Synergistic Mutations Produce Blue-Shifted Bioluminescence in Firefly Luciferase†

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ABSTRACT: Light emission from the North American firefly *Photinus pyralis*, which emits yellow-green (557 nm) light, is widely believed to be the most efficient bioluminescence system known, making this luciferase an excellent tool for monitoring gene expression. In a previous study designed to produce luciferases for simultaneously monitoring two gene expression events, we identified a very promising blue-shifted emitter (548 nm) that contained the mutations Val241Ile, Gly246Ala, and Phe250Ser [Branchini, B. R., Southworth, T. L., Khattak, N. F., Michelini, E., and Roda, A. (2005) Red- and green-emitting firefly luciferase mutants for bioluminescent reporter applications, *Anal. Biochem. 345*, 140−148]. To establish the basis of the unusual blue-shifted emission, we determined that a simple additive effect of the three individual mutations did not account for the spectral properties of the triple mutant. Instead, the bioluminescence emission spectra of two double mutants containing Phe250Ser and either Val241Ile or Gly246Ala very closely resembled that of the triple mutant. Additional mutagenesis results confirmed that the blue-shifted emission of the double mutants was determined by the synergistic behavior of active site residues. Molecular modeling studies of the Gly246Ala and Phe250Ser double mutant supported the notion that the blue-shifted emission was due to localized changes that increased the hydrophobicity at the emitter site as a result of the addition of a single methyl group at position 246. Moreover, the modeling data suggested that the Ala246 side chain remained close to the emitter through an additional H-bond between Ala246 and the hydroxyl group of Phe250, providing a possible structural basis for the synergistic behavior.

The emission of yellow-green light from the North American firefly *Photinus pyralis* is a fascinating phenomenon that results from a well-characterized enzymatic reaction catalyzed by a luciferase enzyme (Luc)[1] (*1*, *2*). Luc first converts substrates firefly luciferin (LH_2), a heterocyclic carboxylic acid, and Mg-ATP into the corresponding luciferyl adenylate (eq 1). This reactive intermediate combines with molecular oxygen at the luciferase active site to produce an electronically excited state product (eq 2), which then emits a photon of visible light (eq 3).

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

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

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

The highly efficient generation of light from LH_2 (*3*) enables the detection of the luciferase protein with great sensitivity using photomultiplier tubes or charged-coupled devices. Additionally, beetle bioluminescence (eqs 1−3) provides a wide dynamic range and low background, features that have led to the development of a wide range of bioanalytical applications that include the detection of bacteria, the measurement of human plasma ATP concentration, and the visualization of tumors (*4*−*9*). Importantly, using bioluminescence to detect the luciferase protein provides an excellent reporter function (*10*) that has been widely applied to investigations of factors regulating gene expression including gene delivery (*11*), gene silencing (*12*), and real-time imaging of protein expression in live animals (*13*−*15*).

Typically, assays using luciferase as a reporter are sensitive, quantitative, rapid, reproducible, and relatively easy to perform (*16*, *17*). Because of the complexity of cellular genetic regulation and the influences of environmental effects, nonspecific interferences can make it difficult to characterize a particular physiological response. To enhance

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[1] Abbreviations: CB, 50 mM Tris−HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA, and 1 mM DTT; GST, glutathione-*S*-transferase; Lcr, *Luciola cruciata* luciferase; LH_2, D-firefly luciferin; LH_2-AMP, D-luciferyl-*O*-adenosine monophosphate; Luc, *Photinus pyralis* luciferase (E.C. 1.13.12.7); MC, Monte Carlo; PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3); PMT, photomultiplier tube; Ppy GR, Ppy WT containing mutations Val241Ile, Gly246Ala, and Phe250Ser; PMSF, phenylmethanesulfonyl fluoride; Ppe2, a *Photuris pennsylvanica* luciferase whose amino acid sequence is 99.6% identical to the deduced sequence of the cDNA of another *Ph. pennsylvanica* luciferase with GenBank accession number U31240; Ppy WT, recombinant *P. pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer−.

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the specificity and precision of genetic regulation measurements, a second reporter can be employed as a control. In the Promega Chroma-Glo luciferase assay system, constructs that encode red- and green-emitting Jamaican click beetle luciferase mutants provide this improvement (*18*). Another dual-color system based on *Phrixothrix* railroad worms (*19*−*21*) and a tricolor system consisting of wild-type and variant railroad worm enzymes (*22*) also have been reported. While some of the advancements in bioluminescence-based bioanalytical applications have been made possible by the various colors and chemistries of native bioluminescence systems, many, like the reporter gene assays, have come about through the use of mutagenesis methods to manipulate firefly bioluminescence color. The latter approach has been an active area of investigation in our laboratory (*23*, *24*), and we are now attempting to create novel Luc mutants with nonoverlapping emission spectra to achieve improved assay sensitivity.

A great deal of effort, including our own, has gone into attempts to elucidate the mechanism of bioluminescence color (emission spectra) from the standpoint of how protein structure determines the wide range of bioluminescence color from a single molecular emitter, oxyluciferin (*23*, *25*−*33*). Even with a wealth of available mutagenesis data and a structural framework provided by several crystal structures (*34*, *35*) including wild-type *Luciola cruciata* luciferase (Lcr) and a red-emitting single-point mutant complexed with an LH_2-AMP analogue (*36*), a convincing mechanistic understanding of bioluminescence color remains elusive.

