FINAL REPORT

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The overall objective of this project is to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. Bioluminescent materials are non-toxic and biodegradable and only emit light and not spontaneously. Furthermore, their emission characteristics may be altered so that their signals are bright and decay after a few seconds or they can be made to last for 30 minutes to hours. Also, the color of the light signal can be varied over the visible range.

The 3 major objectives of this project are: (1) to develop novel bright flash and/or glow emitting sources of red (λ_{max}> 610 nm) and green (λ_{max} < 555 nm) light based on the *P. pyralis* luciferase systems; (2) to correlate the results of the characterization of the selected mutant enzymes to an improved understanding of the specific roles of the changed amino acids in conformational changes essential for catalysis, bioluminescence color and thermostability; and (3) to characterize chromophores, organic structures and proteins from bioluminescent worms supplied by collaborator Dimitri Deheyn.

Summary of Significant Work Accomplished

There were five major accomplishments completed with this grant.

(**1**) We demonstrated the feasibility of performing dual analyte reporter gene assays in bacteria and mammalian cells grown at 37 °C with thermostable red (612 nm) and green (552 nm) light-emitting mutants of firefly luciferase that we developed.

(**2**) The gene for Ppy RE9, a novel thermostable (~6 h at 37 °C) red-light (λ_{max} = 617 nm) emitting firefly luciferase variant, was human codon optimized. The gene provided ~100-fold greater signal intensity than a commercial red luciferase in genetic reporter testing in a Human Embryonic Kidney 293 cell line.

(**3**) Mutagenesis studies of firefly luciferase identified residues responsible for blue-shifted emission, pH and thermal stability. Molecular modeling studies suggest structural basis for color shift.

(**4**) A red-emitting (λ_{max} = 617 nm) luciferase variant Ppy RE10 was made that had optimal properties for BRET. The point mutations Thr169Cys and Ser399Cys introduced surface exposed thiols that were labeled with maleimide containing AlexaFluor nIR dyes. Covalently labeled Ppy RE10 produced light with maxima at 705 nm or 778 nm via intramolecular BRET processes. A fusion protein consisting of Ppy RE10 and a biotin binding domain linked through a flexible peptide containing a factor Xa protease cleavage site was

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14. ABSTRACT

The overall objective of this project was to discover, design and demonstrate the feasibility of bioluminescent materials for use in marking, tagging, and anti-tamper applications. Five major accomplishments were completed. (**1)** The feasibility of performing dual analyte reporter gene assays in mammalian cells grown at 37 °C with thermostable red and green light-emitting mutants of firefly luciferase was demonstrated. (**2**) The gene for Ppy RE9, a novel thermostable red-light emitting firefly luciferase variant, was human codon optimized. The gene provided ~100-fold greater signal intensity than a commercial genetic reporter in a human cell line. (**3**) Mutagenesis studies of luciferase identified residues responsible for blue-shifted emission, pH and thermal stability. (**4**) A red-emitting luciferase variant Ppy RE10 was selectively labeled with near IR fluorescent dyes. Through the BRET process, the enzymes produced light with maxima at 705 nm or 778 nm. Fusion proteins containing these labeled enzymes were made and immobilized onto magnetic microspheres. (**5**) A fluorescent chlorophyll *a* derivative was isolated from the worm *C. variopedatus*. Also, mass spectral studies of a small molecule fraction of *O. phosphorea* mucous advanced the structure identification of a putative bioluminescence stimulating factor.

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labeled with a nIR dye. After immobilization on magnetic microspheres containing avidin, this material was used to assay factor Xa activity in blood with reduced interference from hemoglobin.

(**5**) A fluorescent chlorophyll *a* derivative was isolated from the worm *C. variopedatus* provided by the Deheyn lab. We hypothesize that the luciferin from this worm is structurally related to the dinoflagellate and krill luciferins, whose structures resemble and are derived from chlorophyll *a*. This hypothesis was preliminarily investigated by LC/ESMS. Also, Mass spectral studies of a small molecule fraction of *O. phosphorea* mucous advanced the structure identification of a bioluminescence stimulating factor.

Detailed Descriptions of the 5 Major Accomplishments.

Major Accomplishment 1.

(1) Typically, assays using luciferase as a reporter are sensitive, quantitative, rapid, reproducible and relatively easy to perform [23, 24]. However, because of the complexity of cellular genetic regulation and the influences of environmental effects, non-specific interferences can make it difficult to characterize a particular physiological response. For this reason, a second control reporter should be introduced to improve the specificity and precision of genetic regulation measurements. In the Promega Dual-Luciferase Reporter Assay System, which requires sequential measurement of both firefly and *Renilla* luciferases in one sample, the control and test signals are generated by two separate bioluminescence reactions. Several drawbacks of this system are that two substrates and a stop solution are required and that it involves the expression of two dissimilar enzymes. Recently, two dual color reporter assay systems utilizing red- and green-emitting luciferases from the Jamaican click beetles [25] and *Phrixothrix* railroad worms [26-28], and a tricolor system consisting of wild-type and variant railroad worm enzymes [29] have been reported. While the methods are promising, the sensitivity of these reporters may not be sufficient for some demanding applications.

Our objective is to produce mutants of luciferase with bioluminescent properties suitable for improved dual-color reporter assays, biosensor measurements and imaging techniques based on the *P. pyralis* enzyme generally believed to be the most efficient bioluminescence system [3]. Recently, we developed [30] a set of red (Ppy RE)- and green (Ppy GR)-emitting Luc mutants with bioluminescence emission maxima separated by 67 nm and spectra distinguishable by optical filters. In model microplate luminometer studies performed with luciferases expressed as GST-fusion proteins in *E. coli*, we demonstrated that 20 amol of Ppy GR could be detected in crude cell lysates in the presence of ~10 fmol of Ppy RE serving as a control. The first-generation mutants were expressed at similar levels and provided a set of proteins that could be assayed with a single substrate. While our initial results were encouraging, the Ppy RE and Ppy GR mutants suffered the disadvantages of poor thermostability and emission spectra that red-shifted and broadened below ~ pH 7.4. These problems [31, 32] are common to all true firefly luciferases and many color shifted mutants of these enzymes, including other recently described multicolor Luc variants [33]. While poor thermostability is likely a typical property of all beetle

luciferases, light emission from the railroad worm and click beetle enzymes is not pH dependent and mutants [26, 28, 29] of these enzymes generally retain this characteristic. Several strategies, including gene chimerization [34], directed evolution [35] and random mutagenesis [32, 36-38], have been implemented to produce thermostable luciferase reagents. Of particular interest were the reports [37, 39] of Tisi and coworkers that a *P. pyralis* luciferase variant containing five point mutations ("mutant E") had an ~27-fold increased half-life at 37 °C compared to the wild-type enzyme. To improve the thermostability of Ppy RE and Ppy GR at 37 °C, we introduced the same multiple mutations into the color-shifted mutants. We present here the findings of studies on the development and characterization of thermostable versions of Ppy RE and Ppy GR that retained their suitable bioluminescent properties and became pH insensitive making them highly suitable for demanding imaging and reporter gene applications.

Materials. The following materials were obtained from the sources indicated: Mg-ATP from bacterial source (Sigma-Aldrich, St. Louis, MO); restriction endonucleases (New England Biolabs, Beverly, MA); mutagenic oligonucleotides (Invitrogen, Carlsbad, CA); Glutathione Sepharose 4B and pGEX-6P-2 expression vector (GE Healthcare, Piscataway, NJ); QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The recombinant Ppy WT, Ser284Thr (Ppy RE) and Val241Ile/Gly246Ala/Phe250Ser (Ppy GR) proteins were expressed and purified as previously reported [30, 40, 41]. D-Firefly luciferin and Chroma-Glo Reagent were generous gifts from Promega.

General methods. Detailed descriptions of the methods and equipment used to determine bioluminescence activity-based light assays and steady-state kinetic constants have been described previously [42]. Concentrations of purified proteins were determined with the Bio-Rad Protein assay system using BSA as the standard. The homogeneity of all purified proteins was > 95% based on SDS-PAGE analysis performed according to the method of Laemmli [43], using the equipment and conditions previously described [44, 45]. 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 (in daltons) of the newly reported proteins were: Ppy WT-TS, 61 088; Ppy GR-TS, 61 055; Ppy RE-TS, 61 111 (TS, Thr214Ala/Ala215Leu/Ile232Ala/Phe295Leu/Glu354Lys) and GST-Ppy WT 87 561. The determined mass values were all within the allowable experimental error (0.01 %) of the calculated values.

Site-directed mutagenesis. The QuikChange Site-Directed Mutagenesis kit was used to create the mutants Ppy WT-TS, Ppy GR-TS and Ppy RE-TS. Site-directed mutagenesis was carried out according to the manufacturer's instructions using the Luc wild-type, Val241Ile/Gly246Ala/Phe250Ser and Ser284Thr DNA sequences in the pGEX-6P-2 vector as templates and the following primers with their respective reverse complements: Thr214Ala/Ala215Leu: 5´-CCG CAT AGA **GCT CTC** TGC GTC AGA TTC TCG CAC GCC AGA GAT CCT-3´ [*Spe*I]; Ile232Ala: 5´-GCC AGA GAT CCA ATA TTT GGC AAT CAA ATC **GCT** CCG GAT ACT GC-3´ [*Ssp*I]; Phe295Leu: 5´-CCA ACC CTA TTT TCA TTC **TTG** GCC AAA AGT ACT CTG ATT GAC-3´ [*Sca*I]; and Glu354Lys: 5´-CT GAG ACT ACT AGT GCT ATT CTG ATT ACA CCC **AAG** GGG GAT GAT A-3´ [*Spe*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. The Ppy WT-TS, Ppy GR-TS and Ppy RE-TS proteins were expressed in *E. coli* strain BL21 at 22 °C as GST-fusion proteins. 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 phenylmethylsulfonyl fluoride. Aliquots (5 ml) of a solution of lysozyme in PBS (10 mg/ml) were added and the cells were lysed by sonication. Triton X-100 was added to the lysates (1% final volume) and the whole-cell extracts were isolated by centrifugation at 20,000 *x 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 (CBA).

