (black), its C311T mutant (light gray), and its C311A mutant (dark gray).

only small red shift and broadening of the bioluminescence spectrum.

Macrolampis Firefly Luciferase. In *Macrolampis* firefly luciferase, the S314A mutation resulted in a bimodal spectrum with a broad spectrum at pH 8, indicating an increase in the red emitter population in this pH-sensitive luciferase. Similarly, the S314C mutation also resulted in a bimodal and broad spectrum (Figure 6). Both mutations had little effect on luciferin K_M but decreased the K_M for ATP, indicating an affinity increase for this latter substrate.

Luciferase-like Enzyme. We also analyzed the effect of T306C in *Zophobas* mealworm luciferase-like enzyme, which also displays a spectrum in the red (615 nm). The mutation, however, had an only modest blue shift effect on the spectrum.

To check for major conformational changes caused by the mutations, we analyzed tryptophan fluorescence spectra. All position 311 (314) mutants displayed tryptophan fluorescence spectra essentially identical to those of the respective wild-type enzymes (results not shown), indicating that the mutations did not cause considerable conformational changes in the luciferases structures, and therefore that their effect on bioluminescence spectra could be caused by rather local specific effects.

DISCUSSION

In the past 20 years, many studies that aimed to improve our understanding of the structural determinants and mechanisms of bioluminescence colors were conducted, mainly with firefly luciferases. Several mutations were found to affect bioluminescence colors, usually resulting in red-shifted mutants. Mutations affecting bioluminescence colors pertain to two main classes: those in the luciferin binding site directly affecting the interaction with the excited oxyluciferin and those spread over the three-dimensional structure indirectly affecting the emission spectra through changes in conformation.[42] Branchini et al.[21,24] investigated the effect of several mutations in the luciferin binding site of *Photinus* firefly luciferase, showing that mutation of residues R218, H245, F247, G315, G316, T343, S347, and