The emission maxima of the colors produced by all known cloned beetle luciferases range from 536 nm for the click beetle *Pyrearinus termitilluminans* (*37*) to 623 nm for the railroad worm *Phrixothrix hirtus* (*38*, *39*). Within the Lampyridae family of true firefly luciferases, however, the natural color variation is much smaller, ranging from 538 to 575 nm (*40*, *41*). To achieve the emission maximum extremes in Ppy WT ($\lambda_{max} = 557$ nm) that occur in the wild-type beetle luciferases requires a 66 nm red shift, but only a 21 nm blue shift. Among the Lampyridae luciferases, however, it is reasonably easy to produce enzyme variants (~20 mutagenic sites) that emit significantly red-shifted bioluminescence, although often with much lower efficiency than the corresponding wild-type enzymes. Conversely, it is relatively difficult to blue-shift firefly bioluminescence at the in vitro optimal pH of ~8. Curiously, many firefly luciferase variants are known (*27*) with emission maxima greater than 615 nm. However, to the best of our knowledge, Ppy GR, our *P. pyralis* variant containing the changes Val241Ile, Gly246Ala, and Phe250Ser has the shortest emission maximum (548 nm) produced by mutagenesis of a true firefly luciferase (*23*). Although Ppy GR and a thermostable version of this enzyme are promising reagents for improved dual-color assays (*23*, *24*), creating luciferase mutants with even greater blue-shifted emission has the potential to significantly improve the performance of reporter gene assays. We therefore turned our attention to determining the basis for the unusual blue-shifted light emission from Ppy GR. During the course of this systematic site-directed mutagenesis study, we discovered a remarkable example of an enzyme property determined by the synergistic behavior of active site residues.

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the sources indicated: Mg-ATP from a bacterial source from Sigma-Aldrich (St. Louis, MO), restriction endonucleases from New England Biolabs (Beverly, MA), mutagenic oligonucleotides from Invitrogen (Carlsbad, CA), glutathione Sepharose 4B media, MicroSpin columns, and pGEX-6P-2 expression vector from GE Healthcare (Piscataway, NJ), and QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The recombinant Ppy V241I, Ppy G246A, Ppy F250G, Ppy F250S, Ppy G246A/F250S, Ppy GR, and Ppy WT proteins were expressed and purified as previously reported (*23*, *28*, *32*). D-Firefly luciferin was a generous gift from Promega (Madison, WI).

General Methods. Detailed descriptions of the methods and equipment used to determine bioluminescence activity-based light assays at pH 7.8 have been described previously (*23*, *42*). The same methods were used to measure activity at pH 6.0 and 7.0 except that 25 mM MES buffers were used. Additionally, a new luminometer equipped with a Hamamatsu R928 PMT and C6271 HV power supply socket assembly was constructed and used to make some of the light intensity measurements reported here. The instrument consists of a custom-built aluminum box fitted with an Aminco Chem Glow II sample compartment into which the side-reading PMT was fixed in the central area. The device accommodates 8 × 50 mm polypropylene tubes from Evergreen Scientific (Los Angeles, CA). The socket assembly is powered by a constant 12 V dc (ELPAC Power Systems model FW1812), and the high-voltage output to the PMT was controlled with a variable voltage input of 0−5 V dc (GW laboratory dc power supply model GPS-1850D). Data were acquired from the analog output of the PMT through a National Instruments NI SC-2345 signal conditioning connector block and NI 186623E-02 SCC-A102 isolated analog input connector (50 Hz sampling rate) and were stored on a Dell Dimension computer equipped with a National Instruments (NI) PCI 6221 card. Instrument control and data analysis were accomplished with programs developed in-house using NI-DAQmx and LabVIEW 7 Express software. All measurements were corrected for the spectral response of the Hamamatsu R928 PMT.

Concentrations of purified proteins were determined with the Bio-Rad protein assay system using bovine serum albumin as the standard. The homogeneity of all purified proteins was >95% on the basis of SDS−PAGE analysis performed according to the method of Laemmli (*43*), using the equipment and conditions previously described (*44*). DNA sequencing performed at the W. M. Keck Biotechnology Laboratory at Yale University verified the mutations of all luciferase genes.

Mass spectral analyses of purified proteins were performed by tandem HPLC−electrospray ionization mass spectrometry using a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer. The found molecular masses (Da) of the newly reported proteins were (Ppy F250T) 61 110, (Ppy G246A/F250G) 61 085, (Ppy G246A/F250T) 61 131, (Ppy V241I/F250G) 61 087, (Ppy V241I/F250S) 61 113, (Ppy V241I/F250T) 61 121, and (Ppy V241I/G246A/F250T) 61 134. The determined mass values were all within the allowable experimental error (0.01%) of the calculated values.