The GST-Luc fusion protein was expressed and purified as described above omitting the protease cleavage step. Instead, the fusion protein was eluted directly from the Glutathione Sepharose 4B resin with 10 mM GSH in 50 mM Tris (pH 8.2), 150 mM NaCl, 10 mM EDTA, and 1 mM DTT; dialyzed against CB to remove GSH and stored in CBA at 4 °C.

For model reporter studies, GST-luciferase fusion proteins were expressed in duplicate 5-ml LB cultures of *E. coli* strain BL21 grown at 37 °C to midlog phase (A_{600}= 0.6). Cultures were then kept at 37 °C overnight or transferred to an incubator set to 22 °C and grown overnight at this temperature. Protein expression was not induced in these experiments. Soluble cell lysates were prepared from individual cultures according to the detailed method previously reported [30].

GST activity assays were performed using the substrate 1-chloro-2,4-dintrobenzene as described in the GST Gene Fusion System manual (GE Healthcare, Piscataway, NJ). The determined GST-based specific activity of purified GST-Luc (ΔA_{340nm} = 0.0843 ± 0.0035 min^{-1} μg^{-1}) was used to estimate the concentrations of all GST-luciferase fusion proteins in soluble cell lysates.

Circular Dichroism (CD) spectroscopy. CD spectra (190-260 nm) of luciferases, 0.2 mg/ml in 0.1M sodium phosphate buffer (pH 7.5) containing 0.4 M ammonium sulfate and 0.5% glycerol, were measured with a JASCO J-810 spectropolarimeter using a covered 0.1 mm path-length quartz cell. Data were acquired at 10 °C using a scan rate of 20 nm/min with a bandwidth of 1 nm. Spectra were accumulated over four repeated scans and corrected for solvent background. Mean residue ellipticities were calculated using a mean residue weight of 110.2. Mean thermoaggregation temperatures (T_m) of the luciferases were determined by monitoring CD at 222 nm over the temperature range of 10-90 °C, with a temperature increment of 1 °C/min, a data pitch of 0.2, a response time of 4 s, and a bandwidth of 1 nm. Cuvette temperature was regulated with a JASCO PTC-423S Peltier device. At the end of each experiment, precipitate was observed in the cuvette and protein could not be detected in solution (Bio-Rad assays). Values of T_m were calculated using the protein denaturation feature of the JASCO Spectra Analysis software.

Heat inactivation studies. Enzymes (0.08-0.1 mg) were incubated for 2 h at 37 °C in 0.2 ml of 25 mM glycylglycine buffer (pH 7.8) containing 10 µl CBA. Aliquots (4-6 µl) were taken at regular intervals and assayed for bioluminescence activity as previously described [42] except that no correction was applied for the spectral response of the PMT. Half-lives were calculated using first order rate constants obtained from log plots of percent activity remaining versus time.

Bioluminescence emission spectra. Bioluminescence emission spectra, corrected for the spectral response of the detector, were obtained at 25 °C and 37 °C using previously described methods and equipment [30]. Reactions (0.525 ml) were initiated by adding 2-18 µg of luciferase in 25 µl CBA to cuvettes containing solutions of LH_2 (70 µM) and Mg-ATP (2.0 mM) in 25 mM MES (pH 6.0), 25 mM glycylglycine (pH 7.0) or 25 mM glycylglycine (pH 7.8). The pH values of the reaction mixtures were confirmed before and after all spectra were obtained. The spectral measurements at 37 °C were made 30 min after addition of the luciferases to the buffered reagent solutions maintained at 37 °C.

Antibody cross reactions (Western blot). Soluble protein extracts of GST-luciferases grown at 22 °C and 37 °C were prepared as described above. In duplicate experiments, aliquots of each protein lysate were resolved by SDS-PAGE (7.5% precast gels, JULE, Inc., Milford, CT). One gel was stained with Coomassie Blue R-250 and the second was electroblotted at 4 °C for 70 min at 200 mA onto nitrocellulose (Schleicher and Schuell, Keene, NH) in 25 mM Tris buffer (pH 8.3) containing 0.192 M glycine and 15% methanol [46]. The membrane was incubated overnight at 4 °C in blocking solution, washed according to the manufacturer's directions and incubated with goat anti-*P. pyralis* luciferase antibody conjugated to horseradish peroxidase (Abcam, Inc, Cambridge, MA) (1:100 000 dilution) for 1 h at ~22 °C. After thorough washing with PBS-1% Tween, the bound luciferase-antibody complex was visualized using the 3, 3´, 5, 5´-Tetramethylbenzidine (TMB) Liquid Substrate System (Sigma-Aldrich, St. Louis, MO).

Microplate luminometer assays. Light measurements were performed in triplicate in 96-well plates (LUMITRAC) using a Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). Unless otherwise noted, each well contained 2 – 10 µl of sample in 200 µl of 91 µM LH_2 in 25 mM glycylglycine buffer (pH 7.8) and assays were initiated by the automated injection of 60 µl of Mg-ATP (9 mM) in the same buffer. Light output was integrated for 8 s following a 2 s delay. No corrections were applied for the spectral response of the PMT.

For activity assays of individual GST-fusion proteins from soluble cell lysates (Fig. 4), stock solutions were prepared by diluting lysates with equal volumes of CBA containing 1 mg/ml BSA. The stock solutions were serially diluted and 2 µl aliquots (2.4 amol - 2.4 pmol) were assayed as described above. Light measurements with the green- and red-emitting mutants were made with the corresponding green (Kodak Wratten 44A) or red (Kodak Wratten 29) filters in place as described previously [30]. These values were corrected for background luminescence and the transmittance of the filters [30].

For dual assays of mixtures containing GST-fusion proteins of Ppy GR-TS and Ppy RE-TS either in soluble cell lysates or in suspensions of intact *E. coli* cells (Fig. 6), duplicate plates were assayed sequentially with each filter in place. In the cell lysate protocol, 10 µl of luciferase (1 fmol total) containing varying amounts of the Ppy GR-TS and Ppy RE-TS GST-fusion proteins (Fig. 5) were analyzed as described above. Mixtures of intact cell suspensions containing the red- and green-emitting luciferases (120 µl total) in varying amounts were assayed (8 s integration) 99 s after the automated addition of an equal volume of Chroma-Glo Reagent (Fig. 6). The transmission properties of the filters and the spectral overlap of the bioluminescence emission spectra (normalized) are shown in Fig. 1. The relative contributions of the two enzymes to the total light emission were determined from the assay data using the Promega "calculator" [47] as previously described [30].

Results and discussion

Characterization of luciferase mutants.

The pair of luciferase enzymes Ppy RE and Ppy GR containing the Ser284Thr and Val241Ile/Glt246Ala/Phe250Ser mutations, respectively, were documented [30] to be well-suited for reporter applications having well separated emission spectra, yet similar specific activities and steady state kinetic constants at 25 °C (Tables 1 and 2). The emission maxima of the Ppy GR mutant, however, was broadened and slightly red-shifted at 37 °C (Table 2). Similar changes were observed at pH 7.0 and 25 °C; whereas both significant broadening and red-shifting occurred at pH 6.0. An even more serious problem was the poor thermostability of the color mutants at 37 °C. Ppy GR, like Ppy WT, had an *in vitro* 37 °C half-life of ~ 15 min in 25 mM glycylglycine buffer, pH 7.8 containing 7.1 mM NaCl, 40 mM ammonium sulfate and 0.1% glycerol, while the value for Ppy RE was ~ 48 min (Table 1). Recently, the loss of luciferase activity following even mild heat exposure was noted [48] as a cause for the misinterpretation of reporter activity. For reporter and other practical applications of luciferase proteins, poor thermostability seriously compromises the sensitivity and precision attainable. In addressing the issue of the poor thermostability of Luc, Tisi and coworkers [37, 39] employed a combination of rational and random mutagenesis methods to produce several heat stable variants. "Mutant E", a Luc containing the five changes Thr214Ala, Ala215Leu, Ile232Ala, Phe295Leu and Glu354Lys, had the longest *in vitro* half-life at 37 °C of 82.1 min, a value nearly 27-fold higher than that of the wild-type luciferase (3.06 min). The difference between this value measured in Hanks Balanced Salt Solution and our measurement (15.6 min) probably reflects the stabilizing effect of ammonium sulfate and glycerol, additives that we routinely use to increase the long-term storage of luciferase solutions. However, any differences in protein concentration also would be relevant because we found luciferase half-lives generally increased with increasing protein concentration (data not shown). Although the conditions used in both *in vitro* half-life studies were simple models, it is useful to compare the relative changes in the wild-type values resulting from introduced mutations.

Using four mutagenic primers, we introduced the five mutations found in "mutant E" into Ppy WT, Ppy RE and Ppy GR and expressed the corresponding proteins, Ppy WT-TS ("mutant E"), PpyRE-TS and Ppy GR-TS, as GST fusion proteins. The luciferases were purified by affinity chromatography and contained the additional N-terminal peptide GlyProLeuGlySer- [41] that remained after PreScission protease cleavage to remove GST. Average yields of 10-14mg of purified proteins per 0.5 L culture were obtained. CD spectra of the luciferases were obtained and the summarized results (Table 1) showed no significant differences indicating that the mutations did not affect the wild-type protein backbone conformation. A comparison of the flash-height based specific activities and the K_m values for LH_2 and Mg-ATP of the luciferases Ppy RE to the corresponding TS versions revealed only minor differences (Table 1). With the Veritas microplate luminometer used in these studies, the green/red ratio of the specific activities of the thermostable mutants was 3.9; whereas the ratio determined with the manual single tube instrument was 1.5 (Table 1). Since the integrated activity data were not corrected for the spectral response of the detector, this difference can mainly be attributed to lower spectral sensitivity of the microplate luminometer to the red emission of Ppy RE-TS. It is significant, however, that with the usual concentrations of assay reagents, the additional mutations do not appreciably lower the integration-based specific activities measured in a microplate luminometer format (Table 1).