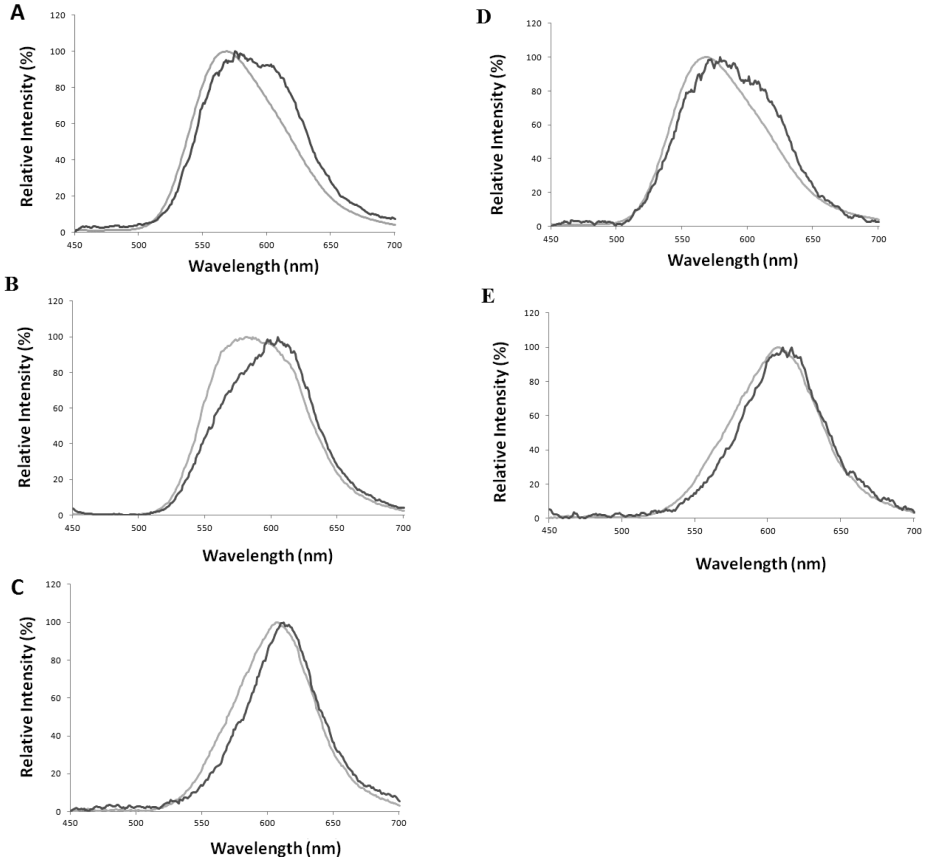

Figure 6. Bioluminescence spectra of *Macrolampis* sp2 firefly luciferase and its mutants: (A) wild type (gray) and S314C (black) at pH 8.0, (B) wild type (gray) and S314C (black) at pH 7.0, (C) wild type (gray) and S314C (black) at pH 6.0, (D) wild type (gray) and S314A (black) at pH 8.0, and (E) wild type (gray) and S314A (black) at pH 6.0.

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Table 1. Summary of the Physicochemical Properties of Beetle Luciferases and Position 311 (314 in *Macrolampis* firefly luciferase) Mutants

		λ_{max}[a] (nm)		K_M[b] (μM)		
luciferase	residue	pH 8.0	pH 6.0	luciferin	ATP	pH
Lampyridae						
Macrolampis sp2	S314	564 [88]	606 [77]	7	83	8.2
S314A	A314	583/613 [89]	613 [62]	9	26	
S314C	C314	578/600 [95]	615 [61]	7	18	
Phengodidae						
P. hirtus	C311	623 [55]	623 [62]	7	150	8.1
C311T	T311	606 [90]		7,5	25	8.1
C311A	A311	623 [54]		4	122	
P. vivianii	T311	546 [70]	546 [75]	64	330	8.1
T313C	C311	551 [93]				
Elateridae						
Py. termitilluminans	C311	534 [65]	534 [78]	80	370	8
C311A	A311	590 [92]	557 [68]	150	260	8.5
Tenebrionidae						
Zophobas luciferase-like enzyme	T306	615 [92]		860	260	8.5
T306C	C306	607 [80]				

[a]Errors associated with the emission peak of ±2.5 nm. [b]Errors associated with K_M values of ±15%.

A348 strongly affects the bioluminescence color. However, most of these luciferin binding site residues are invariant or conserved, and no direct relationship could be found between their identity and the bioluminescence colors in different beetle luciferases. Furthermore, in the pH-insensitive luciferases, few of these luciferin binding site residues were shown to drastically affect bioluminescence colors.[38−40] Only the mutations of the invariant R215 (R218 in *Ph. pyralis* luciferase) were shown to dramatically affect the emission spectra of firefly luciferase and of the green- and yellow-emitting luciferases of *Phrixothrix* railroad worms, but not those of *Phrixothrix* red-emitting luciferases, suggesting that this arginine and its guanidine group could play a major role in green bioluminescence.[38−40] On the other hand, residues outside the active site were often found to dramatically affect bioluminescence colors, mainly in firefly luciferases. Among them, the residues located in the loop of residues 223−235,[41] residues S284, E311, and R337, and the loop of residues 351−360[35,46,47] are connected by a network of hydrogen bonds and salt bridges whose interactions could be affected by mutations, pH, and temperature (in the case of firefly luciferases), affecting the active site conformation and polarity and modulating bioluminescence colors.[42,45]

Previous modeling studies with *Phrixothrix* railroad worm and *Py. termitilluminans* larval click beetle luciferases showed that the residues at position 311 are located within van der Waals contact with oxyluciferin phenolate,[4,37] being one of the few relatively variable positions in the luciferin binding site of beetle luciferases. These residues are located in the well-conserved ^{314}SGGA^{317} luciferin binding motif (*Ph. pyralis* luciferase), a flexible loop in beetle luciferases that also interacts with AMP. In this loop, the G315A and G316A mutations were already shown to red-shift bioluminescence spectra and affect luciferin affinity in *Ph. pyralis* firefly luciferase.[24] However, residues C/T311 and S314 display essentially similar properties with slightly polar side chains that apparently contribute little to differences in the polarity that could be directly associated with the bioluminescence spectrum of beetle luciferases.