Site-Directed Mutagenesis. Starting with the indicated DNA sequences in the pGEX-6P-2 vector as templates, the QuikChange site-directed mutagenesis kit was used to create the following mutants using the indicated primers and their respective reverse complements: Ppy F250T from Ppy WT (*28*), 5′ -C GGT TTT GGA ATG **ACT** ACT ACA CTC GGA TAT CTG ATA TGT GG-3′ [*Eco*RV]; Ppy G246A/F250G from Ppy G246A (*32*), 5′ -CAT CAC GCG TTT GGA ATG **GGG** ACT ACA CTC GGA TAT CTG ATA TGT GG-3′ [*Eco*RV]; Ppy G246A/F250T from Ppy G246A (*32*), 5′ -C GCG TTT GGA ATG **ACT** ACT ACA CTC GGA TAT CTG ATA TGT GG-3′ [*Eco*RV]; Ppy V241I/F250G, Ppy V241I/F250S, and Ppy V241I/F250T from Ppy F250G (*32*), Ppy F250S (*32*), and Ppy F250T, respectively, 5′ -CT GCG ATT TTA AGT GTT **ATT** CCA TTC CAC CAT GGT TTT GGA ATG-3′ [*Nco*I]; Ppy V241I/G246A/F250T from Ppy F250T, 5′ -CT GCG ATT TTA AGT GTT **ATT** CCA TTC CAT CAC **GCG** TTT GGA ATG-3′ [*Mlu*I] (bold represents the mutated codon, underline represents silent changes to create a unique screening endonuclease site, and brackets indicate the screening endonuclease).

Protein Expression and Purification. All enzymes were expressed in *Escherichia coli* strain BL21 at 22 °C as GST-fusion proteins as previously described (*31*). The cells were harvested by centrifugation at 4 °C and then kept at −80 °C for 15 min. Cell pellets were resuspended in 50 mL of PBS containing 0.1 mM PMSF and 0.5 mM DTT. Aliquots (5 mL) of a solution of lysozyme in PBS (10 mg/mL) were added, and the cells were lysed by sonication and were treated with DNase (5 μg/mL) and RNase (10 μg/mL) for 10 min on ice. Triton X-100 was added to the lysates (1% final volume), and the whole-cell extracts were isolated by centrifugation at 20000*g* for 1 h. Proteins were further purified using glutathione Sepharose 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST-fusion proteins by incubation with PreScission protease in CB for 18−20 h at 4 °C with gentle mixing. Proteins were eluted with CB and stored at 4 °C in this buffer containing 0.8 M ammonium sulfate and 2% glycerol.

When smaller quantities of protein were sufficient, purifications were performed with glutathione Sepharose 4B MicroSpin columns. Cultures in *E. coli* strain BL21 (10 mL) were grown in 50 mL flasks at 37 °C in LB broth supplemented with 100 μg/mL ampicillin to mid log phase ($A_{600} = 0.6-0.9$), transferred to a 22 °C incubator, and, after equilibrating for 10 min, induced with 0.1 mM IPTG. Following growth for 18−20 h at 22 °C, the cells were harvested by centrifugation at 4 °C, and the resulting pellets were resuspended in 0.5 mL of PBS containing 0.1 mM PMSF and 0.5 mM DTT. Lysozyme (5 μL of a 10 mg/mL solution) was added, and the cell suspensions were frozen in a dry ice−2-propanol bath and then rapidly thawed in a 37 °C water bath. After 10 additional freeze/thaw cycles, the lysates were treated with DNase (5 μg/mL) and RNase (10 μg/mL) for 5 min on ice. Triton X-100 (1% final volume) was added, and the mixtures were centrifuged at 13000*g* for 20 min at 4 °C. Proteins in the soluble supernatants (~0.5 mL) were bound to MicroSpin columns by incubation with gentle mixing at 4 °C for 45 min, and the columns were then thoroughly washed with PBS and CB. Proteins were released from the columns by adding PreScission protease in CB (20 units in 0.2 mL) and gently mixing for 2 h at 4 °C. Luciferases were eluted by centrifugation (735*g* for 1 min), concentrated to ≥0.5 mg/mL using a Vivaspin 4 10K MWCO ultrafiltration spin column, and stored as described above.

Steady-State Kinetic Constants. Values of K_m for D-LH_2 and Mg-ATP for all luciferases were determined from bioluminescence activity assays in which measurements of maximal light intensities were taken as estimates of initial velocities. Data were collected for reactions in 25 mM glycylglycine buffer, pH 7.8, and were analyzed as described earlier (*42*).

Bioluminescence Emission Spectra. Bioluminescence emission spectra, corrected for the spectral response of the R928 photomultiplier tube used to make the measurements, were obtained at 25 °C using previously described methods and equipment (*23*). Reactions (0.525 mL) were initiated by adding 2−18 μg of luciferase in 25 μL of CB containing 0.8 M ammonium sulfate and 2% glycerol to cuvettes containing solutions of LH_2 (70 μM) and Mg-ATP (2.0 mM) in 25 mM MES (pH 6.0) or 25 mM glycylglycine (pH 7.0 and 7.8). The pH values of the reaction mixtures were confirmed before and after all spectra were obtained.