Table 1

Properties of purified luciferase enzymes at pH 7.8

Enzyme	Relative specific activity [a]		K_m (μM)		Half-life, 37 °C (h)	T_m (°C)[b]	Molar ellipticity [Θ] (deg cm^2/dmol)	
	Integration (8 sec)	Flash Height	LH_2	Mg-ATP			208 nm	222 nm
Ppy WT	100	100	15 ± 2	160 ± 20	0.26	46.6	-8093 ± 182	-7796 ± 208
Ppy WT-TS	95	87	23 ± 2	89 ± 9	11.5	58.3	-8483 ± 310	-7685 ± 211
Ppy GR	67	50	6 ± 1	93 ± 20	0.23	46.7	-8552 ± 210	-7576 ± 148
Ppy GR-TS	58	46	23 ± 3	163 ± 27	10.5	66.0	-8423 ± 443	-8075 ± 425
Ppy RE	18	24	8 ± 1	172 ± 15	0.8	47.3	-8131 ± 430	-7907 ± 452
Ppy RE-TS	15	31	18 ± 2	68 ± 5	8.8	55.0	-8411 ± 148	-7913 ± 160

[a] Enzyme activity values used to calculate integrated specific activities were obtained as described under microplate luminometer assays in Materials and methods and were not corrected for the spectral response of the detector. Assays for flash height-based activities assays as described previously [42] and were corrected for the spectral response of the Turner TD-20e H6199 photomultiplier tube employed. [b] T_m, mean aggregation temperature determined by CD spectroscopy monitored at 222 nm as described in Materials and methods.

Thermal and pH stability of luciferase mutants.

With *in vitro* reporter methods, assay temperature and pH can be carefully controlled; however; the genetic activity being measured by expression of analyte proteins typically occurs at 37 °C. For this reason, and with *in vivo* applications enzyme stability at 37 °C is paramount. The 44-fold increase in the 37 °C half-life of Ppy WT-TS over Ppy WT confirmed the dramatic increase in thermostability that was reported [39] for "mutant E". A similar increase in heat stability was realized with the Ppy GR-TS enzyme (37 °C half-life = 10.5 h), while Ppy RE-TS had a slightly lower 37 °C half-life of 8.8 h (Table 1). To further characterize the heat stability of the luciferases, we measured mean aggregation temperatures (T_m) by CD spectroscopy monitored at 222 nm. Ppy WT, Ppy GR and Ppy RE had T_m values of ~47 °C, and displayed similar tendencies to aggregate [49] as had been reported for Luc using different methods. The 8 °C to 19 °C increases in T_m values for the enzymes containing the TS mutations were striking (Table 1); further substantiating the improved stability, illustrated here by the resistance to heat-induced aggregation, that was achieved with five amino acid changes.

Table 2

Effect of pH and temperature on bioluminescence emission [a]

Enzyme	pH 6.0, 25 °C			pH 7.0, 25 °C			pH 7.8, 25 °C			pH 7.8, 37 °C		
	Maxima	Bandwidth		Maxima	Bandwidth		Maxima	Bandwidth		Maxima	Bandwidth	
		50%	20%		50%	20%		50%	20%		50%	20%
Ppy WT	613	61	129	562, 603	102	148	557	68	113	563, 610 (sh)	92	135
Ppy WT-TS	565, 599 (sh)	95	138	558	73	118	556	68	113	565, 610 (sh)	94	140
Ppy GR	596, 560 (sh)	97	105	551	81	128	548	68	113	553	80	126
Ppy GR-TS	549	72	124	547	67	111	546	66	110	552	75	123
Ppy RE	616	56	107	616	56	112	615	56	110	616	64	120
Ppy RE-TS	614	59	111	612	59	114	610	59	111	612	60	116

[a] Bioluminescence emission spectra of purified luciferases were acquired as described in Materials and methods.

Bandwidths (nm) of emission spectra were measured at 50 and 20% of the intensity at the maximum wavelength (nm).

The underlying assumptions for developing thermostable mutants Ppy RE-TS and Ppy GR-TS for reporter and imaging applications at 37 °C are that greater sensitivity will be realized because the enzymes will be expressed as active folded proteins in higher yield and that they will maintain their activity longer than the corresponding Ppy RE and Ppy GR. The latter point is substantiated by the *in vitro* 37 °C half-life and T_m data (Table 1) discussed above. We expect that similar relative improvements will be realized *in vivo* as was the case with the 5-fold improvement for "mutant E" compared to Luc in mammalian tumor cell lines [39]. The lower *in vivo* 37 °C relative half-life improvement (5-fold versus 27-fold *in vitro*) for "mutant E" compared to Luc was a result of the greater increase (16-fold) in the stability of Luc *in vivo* probably because of the stabilizing effect of chaperone proteins [39, 50, 51].

Western blot analysis (Fig. 2) qualitatively demonstrated the differences in yields of luciferases with and without the TS mutations in soluble cell lysates from 5 ml LB cultures of bacteria grown at 22 °C and 37 °C. The experiments were performed without induction to model difficult conditions that might be encountered with low level reporter signals. While the levels of expression of all the soluble GST-luciferase fusion proteins were similar in bacteria grown at 22 °C (Fig. 2, panels A and B), the expression of the proteins containing the TS mutations at 37 °C (Fig. 2, panels C and D, lanes 5 - 7) far exceeded that of the proteins lacking the mutations (Fig. 2, panels C and D, lanes 2 - 4). The total yields of soluble GST-luciferases from the 5 ml cultures were then estimated from assays of GST activity using GST-Luc as the standard (Materials and methods). The luciferases containing the TS mutations (Fig. 3, lanes 2, 4 and 6) were expressed at similar levels at 22 °C and 37 °C, while those lacking the mutations (Fig. 3, lanes 1, 3 and 5) expressed well at 22 °C, but at ~10-fold lower levels at 37 °C. The specific activities of all the TS versions of the luciferases were approximately the same at both temperatures, whereas the other luciferases had the expected specific activities at 22 °C and ~100-fold lower values at 37 °C (data not shown). These data taken together confirmed that the

Ppy RE-TS and Ppy GR-TS enzymes were expressed in bacteria at 37 °C as active folded proteins in higher yield than were their counterparts with no mutations. For dual-color bioluminescent reporter assays, minimal overlap between the two signals is highly advantageous. Therefore, the emission maxima should be well separated and the spectral bandwidths should be narrow. Ppy GR and Ppy RE provided a very good example of a suitable set of emitters having emission maxima at pH 7.8 and 25 °C of 548 nm and 615 nm, a 67 nm separation [30]. The bandwidth of the green emission was similar to Ppy WT, while the red emission was somewhat sharper (Table 2). These desirable characteristics were essentially maintained in Ppy RE-TS and Ppy GR-TS, whose emission maxima at pH 7.8 and 25 °C were 546 nm and 610 nm, respectively (Fig. 1, Table 2). While the TS mutations provided enhanced, but incomplete, pH stability of bioluminescence emission to Ppy WT, Ppy GR-TS became nearly completely pH resistant displaying only very minor peak broadening at pH 6.0. The already pH resistant Ppy RE maintained this favorable attribute. The TS mutations stabilized luciferase activity at 37 °C and bioluminescence color at 25 °C; however; they had very little effect on the latter at 37 °C, evidence that bioluminescence red-shifting occurs by multiple mechanisms. Based on the physical characterization data presented here, Ppy RE-TS and Ppy GR-TS are significantly stabilized versions of the previously described [30] red- and green-emitting luciferases, Ppy RE and Ppy GR.

Feasibility of using Ppy RE-TS and Ppy GR-TS in dual-analyte assays. To further evaluate the potential of Ppy RE-TS and Ppy GR-TS as reporters of dual-analyte concentrations, a model system was developed using *E. coli* cell lysates from cells grown at 37 °C without induction. Microplate luminometer assays of individual thermostable luciferases expressed as GST-fusion proteins demonstrated that light emission was linearly proportional to the amount of each enzyme over 6 orders of magnitude from 2 amol to ~ 2 pmol for Ppy GR-TS and 5 amol to ~2 pmol for Ppy RE-TS (Fig. 4). These detection limits are quite similar to those previously reported [30] in similar experiments with the Ppy GR (1 amol) and Ppy RE (4 amol) enzymes grown at 22 °C. The concept of detection limits in dual-color assays is complex because noise is contributed by any luminescence from one mutant transmitted through the filter used to monitor the other (Fig. 1). Moreover, this phenomenon is concentration dependent so that the detection limits and working ranges for each emitter are dependent on the concentration of the other. A more detailed discussion of this concept has been advanced elsewhere [30].

We next demonstrated that the relative amount of each thermostable luciferase variant grown at 37 °C in mixtures containing 1.0 fmol total luciferase could be quantified from measurements of simultaneously emitted red and green light (Fig. 5). The results were significant because they indicated that it is feasible to monitor two distinct activities at 37 °C with Ppy-RE-TS and Ppy GR-TS. An additional model study was undertaken to demonstrate that the *P. pyralis* thermostable luciferase mutants could be adapted for use with a single commercial reagent in a standard microplate luminometer protocol. For this experiment, we used the Veritas microplate luminometer with the Promega Chroma-Glo Luciferase Assay System protocol [47] and the Chroma-Glo Reagent, a solution designed to lyse mammalian cells and produce a stable luminescent signal. As shown in Fig. 6, the relative amount of Ppy RE-TS and Ppy GR-TS in mixtures of intact *E. coli* BL21 cells grown at 37 °C could be quantified from measurements of simultaneously emitted red and green light in a standard commercial format.