One of the major findings of this study is that the main-chain carbonyls of residues C/T311 (S314) face the oxyluciferin phenolate, instead of the side chains. The side chains are rather involved in packing the residues against second-shell residues and may only indirectly affect the orientation and distance of the main-chain carbonyls around phenolate. This observation may explain the lack of a clear relationship between the identity of luciferin binding site residues and bioluminescence colors and agrees with the mechanistic hypothesis of Hirano et al.,[31] who first proposed that main-chain carbonyls could act as a base-accepting phenolate proton during the emission step. Although in our models the residue 311 (314) main-chain carbonyls are more distant than expected for a polar interaction with oxyluciferin, we believe that during the critical emitting step the active site of beetle luciferases may tighten around the emitter, promoting such specific interaction, affecting bioluminescence spectra.

In the green-yellow-emitting luciferases, mutations of the larger Ser and Cys to Ala, with its smaller methyl side chain, resulted in red shifts and broadening of the spectra (Figures 4 and 6), the C311A substitution in *Pyrearinus* luciferase resulting in the largest ever reported red shift for this luciferase (Figure 4). In *Macrolampis* firefly luciferase, the S314A and S314C mutations resulted in broad bimodal spectra with the appearance of a second peak in the red region of the spectra (Figure 6), suggesting an increase in the population of the red-emitting species.

As expected, the C311A mutation in *P. hirtus* railroad worm luciferase, which already displays red emission, had no effect on the spectrum. However, the C311T substitution, which is naturally found in green-emitting railroad worm luciferases (*P. vivianii* and *R. ohbai*), resulted in the largest blue shift ever observed in the emission spectrum of this red-emitting luciferase and a considerable broadening in the green-yellow region (Figure 5). The effect of this substitution agrees with the importance of threonine for blue shifting in the bioluminescence spectrum of railroad worm luciferases.

The mutations affected differently the K_M values for luciferin in different beetle luciferases. The C311A mutation considerably increased the K_M in *Py. termitilluminans*. The C311A mutation decreased the value for *P. hirtus* red-emitting luciferase, and the S314A mutation in *Macrolampis* luciferase and the C311T mutation in *P. hirtus* had little effect. On the

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other hand, there was a general trend to decrease the K_M for ATP in different beetle luciferases upon introduction of these mutations. Altogether, these kinetic results suggest that the residues at position 311 have a variable degree of influence in the luciferin binding of different beetle luciferases, but in all cases, they affect the ATP and AMP binding site.

Altogether, modeling and mutagenesis studies show that substitutions that increase the size of the cavity around phenolate, such C311A and S314A, tend to red-shift the bioluminescence spectra, whereas those mutations that decrease the size of the cavity (C311T) have the opposite effect. Furthermore, these results suggest that mutations that stabilize the position 311 residue side chains tend to blue-shift the spectra, whereas those that destabilize the side-chain packing tend to red-shift the spectra. The stabilization may turn the environment more rigid and less prone to polarization, blue-shifting the spectra. Destabilization, on the other hand, may make the surrounding environment more flexible and eventually polar, resulting in red shifts and, in the case of firefly luciferases, increasing the population of the red emitter resulting in bimodal spectra.