Molecular Modeling. The coordinates of Lcr complexed with oxyluciferin and AMP (PDB ID 2d1r) (*36*) were obtained from the Protein Data Bank (*45*). Maestro v7.5116 was used to add hydrogen atoms where needed and to graphically obtain the G246A/F250S mutant,[2] and the AMBER* force field of MacroModel v9.1113 (*46*) was used in all calculations. A "hot" area with a radius of 8.0 Å (complete residues) from oxyluciferin, AMP, Ala246, and Ser250 was used. It was held in place with two subsequent subshells each extending an additional 2.00 Å with increasing atomic restraints of 100 and 200 kJ/Å. The Polak−Ribiere conjugate gradient minimization mode was used with a derivative convergence criterion of 0.05 kJ/mol. Conformational searches were conducted using the mixed Monte Carlo torsional and molecular position variation method coupled with large-scale low-mode conformational searching (*47*−*51*). The flexible dihedral angles of all the side chains of residues that were within 6.00 Å of Ala246 and Ser250 were randomly rotated by between 0 and 180°, and all solvent molecules in that sphere (9 water molecules) were randomly rotated and translated by between 0 and 1.00 Å in each Monte Carlo (MC) step. Five thousand MC steps were undertaken during the search, and structures within 50 kJ/mol of the lowest energy minimum were kept. A usage-directed method (*50*) was used to select structures for subsequent MC steps. Structures found in the conformational search were considered unique if the least squared superimposition of equivalent non-hydrogen atoms found one or more pairs separated by 0.25 Å or more.

RESULTS

Rationale for Mutagenesis. In our initial effort to produce *P. pyralis* mutants emitting different colors of light for simultaneously monitoring two gene expression events, we identified the very promising blue-shifted emitter Ppy GR,

[2] On the basis of amino acid sequence alignments; equivalent residue position numbers are expressed for all luciferases according to the numbering of *P. pyralis* luciferase.

Table 1: Bioluminescence Activity of Luciferase Enzymes at 25 °C

enzyme	relative integrated specific activity[a] (15 min)			K_m (μM)		bioluminescence emission maximum[b] (nm)								
						pH 7.8			pH 7.0			pH 6.0		
	pH 7.8	pH 7.0	pH 6.0	LH_2	Mg-ATP	λ_{max}	sh	fwhm	λ_{max}	sh	fwhm	λ_{max}	sh	fwhm
Ppy WT	100	53	42	15 ± 2	160 ± 20	557		68	562	603	102	613		61
Ppy GR	73	22	9	6 ± 1	93 ± 20	548		68	551		81	596	560	97
Ppy V241I/G246A/F250T	83	24	6	9 ± 1	41 ± 6	547		69	549		77	589	550	101
Ppy V241I	136	76	41	8 ± 1	283 ± 28	555		63	557		70	609	560	83
Ppy G246A	104	48	29	4 ± 1	29 ± 3	555		65	556		81	611	550	69
Ppy F250S	49	23	12	22 ± 1	133 ± 13	557	607	98	603	555	102	609		71
Ppy F250T	37	19	8	14 ± 2	215 ± 11	554	612	102	594	549	108	605		72
Ppy F250G	23	nd	nd	16 ± 2	166 ± 20	560		70	562	606	91	610		67
Ppy G246A/F250S	42	13	10	10 ± 1	60 ± 6	549		66	554		88	599		79
Ppy G246A/F250T	40	13	4	11 ± 2	60 ± 8	549		72	558	600	95	598		83
Ppy G246A/F250G	33	19	12	11 ± 1	61 ± 6	558		68	558		74	605	556	89
Ppy V241I/F250S	32	15	8	12 ± 2	160 ± 25	550		71	600	570	108	606		76
Ppy V241I/F250T	64	16	8	24 ± 3	151 ± 20	548		78	595	550	110	605		72
Ppy V241I/F250G	13	nd	nd	24 ± 4	182 ± 33	559		69	607	560	106	nd		

[a] Specific activity assays were performed as described in the Experimental Procedures. The values are expressed relative to that of Ppy WT at pH 7.8, defined as 100. nd = data not determined due to insufficient available materials. [b] Bioluminescence emission spectra were measured at pH 6.0, 7.0, and 7.8 as described in the Experimental Procedures. Bandwidths at full-width at half-maximum (fwhm) and the presence of a shoulder (sh) are indicated. The bioluminescence emission spectrum of Ppy V241I/F250G at pH 6.0 was not determined (nd) due to low signal strength.

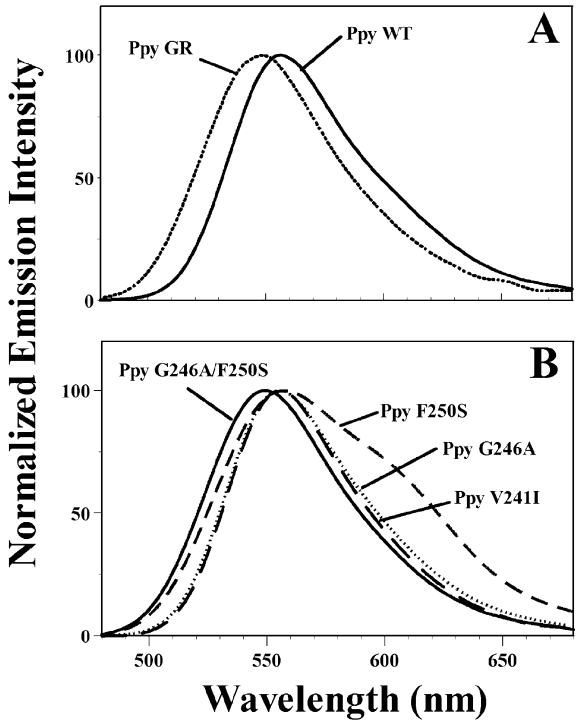

FIGURE 1: Bioluminescence emission spectra at pH 7.8 and 25 °C. The spectra generated by the indicated luciferases were recorded using equipment and conditions previously described (*23*).

which contains the mutations Val241Ile, Gly246Ala, and Phe250Ser (*23*). To assess the individual contributions of each point mutation to the blue-shifted bioluminescence color of Ppy GR, the emission spectra of the Ppy V241I, Ppy G246A, and Ppy F250S proteins were examined (Table 1 and Figure 1B). While a simple additive effect of the three individual mutations does not account for the blue-shifted emission of Ppy GR, the bioluminescence emission spectrum of the double mutant Ppy G246A/F250S (*23*) very closely resembled that of Ppy GR (Figure 1). To determine the structural basis of the unusual color shift, we then made the additional point mutant Ppy F250T and double mutants Ppy G246A/F250T and Ppy G246A/F250G.