The results of the proof-of-concept experiments reported here indicate that the Ppy RE-TS enzyme has great potential as a bioluminescent reporter as well as in imaging applications.

In conjunction with Ppy GR-TS, dual-color reporter systems for genetic monitoring or dual-analyte environmental screening methods could be developed. The *P. pyralis* variants have excellent thermal and pH stability providing a set of proteins that have similar expression levels, require only a single synthetic substrate, have similar specific activities and offer good signal separation. While we believe that the proteins described here may be used in more demanding mammalian cell applications, we are currently working to develop reagents with non-overlapping emission spectra.

[1]*Abbreviations used*: BSA, bovine serum albumin; CB, 50 mM Tris-HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT; CBA, CB containing 0.8 M ammonium sulfate and 2% glycerol; GST, glutathione-S-transferase; LB, Luria-Bertani; LH_2, D-firefly luciferin; LH_2-AMP, D-luciferyl-O-adenosine monophosphate; Luc, *Photinus pyralis* luciferase (E.C. 1.13.12.7); PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3); Ppy WT, recombinant *Photinus pyralis* luciferase containing the additional N-terminal peptide GlyProLeuGlySer-; Ppy-GR, Ppy WT containing Val24Ile/Gly246Ala/Phe250Ser; Ppy-RE, Ppy WT containing Ser284Thr; RLU, relative light units; TS, Thr214Ala/Ala215Leu/ Ile232Ala/Phe295Leu/Glu354Lys.

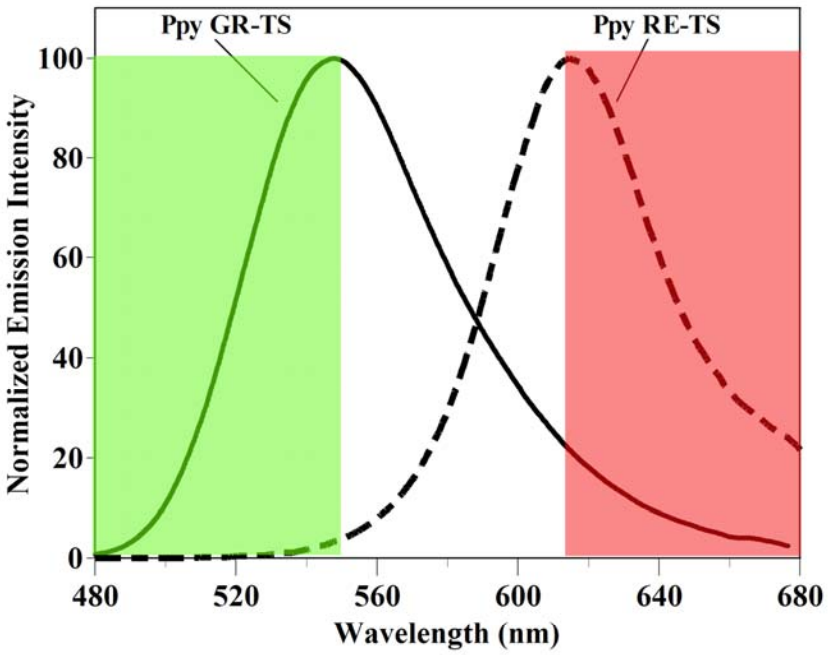

Fig. 1. Normalized bioluminescence emission spectra produced by the Ppy GR-TS and Ppy RE-TS luciferases at pH 7.8. Emission spectra were obtained with purified proteins as described in

Materials and methods. The red and green shadings indicate the spectral regions transmitted through the green (Kodak Wratten 44A) and red (Kodak Wratten 29) filters.

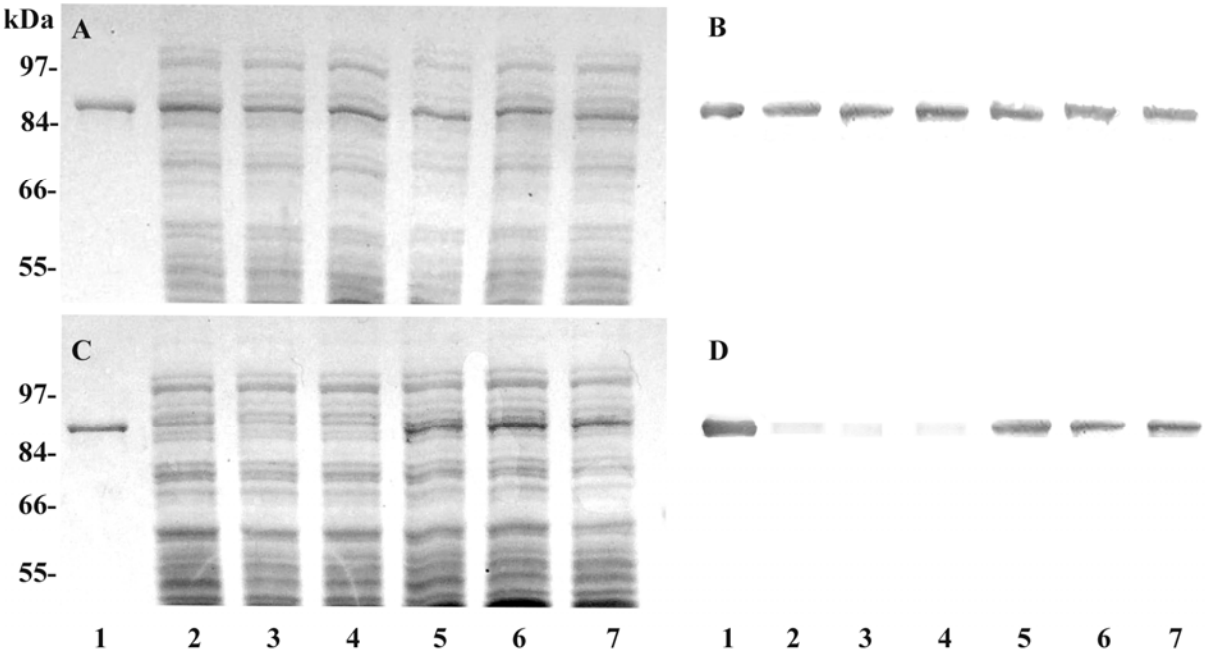

Fig. 2. SDS-PAGE and Western blot analysis of GST-luciferase fusion proteins grown at 22 °C (Panels A and B) and 37 °C (Panels C and D). Soluble cell lysates were isolated from duplicate liquid cultures (5 ml) of *E. coli* BL21 cells expressing GST-fusion proteins grown at 22 °C and 37 °C without induction. Aliquots of lysates (4 µl) were separated by duplicate SDS-PAGE (7.5% gels). One set of gels was stained with Coomassie R-250 to visualize protein bands (Panels A and C) and the other set was electroblotted onto nitrocellulose, followed by reaction with anti-*P. pyralis* luciferase antibody (Panels B and D). The gel lanes represent: 1, purified GST-Luc fusion protein; and the soluble cell lysates of GST-fusion proteins: 2, Ppy WT; 3, Ppy GR; 4, Ppy RE; 5, Ppy WT-TS; 6, Ppy GR-TS; and 7, Ppy RE-TS. The migration of molecular weight standards is indicated.

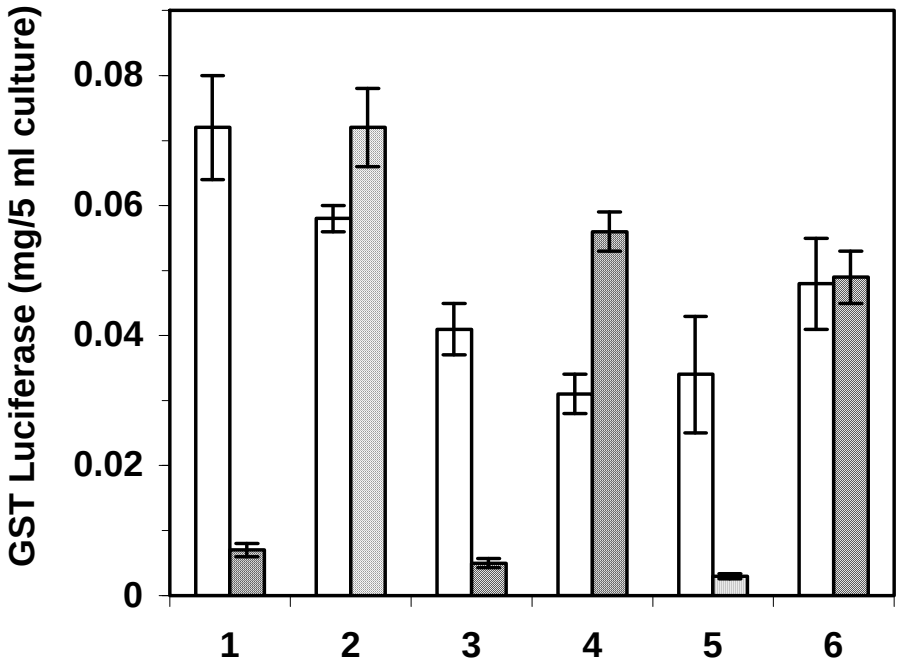

Fig. 3. Effect of growth temperature on the expression of soluble GST-luciferase fusion proteins. At 22 °C and 37 °C, triplicate liquid cultures (5 ml) of *E. coli* BL21 cells were grown without induction expressing GST-fusion proteins of: 1, Ppy WT; 2, Ppy WT-TS; 3, Ppy GR; 4, Ppy GR-TS; 5, Ppy RE; and 6, Ppy RE-TS. Soluble cell lysates were isolated from cultures grown at 22 °C (open bars) and 37 °C (hatched bars) and the levels of the soluble fusion proteins were estimated from GST activity assays performed as described in Materials and methods. Mean values are plotted and SD is indicated by error bars.