In all cases, the effect of the mutations is consistent with the change of the environment around oxyluciferin phenolate. These changes could involve (I) a change in a direct specific interaction between the residue 311 (314) main-chain carbonyl and oxyluciferin phenolate, (II) the entrance of a water molecule into the cavity left by the mutation, or (III) a change in the hydrogen bonding pattern among oxyluciferin, active site residues, and second-shell residues, affecting the overall polarity around phenolate. Alternatively, it is possible that the mutations at position 311 (314) may indirectly affect the position of the residues corresponding to residue I288 of *L. cruciata* firefly luciferase (L287 for *Ph. pyralis* and *Macrolampis* sp2, V286 for *P. hirtus* and *P. vivianii*, and I286 for *Py. termitilluminans*), which according to Nakatsu et al.[20] is critical for the creation of a hydrophobic environment around oxyluciferin phenolate modulating bioluminescence colors. However, because all railroad worm green- and red-emitting luciferases display the same V286 residue at this position, and several firefly luciferases display leucine in a manner that is independent of their bioluminescence color, it seems unlikely that such residues could determine bioluminescence color by a polarity effect. Rather, the size of the side chain could affect the size and tightness of the cavity, influencing other interactions with phenolate.

Chemiluminescence studies with luciferyl adenylate and oxyluciferin showed that aqueous environments are consistent with red chemiluminescence, whereas aprotic environments are consistent with both efficient green and red bioluminescence.[48] In bioluminescence, red shifts and broadening of the spectra in the red region could be explained by the increase of the orientation polarizability of the environment around oxyluciferin phenolate, or increasing the proportion of the red-emitting species during the emitting step. Green light emission, on the other hand, requires aprotic and more hydrophobic environments and, additionally, the presence of a strong base and possibly a countercation. It is possible that the residue 311 main-chain carbonyl contributes to the creation of such an aprotic environment around excited oxyluciferin phenolate, and perhaps working as a base that accepts an oxyluciferin phenolate proton during chemiexcitation upon luciferase conformational changes. Failure to tighten such an interaction with phenolate caused by mutations or indirectly by pH- and temperature-mediated conformational changes (in the case of pH-sensitive firefly luciferases) may relax the environment around oxyluciferin phenolate, allowing the polarization and red light emission. In the case of the pH-sensitive luciferases, upon the introduction of mutations, the already flexible active site could be partially filled by water, changing the proportion of red and green light emitters, whereas in the pH-insensitive green-yellow-emitting luciferases, the magnitude of the effect of the mutation could be limited to an increase in the mobility and slight polarization of the surrounding environment, red-shifting and broadening the spectra.

■ CONCLUDING REMARKS

Through homology modeling, site-directed mutagenesis, and kinetic studies using beetle luciferases eliciting different bioluminescence colors, we showed that residues C/T311 (S314) participate in the oxyluciferin phenolate binding pocket of beetle luciferases. The results indicate that stabilizing substitutions that decrease the size of the cavity around oxyluciferin phenolate tend to blue-shift the bioluminescence spectra, whereas destabilizing substitutions that increase the size of the cavity tend to red-shift the spectra. The results suggest the occurrence of a critical interaction between main-chain carbonyls of residues T/C311 (S314) and oxyluciferin phenolate that could determine bioluminescence colors. These results may also explain the lack of a clear relationship between the physicochemical identity of the luciferin binding site residues and bioluminescence colors. Current studies aim to elucidate how these interactions may affect bioluminescence spectra in different beetle luciferases.

■ ASSOCIATED CONTENT

Supporting Information

Homology models with the surrounding environment of position 311 (314) residue side chains and calculated atomic distances between side chains of residue 311 (314) and surrounding residues. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

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

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Notes

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■ ABBREVIATIONS

Ppy, *Ph. pyralis* luciferase; Pte, *Py. termitilluminans* larval click beetle luciferase; PxGR, *P. vivianii* railroad worm green-emitting

luciferase; PxRE, *P. hirtus* railroad worm red-emitting luciferase; Mac, *Macrolampis* sp2 firefly luciferase; Zop, *Z. morio* luciferase-like enzyme; LH2, D-luciferin; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid; DLSA, 5′-*O*-[*N*-(dehydroluciferyl)-sulfamoyl]adenosine; AMP, adenosine monophosphate.

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