Ppe2, a wild-type luciferase from *Photuris pennsylvanica* whose emission maximum of 538 nm (*40*) makes it the shortest wavelength emitter among all true fireflies, has Ile and Thr, at equivalent Ppy positions[2] 241 and 250, respectively; a combination that is unique among all known beetle luciferase amino acid sequences (Figure 2). We next investigated whether this combination in Ppy might also produce blue-shifted luminescence by making the corresponding Ppy V241I/F250T. To complete the investigation, the additional variants Ppy V241I/F250S, Ppy V241I/F250G, and Ppy V241I/G246A/F250T were produced and studied.

Expression and Purification of Luciferase Proteins. Ppy WT and the modified luciferases listed in Table 1 were expressed as GST-fusion proteins and contained the additional N-terminal peptide GlyProLeuGlySer-, which remained after PreScission protease cleavage from GST. Average yields of newly reported purified proteins (mg/0.5 L of culture) were (Ppy F250T) 7, (Ppy G246A/F250G) 6, (Ppy G246A/F250T) 16, (Ppy V241I/F250T) 7, and (Ppy V241I/G246A/F250T) 9. The Ppy V241I/F250G and Ppy V241I/F250S enzymes were purified using a small-scale spin column procedure that yielded ~0.2 mg of protein from 10 mL of culture. All enzymes generally remained greater than 90% active for up to 6 months when stored at 4 °C. If required, additional amounts of fully active enzymes could be obtained by thawing aliquots of proteins that had been flash frozen in liquid N_2 immediately after isolation.

Characterization of Luciferase Mutants. As reported (*23*) previously, Ppy GR is a luciferase variant with excellent specific activity and affinity for the natural substrates LH_2 and Mg-ATP (Table 1). The integration-based specific activities and K_m values of the other luciferase variants used in this study also were assessed (Table 1). The activity measurements are based on the total amount of light emitted with saturating concentrations of substrates over the pH range from 6.0 to 7.8 and were corrected for the differences in the colors and shapes of the emission spectra produced by each enzyme. At the pH optimum of ~7.8, the specific activities

Luciferase	λ_{max} (nm)	Partial Amino Acid Sequence[2]			
FIREFLIES		241	246	250	286
P. pennsylvanica (Ppe2)	538	Ile	Gly	Thr	Leu
PpyGR	548	Ile	Ala	Ser	Leu
C. distinctus (Cdist)	548	Ile	Gly	Phe	Leu
P. miyako (Pma)	550	Ile	Val	Phe	Leu
L. noctiluca (Lno)	550	Ile	Gly	Phe	Leu
L. lateralis (Lla)	552	Val	Gly	Phe	Ile
P. pyralis (Ppy)	557	Val	Gly	Phe	Leu
P. pennsylvanica (Ppe1)	558	Val	Ala	Phe	Leu
L. cruciata (Lcr)	562	Val	Gly	Phe	Ile
L. italica (Lit)	566	Val	Gly	Phe	Ile
H. parvula (Hpa)	568	Val	Gly	Phe	Ile
L. mingrelica (Lmi)	570	Val	Gly	Phe	Ile
CLICK BEETLES					
P. termitilluminans (Ptm)	536	Leu	Ala	Ser	Ile
P. plagiophthalamus (PplGR)	546	Leu	Ala	Ser	Ile
P. plagiophthalamus (PplYG)	560	Leu	Ala	Ser	Ile
P. plagiophthalamus (PplYE)	578	Val	Ala	Ser	Val
P. plagiophthalamus (PplOR)	593	Val	Ala	Gly	Val
RAILROAD WORMS					
Phengodes sp. (Phg)	546	Val	Ala	Phe	Val
P. vivianii (PvGR)	548	Val	Ala	Phe	Val
P. hirtus (PhRE)	623	Ala	Ala	Phe	Val

FIGURE 2: Bioluminescence emission maxima and partial amino acid sequence comparison of a selected region of the beetle luciferases. The spectral data are from ref *27* except for Ppy (*23*) and Cdist (*59*). The sequences of Phg and Ppe2 were obtained from Keith V. Wood, personal communication. All other amino acid sequences were deduced from GenBank submissions except for Ptm (*37*).

of the single-point mutants at Ppy position 250 were reduced ~2−4-fold. The Val241 to Ile change significantly enhanced activity above that of Ppy WT, while the Gly246 to Ala substitution had no effect. Only minor changes in specific activity were observed when either the Val241 to Ile or the Gly246 to Ala mutation was added to the position 250 variants to create the corresponding sets of double mutants (Table 1). A notable change was the 1.7-fold increase in the specific activity of Ppy V241I/F250T. In Ppy GR, with both the Val241 to Ile and Gly246 to Ala changes present, the specific activity of Ppy F250 was improved 1.5-fold. Compared to the maximum value at pH 7.8, the specific activity of Ppy WT was ~2 and ~2.4 times lower at pH 7.0 and 6.0, respectively. Similar results were obtained with the single-point mutants, while a greater deal of variability was observed with the luciferases containing two or three amino acid changes (Table 1).