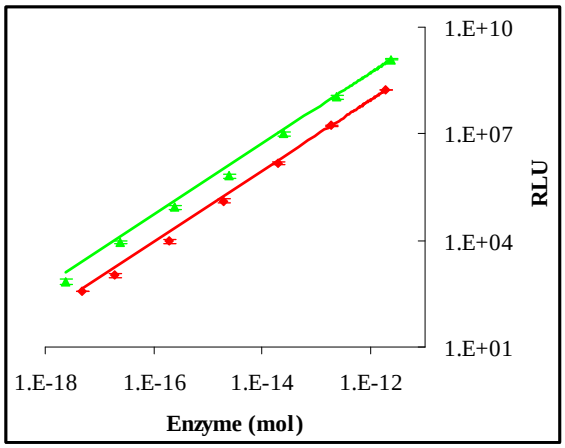

Fig. 4. Relationship of the relative bioluminescence activity of the thermostable green- and red-emitting GST-luciferases to enzyme concentration. Luciferase activity (RLU) of the GST-fusion proteins in soluble cell lysates (from *E. coli* BL21 cells grown at 37 °C without induction) containing Ppy GR-TS (green line, R^2=0.99) or Ppy RE-TS (red line, R^2=0.99) were measured with the green and red filters in place. Assays (0.262 ml) were performed at pH 7.8 as described under microplate luminometer assays in Materials and methods. Mean values are plotted and SD is indicated by error bars.

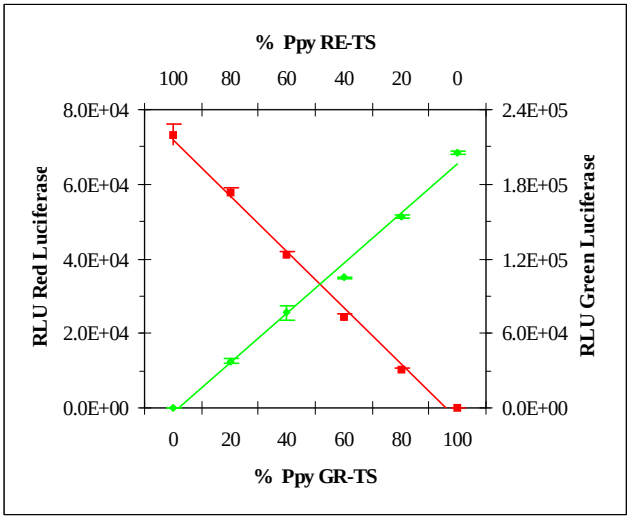

Fig. 5. Relative bioluminescence activity of Ppy GR-TS (green line, R^2=0.99) and Ppy RE-TS (red line, R^2=0.99) GST-fusion proteins in soluble cell lysates (from *E. coli* BL21 cells grown at 37 °C without induction). The luciferases were present in the varying percentages indicated in assays containing 1.0 fmol total enzyme. Assays (0.270 ml) were performed at pH 7.8 as described under microplate luminometer assays in Materials and methods. Mean values are plotted and SD is indicated by error bars.

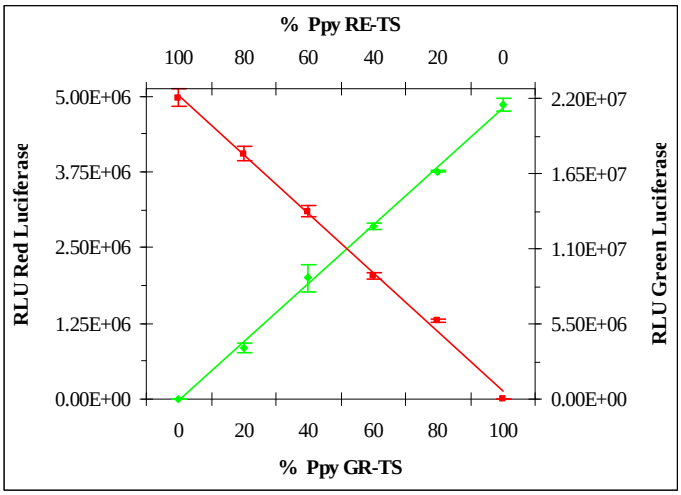

Fig. 6. Relative bioluminescence activity of Ppy GR-TS (green line, R^2=0.99) and Ppy RE-TS (red line, R^2=0.99) GST-fusion proteins in *E. coli* BL21 cells grown at 37 °C without induction. The luciferases were present in the varying percentages indicated in assays containing 0.120 ml of intact *E. coli* BL21 cells. Assays (0.240 ml) were initiated by the automated injection of Chroma-Glo Reagent as described under microplate luminometer assays in Materials and methods. Mean values are plotted and SD is indicated by error bars.

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Major Accomplishment 2.

(2) A more accurate correlation between two gene expression measurements may be achieved by using the same reagents to initiate multiple reactions simultaneously. Promega's Chroma-Glo™ Luciferase Assay System [26] for dual-color assays uses one substrate to activate green- and red-emitting (CBR) luciferases derived from the Jamaican click beetle *Pyrophorus plagiophthalamus*. By employing a luminometer equipped with optical filters, it is possible to sufficiently separate the green and red signals. While the detection sensitivity is quite good, the spectral overlap caused by the long wavelength tail of the green emitter does compromise this parameter. Unfortunately, this emission tailing appears to be an intrinsic property of beetle luciferases that emit green light [3].

Building on our experience producing *P. pyralis* luciferase variants with altered physical and spectral properties, we sought to develop Luc enzymes that would provide improved performance in dual-color assays. An improved red emitter would also have great potential for *in vivo* imaging applications, since light greater than 600 nm penetrates living tissue more effectively [27]. Previously, we developed the *P. pyralis* variants Ppy GR-TS and Ppy RE-TS that displayed bioluminescence emission maxima of 546 nm and 610 nm, respectively [28]. The improved thermostability of these proteins over an earlier pair of *P. pyralis* mutants [29] enhanced expression at 37 °C, making them potentially more suitable for applications in mammalian cells. In model systems using pure proteins or bacterial cell lysates, fmol levels of total luciferase could be quantified from measurements of simultaneously emitted red and green light using a luminometer equipped with optical filters.

The study reported here focuses on the further optimization of Ppy RE-TS with the aim of improving the sensitivity of dual-color reporter assays and imaging applications. We describe the development of Ppy RE8 and Ppy RE9, two novel *P. pyralis* red emitters that were made by altering Ppy RE-TS with four and five additional amino acid changes, respectively. We present a systematic comparison of the spectral and physical properties of the new proteins with CBR and PhRE, the red-light producing luciferase from the railroad worm *Phrixothrix hirtus* [30]. Because Ppy RE8, Ppy RE9, and CBR exhibited promising qualities as pure proteins, these enzymes subsequently were evaluated in model reporter assays using mammalian cell lysates. Noting that among other enhancements, the CBR gene has been codon-optimized for improved expression in mammalian cells [31], we developed codon-optimized versions of Ppy RE8 and Ppy RE9. The results from testing in HEK293 cells suggest that Ppy RE8 and Ppy RE9 may be improved alternatives to Promega's CBR for dual-color reporter and *in vivo* imaging applications, and illustrate the importance of codon-optimization for assays in mammalian cells.

Materials and methods

Materials

The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) from Sigma-Aldrich (St. Louis, MO); restriction endonucleases and Phusion polymerase from New England Biolabs (Ipswich, MA); oligonucleotides from Invitrogen and Integrated DNA Technologies (Carlsbad, CA and Coralville, IA); Glutathione Sepharose 4B media and pGEX-6P-2 expression vector from GE Healthcare (Piscataway, NJ); QuikChange® Site-

Directed Mutagenesis kit from Stratagene (La Jolla, CA); QIAquick PCR purification and gel extraction kits from Qiagen (Valencia, CA); DMEM and Opti-MEM® I reduced-serum medium from Invitrogen (Carlsbad, CA); FBS from Thermo Scientific Hyclone (Logan, UT); *Trans*IT®-LTI transfection reagent from Mirus Bio (Madison, WI). The recombinant Ppy RE-TS protein was expressed and purified as previously reported [28]. The pCBR-Basic Vector, LH_2, Luciferase Assay Reagent (LAR), and Flexi® Vector pF9a CMV were from Promega (Madison, WI). The PhRE gene in the pTrc-HisC vector was provided by Dr. Ariane Söling from Martin-Luther-Universität Halle-Wittenberg.

General methods

Concentrations of purified proteins were determined with the Bio-Rad Protein Assay system using bovine serum albumin as the standard. Luciferase genes in the pGEX-6P-2 and Flexi®Vector pF9a CMV vectors were verified by DNA sequencing at the W. M. Keck Biotechnology Laboratory (Yale University, New Haven, CT) and at Agencourt Bioscience Corporation (Beverly, MA), respectively. 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.

Site-directed mutagenesis

Starting with the Ppy RE-TS gene in the pGEX-6P-2 vector [28], the QuikChange® Site-Directed Mutagenesis kit was used to introduce the following mutations using the indicated primers and their reverse complements: Arg330Gly, 5´ - G GTT GCA AAA **GGC** TTC CAT CTT CCA GGG ATA CGC CAA GGA TAT G- 3´ [*Sty*I]; Ile351Val/Lys354Glu, 5´ - GAG ACT ACT AGC GCT ATT CTG **GTA** ACA CCC **GAG** GGG GAT GAT AAA C- 3´ [*Spe*I]; Phe465Arg, 5´ - GAA TTG GAA TCC ATA TTG TTA CAA CAC CCC AAC ATC **CGG** GAC GCG GGC- 3´ [*Cla*I] (bold represents the mutated codon, underline represents silent changes to create a unique screening endonuclease site and brackets indicate the screening endonuclease). The gene for the resulting luciferase variant, named Ppy RE8, was further modified using the following primer and its reverse complement, producing a mutant named Ppy RE9: Glu354Ile, 5' - GAG ACT ACT AGT GCT ATT CTG GTA ACA CCC **ATC** GGG GAT GAT AAA C- 3' [*Spe*I].

Insertion of CBR and PhRE cDNA into the pGEX-6P-2 vector

The following primer sets were used to amplify CBR*luc* from Promega's pCBR-Basic vector and *PhRE* cDNA from the pTRC-HisC vector: CBR, 5´- GGT AAA GGA TCC ATG GTA AAG CGT GAG AAA AAT GTC-3´ [*Bam*HI] and 5´-ACT CAT CTC GAG ATC TTA TCA TGT CTG CTC GAA G-3´ [*Xho*I]; P. hirtus, 5´- GGA GGA TCC ATG GAA GAA GAA AAC GTT GTG AAT G-3´ [*Bam*HI] and 5´- GC AAT AGC GGC CGC TTA TAA TTT TGA TTT TGC CTG-3´ [*Not*I] (underline represents the endonuclease site introduced for cloning into the pGEX-6P-2 vector and brackets indicate the endonuclease). PCR amplification was performed by initial denaturation at 95 °C for 2 min, a 30-cycle amplification (95 °C for 30 s, 55 °C for 30 s and 68 °C for 1.75 min) and a final extension at 68 °C for 5 min. PCR products were cleaned with the QIAquick PCR purification kit and digested with the above restriction endonucleases. Digested products were purified from an agarose gel using the QIAquick gel extraction kit and ligated into the corresponding sites on the pGEX-6P-2 vector.

Protein expression and purification

Glutathione-S-transferase (GST) fusion constructs of all enzymes were expressed in *E. coli* strain BL21. Cultures (250 ml in Luria-Bertani media with 100 μg/ml ampicillin) were grown in 1 L flasks at 37 °C to mid log phase (A_{600} = 0.5-0.7), then induced with 0.1 mM isopropyl-β-d-thiogalactopyranoside and incubated at 22 °C for 18-20 h. Cells were harvested by centrifugation at 4 °C and then frozen at -80 °C for 15 min. Cell pellets were resuspended in 25 ml of PBS containing 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol. After the addition of lysozyme (2.5 ml of 10 mg/ml solution in PBS), 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 20,000 *x g* for 45 min. Proteins were further purified using Glutathione Sepharose® 4B affinity chromatography according to the manufacturer's instructions. During the purification, luciferases were released from GST by incubation with PreScission protease in CB (cleavage buffer) for 18-20 h at 4 °C with gentle mixing. Proteins were eluted with CB and were either flash frozen in liquid N_2 for long-term storage at -80 °C or were stored at 4 °C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).

Transfection of HEK293 mammalian cells with Luc mutants

Before transfection, all luciferase genes were PCR amplified and cloned into the *Sgf*I/*Pme*I sites of the mammalian expression Flexi® Vector pF9a CMV. HEK293 (ATCC) cells were aliquoted into 6-well plates at a concentration of ~450,000 cells per well in DMEM + 10% FBS. Cells were allowed to recover overnight at 37 °C in 5% CO_2. For each well of mammalian cells to be transiently transfected, 95 μl Opti-MEM® I reduced-serum medium was combined with 6 μl *Trans*IT®-LTI transfection reagent. The mixture was vortexed at the highest setting for 1 s and incubated for 20 min at ambient temperature, then combined with 2 μg of plasmid DNA. The mixture was again incubated at ambient temperature for 20 min before it was added to the mammalian cells. Transfections were allowed to proceed for ~24 h at 37 °C in the presence of 5% CO_2.

Preparation of soluble cell lysates from Luc mutants expressed in HEK293 cells

Following transfection with luciferase mutants, HEK293 cells (200,000 cells) were harvested by trypsinization and pelleted by centrifugation at 1000 rpm for 5 minutes. The cell pellets were then resuspended in 0.1 ml PBS. Cells were lysed by 5 freeze-thaw cycles at -80 °C and 30 °C, and 0.01 ml of 10% Triton X-100 was added.

Bioluminescence specific activities

Bioluminescence activity assays were performed with purified luciferases using a custom-built luminometer assembly, containing a Hamamatsu R928 photomultiplier tube and a C6271 HV power supply socket assembly, which has previously been described in detail [32]. Reactions were initiated by the injection of 120 μl of 9.0 mM ATP into 8 x 50 mm polypropylene tubes containing 0.4 ml of 25 mM glycylglycine buffer (pH 7.8) with 0.4 mM LH_2 and 0.5–2 μg of enzyme. The final concentrations of LH_2 and Mg-ATP were 0.3 mM and 2.0 mM, respectively, in a volume of 0.525 ml. Light output was monitored for 60 s and peak

height and integrated intensity values were recorded and corrected for the spectral response of the Hamamatsu R928 photomultiplier tube.

Steady-state kinetic constants

The K_m values for LH_2 and Mg-ATP for all luciferases were determined using the bioluminescence activity assays described above, taking maximal light intensities as estimates of initial velocities. Data were analyzed using Enzyme Kinetics Pro software (Syntex) as previously described [33].

Heat inactivation studies

Enzymes in CB were diluted to 0.08 mg/ml in 0.4 ml of 25 mM glycylglycine buffer (pH 7.8) and incubated at 37 °C. Aliquots (2-3 µl) were removed over an 8 h period and assayed for bioluminescence activity as described above.

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 and 37 °C using previously described equipment and settings [33]. Reactions (0.525 ml) were initiated by adding 2-18 µg of luciferase in 25 µl CBA to a cuvette containing solutions of LH_2 (0.3 mM final) and Mg-ATP (2.0 mM final) in 25 mM glycylglycine pH 7.8. The spectral measurements at 37 °C were obtained 30 min after the addition of the luciferases to the buffered reagent solutions maintained at 37 °C.

Model reporter assays in mammalian cell lysates

Luminescence measurements of soluble cell lysates from HEK293 mammalian cells were performed in triplicate assays in 96-well tissue culture plates (Costar) using a Varioskan Flash spectral scan multimode plate reader (Thermo Fisher Scientific, Waltham, MA) programmed with SkanIT Software version 2.4. Each well contained 10 µl of soluble cell lysate in 200 µl of 0.4 mM LH_2 (0.3 mM final) in 25 mM glycylglycine buffer (pH 7.8). Assays were initiated by injection of 60 µl of 9 mM Mg-ATP (2 mM final) in the same buffer (Fig. 4). The assays (270 µl) were optimized by including CoA (0.1 mM final) and by reducing the concentrations of LH_2 (0.2 mM final) and Mg-ATP (400 mM final) (Fig. 5A). For studies using LAR, each well contained 10 µl of soluble cell lysate, and assays were initiated by the injection of 100 µl LAR (Fig. 5B). Bioluminescence signals, integrated over 60 seconds, were measured using a 600 nm long pass filter and a PMT voltage automatically adjusted using the AutoRange default setting. The Varioskan Flash spectral scan multimode plate reader has a built in delay of 0.8 s before the first data point is recorded.

Codon-optimization of Ppy RE8 and Ppy RE9

An optimized DNA sequence for the wild-type *P. pyralis* luciferase gene was composed for improved expression in mammalian cells. The sequence was altered to eliminate repeats, local hairpins and cryptic splice sites while maximizing the optimal codon usage for human cells, as reported in the codon usage database [34]. To enhance the transcript stability and translation efficiency, a GC content of ~ 70% was maintained. This codon-optimized Luc DNA sequence was synthesized by PCR of overlapping oligonucleotides and ligation with Phusion polymerase,

performed using a previously reported protocol [35]. The Luc gene was inserted into a shuttle vector, and the DNA sequence was verified by 3X capillary sequencing.

To create codon-optimized versions of Ppy RE8 and Ppy RE9, point mutations were introduced into the optimized Luc sequence by overlap PCR with mutagenic oligonucleotides and Phusion polymerase. The codons containing the mutations were optimized for mammalian cell expression, as described above. After confirmation of the mutations by capillary sequencing, the codon-optimized Ppy RE8 and Ppy RE9 gene constructs were digested with the *BamH*I/*Not*I restriction endonucleases and subcloned into the pGex-6p-2 vector. The DNA sequences were deposited in GenBank, under accession numbers GQ404465 (Ppy RE8) and GQ404466 (Ppy RE9).

Results and Discussion

Rationale for mutagenesis

Aiming to develop thermostable Luc mutants with the reddest possible spectral emissions and high specific activities, we chose our best previous entry, Ppy RE-TS (λ_{max}= 610 nm), as a template for modification [28]. This luciferase contains 6 amino acid changes that red shift bioluminescence (Ser284Thr) and augment stability at 37 °C (Thr214Ala, Ala215Leu, Ile232Ala, Phe295Leu and Glu354Lys) while retaining 31% of the integration-based activity [28]. When the change Arg330Gly, which was discovered by extensive random mutagenesis (data not shown) was added to Ppy RE-TS, a desirable + 4 nm change in bioluminescence was produced. This red shift was accompanied by losses in thermostability and enzyme activity that were partially remedied by adding the respective Phe465Arg [36] and Ile351Val changes. Subsequently, Glu was restored to position 354 by reverting the Glu354Lys mutation, and the emission maximum was shifted an additional 3 nm from 614 nm to 617 nm (Table 1, Fig. 1). With respect to the wild-type *P. Pyralis* enzyme, the new luciferase, Ppy RE8, contained 8 amino acid changes. While the additional change Glu354Ile did not further red-shift bioluminescence as expected [37], the increased thermostability (Table 1) distinguished this new variant, named Ppy RE9, as the most promising luciferase mutant for further evaluation.