With the exception of the ~4- and ~6-fold reductions in the K_m values of Ppy G246A for LH_2 and Mg-ATP, respectively, the single amino acid changes produced only minor effects on the K_m values of the corresponding luciferase variants for the natural substrates (Table 1). As previously reported (*40*, *52*), the K_m values of several click beetle and railroad worm luciferases are also strongly influenced by the occurrence of Gly or Ala at Ppy position 246. It appears too that adding the Gly246 to Ala change to the Ppy Phe250 variants favorably influences the K_m values of the respective double mutants. The additional Val241 to Ile change in the two triple mutants produced minor improvements in the K_m values of these enzymes for LH_2.

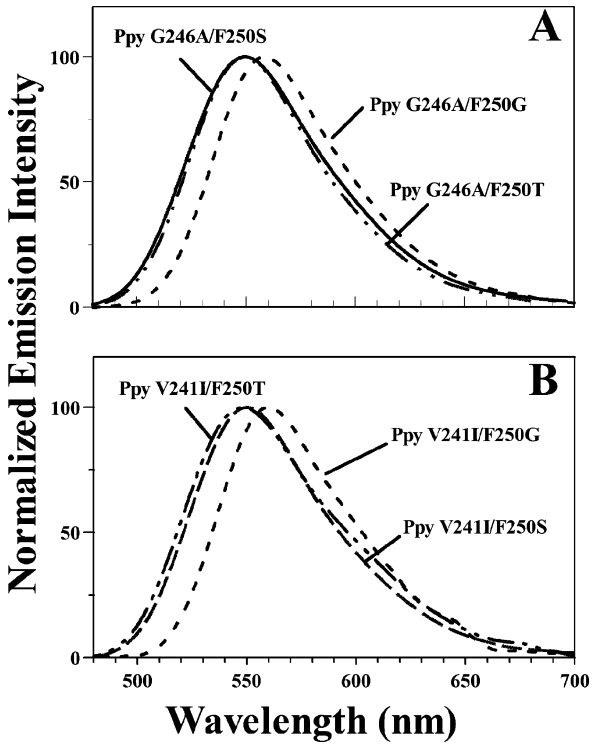

FIGURE 3: Bioluminescence emission spectra at pH 7.8 and 25 °C. The spectra generated by the indicated luciferases were recorded using equipment and conditions previously described (*23*).

Bioluminescence Emission Spectra. The bioluminescence emission spectra of the luciferase enzymes were measured over the pH range from 6.0 to 7.8, and the results are summarized in Table 1. Unlike the click beetle and railroad worm luciferases, the firefly enzymes are usually influenced in vitro by conditions below their pH optima of ~8. As illustrated by the behavior of Ppy WT (Table 1), this results in the emission shifting to longer wavelengths accompanied by significant peak broadening at pH 7, sometimes producing bimodal spectra, and eventually in fully red-shifted emission at pH 6. While all of the enzymes studied retained this sensitivity to acidic pH, resistance to peak broadening under neutral conditions was observed in Ppy V241I and all of the enzymes that contained the Gly246 to Ala mutation except Ppy G246A/F250T.

At pH 7.8, the single-point mutants at positions 241, 246, and 250 failed to produce blue-shifted emission maxima (Table 1) that could account for the bioluminescent properties of Ppy GR alone or additively. In marked contrast, luciferase variants in which Phe250 was changed to either Ser or Thr, produced bioluminescence spectra very similar to that of Ppy GR when either the Gly246Ala or Val241Ile (Figure 3) substitution was also present. Apparently, a side hydroxyl group at position 250 is a critical element because blue-shifted bioluminescence was not observed in the corresponding double mutants Ppy G246A/F250G and Ppy V241I/F250G (Figure 3) that lack this functionality. The triple mutants Ppy GR and Ppy V241I/G246A/F250T, when viewed as the double mutants Ppy G246A/F250S and Ppy G246A/F250T to which the third change Val241Ile has been added, have slightly enhanced emission spectra and improved specific activity.

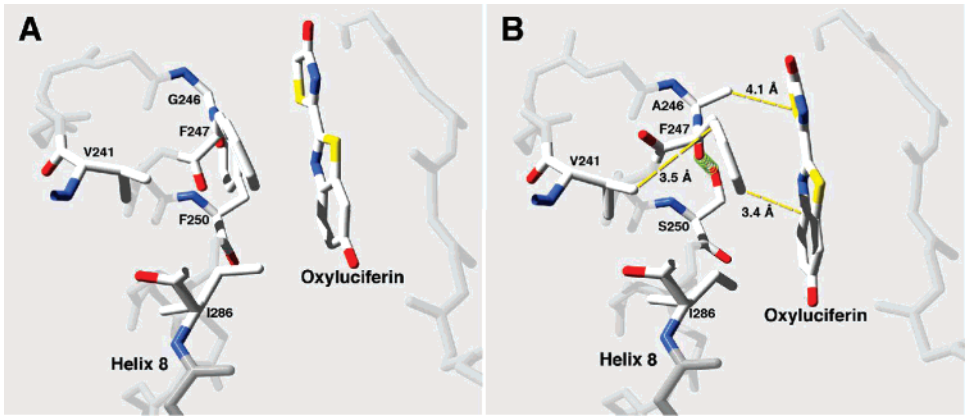

FIGURE 4: (A) Diagram created from the Lcr·oxyluciferin·AMP complex 2d1r (*36*) showing oxyluciferin and residues Val241 to Leu256, Val285 to Ile286, and Val336 to Leu342 (numbering according to the Ppy sequence[2]). (B) Diagram representing the lowest energy structure created, as described in the Experimental Procedures, by molecular modeling starting with the Lcr structure 2d1r (*36*) containing in silico mutations Gly246Ala and Phe250Ser. Oxyluciferin and residues Val241 to Leu256, Val285 to Ile286, and Val336 to Leu342 (numbering according to the Ppy sequence[2]) are shown.

DISCUSSION

Structural Basis of Blue-Shifted Bioluminescence in Ppy G246A/F250S and Ppy G246A/F250T. The variation in the kinetic properties of the *P. pyralis* luciferase variants at positions 241, 246, and 250 examined in this study (Table 1) were not suggestive of major structural changes. These three residues are found at or in close proximity to the oxyluciferin binding site identified in the crystal structures of the homologous *L. cruciata* (Lcr) firefly luciferase (Figure 4A) (*36*). It is therefore likely that local effects account for the observed blue-shifted bioluminescence.

Initially, we determined that the double mutant Ppy G246A/F250S was sufficient to produce the blue-shifted emission maxima of Ppy GR (Table 1 and Figure 1). This result was surprising because the Phe250Ser change alone produced the opposite effect (a red shift) while the Gly246Ala mutation resulted in only a 2 nm change. On the basis of the homologous Lcr structures (*36*), helix 8 of Luc (residues 246−258) is a structural element of the active site with residues Gly246 and Phe247 constituting one side of the substrate/emitter-binding pocket (*34*, *35*). The Lcr structure containing the emitter oxyluciferin provides an excellent model for our results with Ppy because all of the helical residues and Val241 are identical in both enzymes.

It occurred to us that the basis for the synergistic effect of the two mutations might be the formation of a new H-bond between the side chain hydroxyl group of Ser250 and the main chain amide of Ala246. Further, this hypothesis is also consistent with the blue shift occurring in the double mutant containing Phe250Thr and not with the Phe250Gly substitution. We found numerous occurrences of this helical H-bonding interaction in the Protein Data Bank (*45*). One example, from a crystal structure of NikA, a nickel-binding periplasmic protein (*53*), shows the main chain carbonyl to side chain hydroxyl H-bond between the helix N1 (Ala351) and N1 + 4 (Ser355) residues. To evaluate the likelihood of this interaction occurring and producing local structural perturbations in Ppy G246A/F250S, we undertook molecular modeling experiments starting with the crystal structure of the Lcr·oxyluciferin·AMP complex containing the in silico changes Gly246Ala and Phe250Ser.[2] As predicted, the lowest energy structures contained the H-bonding interaction as represented by the single lowest energy structure shown in Figure 4B. Additionally, the model predicts that the β-methyl of Ala246 is positioned close to the C5 carbon (4.5 Å) and S1 sulfur (4.1 Å) atoms of oxyluciferin. A comparison of the model of the double mutant to the starting Lcr crystal structure without the mutations did not reveal any significant difference in the positions of other Lcr residues (data not shown). Overall, the modeling data support the notion that the blue-shifted emission in Ppy G246A/F250S is a result of the localized changes at the emitter site as shown in Figure 4B. It is likely that, in both Ppy G246A/F250S and Ppy G246A/F250T, the addition of a single methyl group at Luc position 246 produces a very localized increase in the hydrophobicity at the emitter site. Further, this interaction is maintained through an additional H-bond between the main chain carbonyl of Ala246 and the side chain hydroxyl of the position 250 substituent, possibly by stabilizing the first turn of the α-helix.

While no single mechanism provides a convincing explanation for the wide range of colors produced by bioluminescent beetles, it is generally accepted that small shifts in emission maxima, like the ones observed in this study, can result from changes in the local polarity of the emitter site (*25*, *54*). The most straightforward interpretation of our results is that a localized decrease in the polarity of the emitter site is responsible for the blue-shifted bioluminescence. This would be similar to the usual hypsochromic shift induced by solvents of decreasing polarity typical of $\pi \rightarrow \pi^*$ transitions in which the dipole moment increases upon excitation (*55*). In fact, the fluorescence emission maximum of the LH_2 analogue dehydroluciferinol exhibits solvent-induced blue shifts of up to 28 nm (*56*). Additionally, the proposals that bioluminescence color determination is based on the polarization of the luciferase·oxyluciferin complex (*57*) or the "molecular rigidity of the excited state of oxyluciferin" (*36*) are supported by our findings.