Table 1. Properties of purified luciferases at pH 7.8

Enzyme	Relative specific activity[a]		K_m (μM)		Thermal Inactivation (h)[b]	Bioluminescence emission maximum (nm)[c]	
	Peak	Integration (60 s)	LH_2	Mg-ATP		25 °C	37 °C
Ppy RE-TS	100	100	18 ± 2	68 ± 5	4.5	610 (59)	612 (60)
Ppy RE8	70	78	77 ± 8	391±39	3.5	617 (60)	618 (62)
Ppy RE9	77	71	84 ± 5	222 ± 24	6.0	617 (57)	617 (59)
CBR	9	41	22 ± 3	5.1 ± 0.8	5.5	617 (62)	619 (66)
PhRE	52	12	4.7 ± 0.6	119 ± 23	0.01	622 (60)	–

[a] Peak bioluminescence-based and 60 s integrated specific activities were measured as described in Materials and methods. All activity values are expressed relative to Ppy RE-TS, defined as 100. [b] Thermal inactivation is reported as time to 50% initial activity when incubated at 37 °C. [c] Bioluminescence emission spectra of purified proteins were obtained as described in Materials and methods, with bandwidths at full-width half-maximum (fwhm) indicated in parentheses. Due to the rapid inactivation of PhRE at 37 °C, a bioluminescence spectrum could not be recorded.

Expression and Purification of Luciferase Proteins.

The 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, and were obtained in average yields of 8.0 (Ppy RE8), 10.4 (Ppy RE9), 2.5 (CBR), and 2.1 (PhRE) mg/0.25 L culture. All enzymes generally remained greater than 90% active for up to 6 months when stored at 4 °C in CBA. 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.

Amino acid sequences, deduced from our DNA sequences results, were used to predict the protein masses (Da), which were all found to be within allowable experimental error (0.01%) of the mass spectral analysis: CBR, 60 596; PhRE, 61 154; Ppy RE8, 61 000; and Ppy RE9, 60 988. Our sequencing data for PhRE, however, predicted a single amino acid deviation from the GenBank entry (accession number AF139645.2) at position 6 (Val to Ile) that was confirmed by the +14 Da difference.

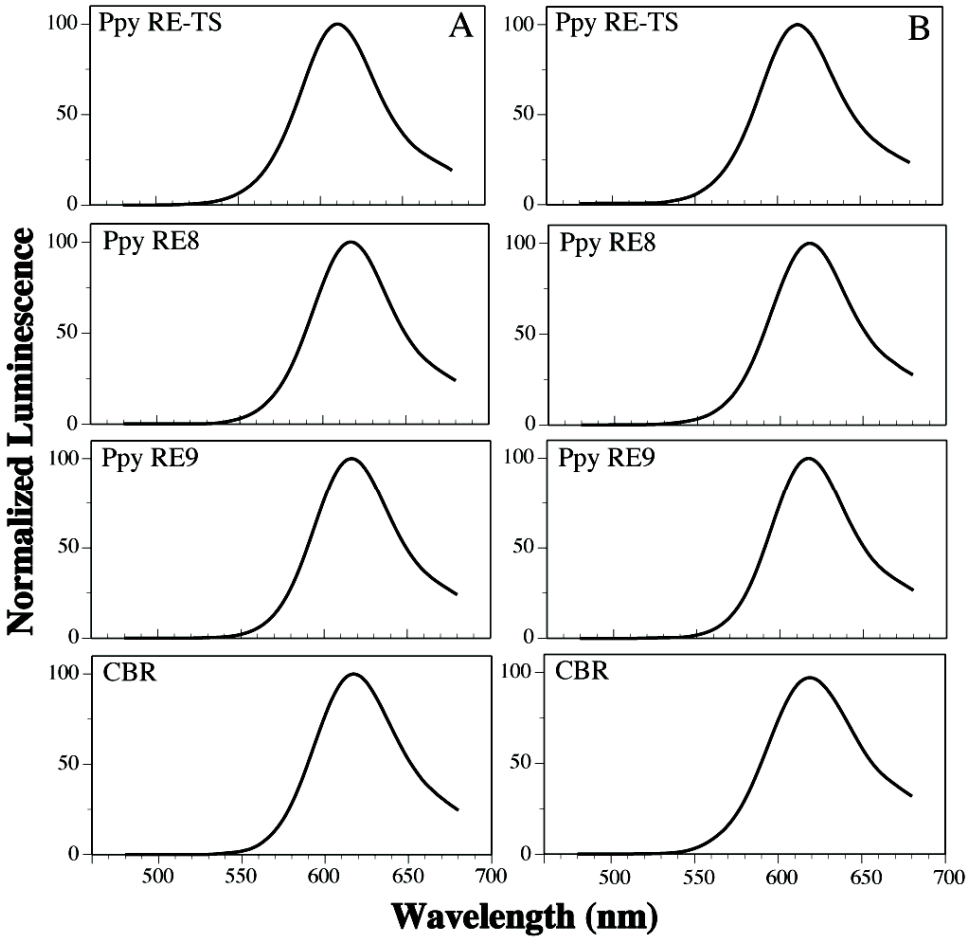

Fig. 1. Normalized bioluminescence emission spectra of purified luciferase enzymes measured at pH 7.8 and (A) 25 °C or (B) 37 °C, as described in Materials and methods.

Bioluminescence Color

For dual-color assays and imaging applications in mammalian cells, red shifted bioluminescence emission spectra, as well as high specific activity, thermostability and yield of expressed protein at 37 °C are all necessary characteristics of highly suitable reporter enzymes. Red shifted bioluminescence color was the primary objective in engineering Ppy RE8 and Ppy RE9, and this goal was met (Table 1, Fig. 1). Although the bioluminescence color of PhRE remained unrivaled (λ_{max}=622 nm at 25 °C), Ppy RE8 and Ppy RE9 emitted light nearly matching the impressive color of the CBR signal (λ_{max}=617 nm at 25 °C). Based solely on their bioluminescence color and emission profiles, the four luciferases studied are very good candidates for the intended applications. A serious challenge of detecting light from within living tissue is scattering and absorption by pigmented macromolecules such as hemoglobin and myoglobin. Light greater than 600 nm in wavelength exhibits greater transmission through these molecules compared to green light [27]; accordingly, red-emitting luciferases are reportedly detectable at lower levels than green-emitting luciferases *in vivo* [38]. For dual-color assays, red-emitting luciferases with narrow emission profiles are the best candidates, because overlap with a simultaneous green signal is minimized.

Thermostability and steady-state kinetic constants

For studies in mammalian cells, luciferases must be expressed at 37 °C. Thermostable luciferases have been shown to accumulate more rapidly within cells and remain active for a longer duration, thereby lending greater sensitivity to the detection method [39]. We estimated the thermostability of the luciferases by incubating them at 37 °C in a low ionic strength buffer and monitoring the decrease in bioluminescence activity. The results presented in Table 1 and Fig. 2 were obtained under identical conditions and are useful for making relative comparisons, however, luciferase stability is very dependent on buffer choice and ionic strength. For example, the inclusion of ~50 mM ammonium sulfate is sufficient to increase the time it takes Ppy RE-TS activity to decline 50% from 4.5 to 8.8 h [28]. Ppy RE8 and Ppy RE9 remained 50% active for 3.5 h and 6.0 h of incubation, respectively, while after 8 h at 37 °C the enzymes retained 28% and 42% of their initial activity (Table 1, Fig. 2). Over an 8 h time period, the stability of the bioluminescence activity of CBR and Ppy RE9 at 37 °C is quite similar and exceeds that of Ppy RE8 (Fig. 2). Compared to the already impressive thermostability of Ppy RE-TS, Ppy RE9 is somewhat more stable at physiological temperature (Table 1), an improvement that is attributable to the Glu354Ile mutation.

The heat inactivation assays also revealed a weakness of PhRE, which lost 50% activity after less than a minute of incubation at 37 °C (Table 1). However, PhRE has been paired with green-emitting luciferases from the same species (*Phrixothrix hirtus*) [40] and from the beetle *Rhagophthalmus ohbai* [41] to study circadian rhythms in whole cells. Possibly, protection against thermal denaturation in these studies was afforded by chaperone proteins in the cellular environment, as proposed by Baggett based on studies with Luc variants [39].

The amino acid changes that red-shifted the emission of Ppy RE8 and Ppy RE9 relative to Ppy RE-TS were also responsible for elevating the enzymes' K_m values for LH_2 (4- and 5-fold, respectively) and Mg-ATP (6- and 3-fold, respectively) (Table 1). Still, 2 mM standard Mg-ATP is sufficient for *in vitro* assays, and LH_2 concentrations can readily be adjusted to ensure saturation. For *in vivo* applications in which substrate availability is a concern, the 4.7 μM K_m

value of PhRE for LH_2 could offer an advantage. Likewise, the exceptionally low 5.1 μM K_m value of CBR for Mg-ATP could be exploited for ultrasensitive measurements of this analyte.