Luc Position 246 and 250 Mutagenesis and Luciferase Sequence Comparisons. Partial sequence comparisons of the beetle luciferases (Figure 2) revealed naturally occurring examples in the click beetles of the Ala246/Ser250 and Ala246/Gly250 combinations that were introduced as mutations into Luc. In the click beetle isozymes, a few amino

acid changes account for the incremental shift in emission maxima from 546 nm (Ppl GR) to 560 nm (Ppl YG) to 578 nm (Ppl YE). An additional 15 nm shift converts Ppl YE to the orange-emitting enzyme Ppl OR (593 nm), requiring only the single change Ser250 to Gly. This latter shift is reassuringly similar to what we observed with Ppy G246A/F250S and Ppy G246A/F250G. Moreover, in the click beetle isozymes, the shifts resulting from the amino acid substitution are largely independent of each other (*25*). Another click beetle (Ptm), which has the shortest reported luciferase emission maximum wavelength (536 nm), contains the same Ala246/Ser250 combination, indicating relatively good consistency within the fireflies and click beetles that this combination of residues is associated with shorter wavelength bioluminescence.

The combination Ala246/Phe250 that occurs naturally in the firefly Ppe1 and all railroad worm luciferases is associated with a wide range of colors of emitted light (Figure 2) and is therefore not a reliable determinant of bioluminescence color, consistent with our finding with the point mutant (Ppy G246A). There are other reported examples (*52*) where the Gly246 to Ala mutation in fireflies with Phe250 does not alter bioluminescence color, and this is likely to be the general case for the firefly enzymes. There are, however, examples among click beetles and railroad worms (*33*, *52*) where changing Ala246 to Gly produces red-shifted emission, indicating a clear difference in the way Phe250 influences color determination among the three beetle families. In Pma, Val is reported (*58*) to be present along with Phe250, and this is the only occurrence of a residue other than Ala or Gly at position 246 (Figure 2). We made the point mutant Ppy G246V and determined that its emission maximum was unchanged from that of Ppy WT. However, it was a very poor luciferase with specific activity ~1% that of the Luc (data not shown). It is surprising that Val can be tolerated in Pma, and it is not clear whether the hydrophobic side chain is influencing the emission maximum.

Luc Position 241 and 250 Mutagenesis and Luciferase Sequence Comparisons. Ppe2, a luciferase from *Ph. pennsylvanica*, is an enzyme that emits light with a 538 nm maximum, the shortest wavelength emission among the true fireflies, and contains the unique combination of residues Ile241, Gly246, and Thr250 (*40*) (Figure 2). We constructed a *P. pyralis* variant containing the same combination of residues (Ppy V241I/F250T) and recorded an emission spectrum with a 9 nm blue-shifted maximum (Table 1 and Figure 3B). Additionally, the related Ppy V241I/F250S and Ppy V241I/G246A/F250T enzymes had similar maxima; however, the emission of Ppy V241I/F250G remained essentially unchanged (Table 1 and Figure 3B). The results with the three double mutants containing the Val241 to Ile change provided a second example of a synergistic hypsochromatic shift dependent on a hydroxyl substituent at position 250. Molecular modeling studies subsequently were carried out, and all the low-energy structures within 5 kJ/mol contained a new H-bond either between Ser250 and Gly246 or between Ser250 and Thr346. Unfortunately, comparisons of these structures to the starting Lcr crystal structure without the mutations did not reveal any significant difference in the positions of any Lcr residues (data not shown). In this case, the molecular modeling results do not suggest a structural basis for the synergistic blue shift. However, we speculate that a H-bonding interaction and the increased size of the position 241 residue act together to force the phenyl side chain of Phe247 closer to the benzothiazole ring of the emitter. In the Lcr crystal structure, the Phe247 side chain is in van der Waals contact with the heterocyclic ring of the emitter (Figure 4A). The movement of the phenyl ring closer to the emitter is one aspect of a conformational change proposed to make the active site more hydrophobic and favoring green light emission (*36*). Additionally, we had previously demonstrated the importance of the aromatic ring of Phe247, an absolutely conserved residue among the beetle luciferases, in mutagenesis studies with *P. pyralis* point mutants F247A, F247L, and F247Y (*32*). While the Tyr replacement had no effect on bioluminescence, the Leu and Ala replacements produced 8 and 30 nm red shifts, respectively. As discussed above in more detail, a decrease in the local polarity of the emitter site appears to be related to blue-shifted bioluminescence.

While the Val241 to Ile change alone in Luc causes only a modest 2 nm blue shift, the residue is important to color in the click beetle isozymes with serine at position 250 (Figure 2). In fact, the change from Val241 to Leu, in conjunction with the change Arg226 to Glu results in an 18 nm blue shift transforming Ppl YE (578 nm) into Ppl YG (560 nm) (*25*). Likewise, the green-emitting (536 nm) Ptm has Leu at position 241, while the red-emitting Ph RE (623 nm) is the only luciferase with Ala at this position. These sequence comparison and bioluminescence data are consistent with the importance of the size and/or hydrophobicity of the side chain at position 241 in the luciferases.

While the three residues investigated in this study are not solely responsible for the color properties of the beetle luciferases or even a single family of these enzymes, the effects of the mutations on Luc and the naturally occurring click beetle residues are reasonably consistent. Moreover, we have discovered two examples of an enzyme property, in this case the color of bioluminescence, being determined by the synergistic effect of two point mutations. Molecular modeling studies have been helpful in understanding the basis of one of these examples, and additional studies are in progress to better understand the documented phenomena.

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