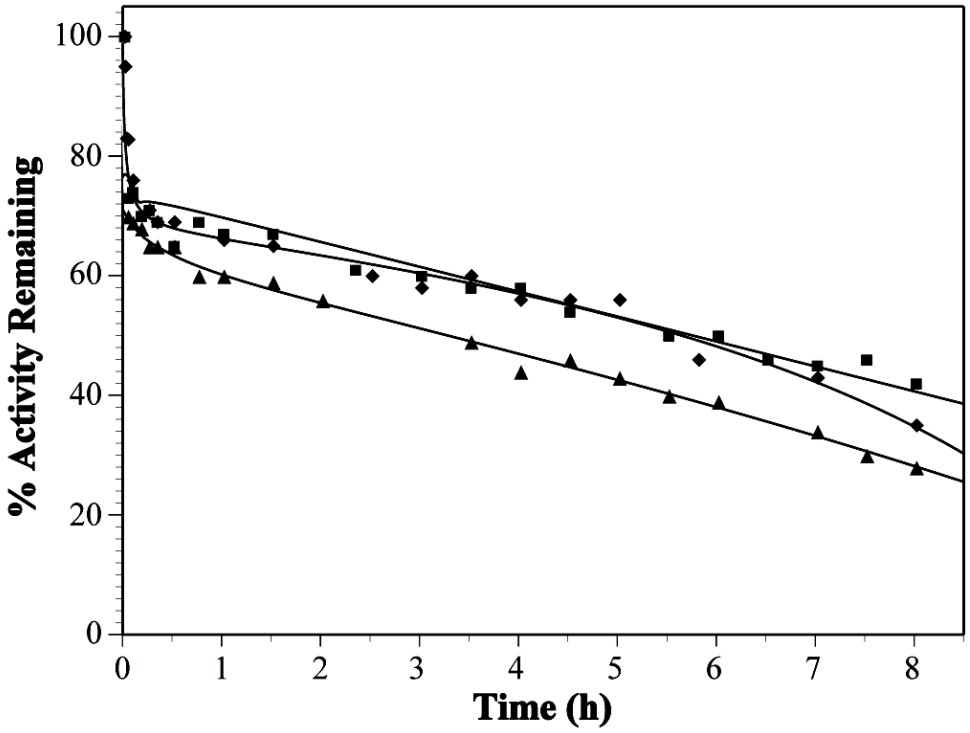

Fig. 2. Heat inactivation of purified luciferases Ppy RE8 (▲), Ppy RE9 (■) and CBR (◆) at 37 °C in 25 mM glycylglycine buffer (pH 7.8). Bioluminescence activities were monitored over 8 h as described in Materials and methods, and data were collected in duplicate.

Bioluminescence Specific Activities

The specific activities of the purified luciferases were determined from bioluminescence activity measurements using saturating levels of LH_2 and Mg-ATP, and data were corrected for the spectral response of the PMT. It is difficult to red-shift the emission spectra of luciferases without compromising specific activity. Fortunately, the Ile351Val mutation in Ppy RE8 and the additional Glu351Ile mutation in Ppy RE9 appeared to fortify the enzymes against dramatic activity loss. Relative to the template Ppy RE-TS, Ppy RE8 and Ppy RE9 retained 78% and 71% integration-based and 70% and 77% flash height-based specific activity, respectively (Table 1, Fig. 3). While CBR had only a 9% flash height-based specific activity, the slow signal decay resulted in a 60 s integrated activity of 41%, a value ~ 2-fold lower than the Ppy RE8 and Ppy RE9 activities. In contrast, PhRE emitted a bright flash (52% of Ppy RE-TS) but its rapid decay rate lowered the integrated activity to only 12% (Table 1, Fig. 3). Since PhRE exhibited relatively low integrated specific activity and poor thermostability at 37 °C (Table 1), we did not perform further studies with this enzyme. It is likely, however, that improved PhRE variants can be developed, though this undertaking might compromise the outstanding red bioluminescence color.

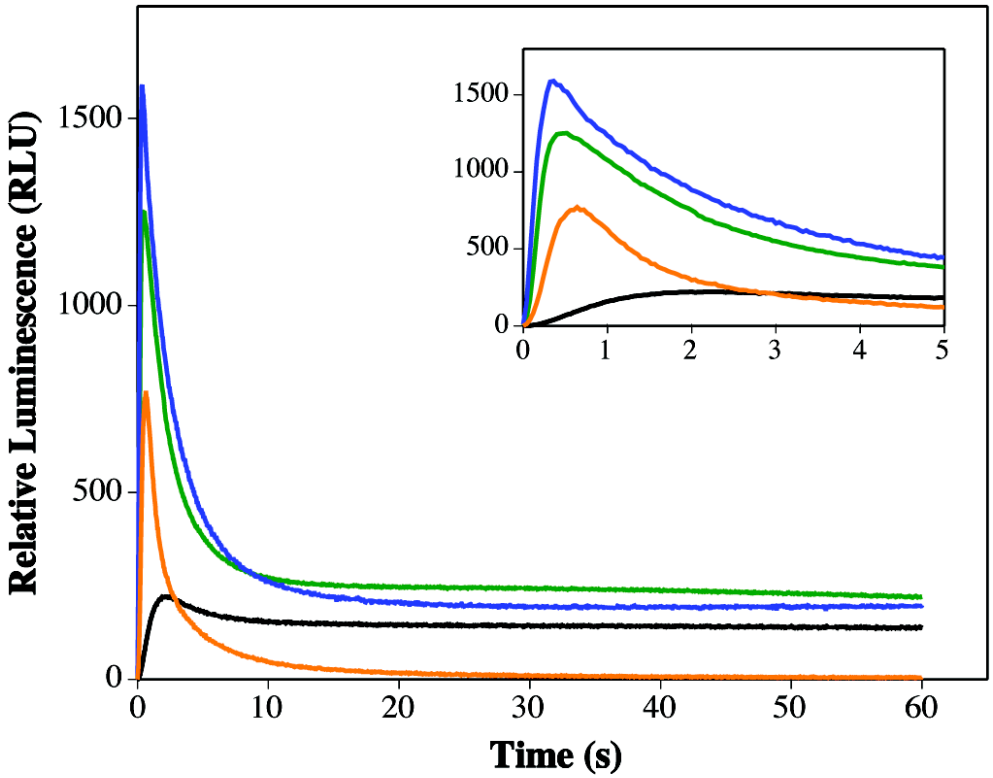

Fig. 3. Relative bioluminescence activities from reactions of equivalent quantities of purified luciferases Ppy RE8 (green line), Ppy RE9 (blue line), CBR (black line) and PhRE (orange line) using 0.3 mM LH_2 and 2 mM Mg-ATP were recorded with a custom-built luminometer as described in Materials and methods.

Model Reporter Assays in Mammalian Cells

The promising results from the characterization of the pure Ppy RE8 and Ppy RE9 proteins suggested these enzymes might provide greater sensitivity than CBR in reporter assays and *in vivo* imaging applications. Therefore, we elected to further evaluate the proteins by expressing them in mammalian cells. The luciferases were expressed at 37 °C in HEK293 cells, and lysates of equivalent cell count were prepared and assayed in 96-well tissue culture plates using a Varioskan Flash plate reader and substrate concentrations identical to those used to evaluate the purified enzymes. The light emission profiles were integrated over 60 s and the results indicated that Ppy RE9 and CBR signals were equivalent, while the integrated activity of Ppy RE8 was 1.3-fold greater (Fig. 4). A possible reason that we did not realize the expected 2-fold enhanced light output of the *P. pyralis* variants over CBR was that the Ppy RE8 and Ppy RE9 proteins were expressed in lower yield. The click beetle gene, unlike the insect genes, had been mammalian codon-optimized [31]. And in fact, recent reports demonstrated that codon-optimized *P. pyralis* luciferase genes enhanced the sensitivity of *in vivo* imaging studies in mice [38,42]. Codon-optimized versions of Ppy RE8 and Ppy RE9 were tested in HEK293 cell lysates and the results confirmed the importance of codon-optimization, as the 60 s integration-based activities were enhanced 6.7-fold and 9.5-fold, respectively (Fig. 4). Since the assay conditions

were similar to those used to determine the integration-based specific activities of the purified proteins (Table 1), these results suggest that the codon-optimized firefly luciferases were expressed at 4- to 5-fold higher levels than CBR. We did not, however, verify this by direct measurement of the individual enzyme concentrations in the lysates.

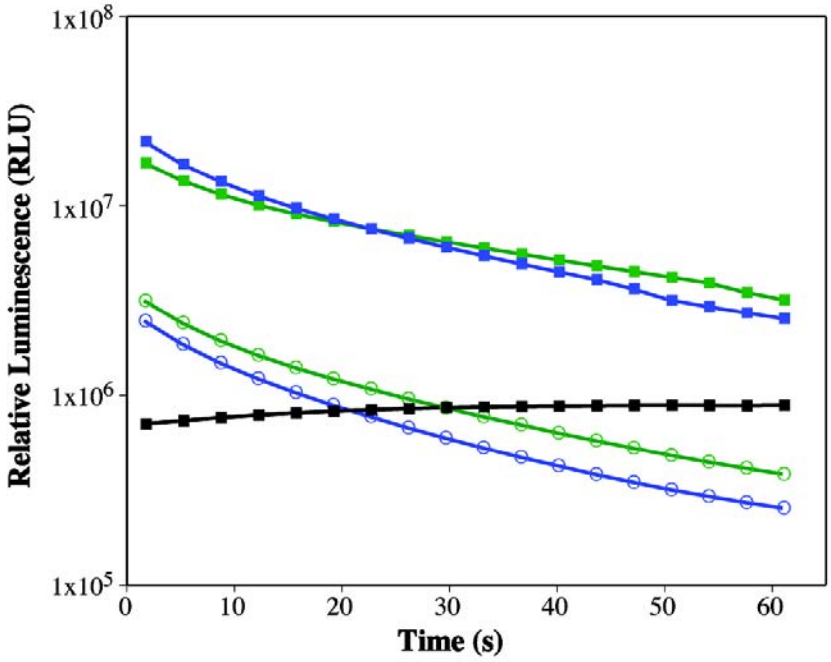

Fig. 4. Relative bioluminescence activities of soluble cell lysates from equivalent numbers of HEK293 cells expressing Ppy RE8 (green **0**), Ppy RE9 (blue **0**), human codon-optimized Ppy RE8 (green ■), human codon-optimized Ppy RE9 (blue ■), and mammalian codon-optimized CBR (black ■) at 37 °C. Assays of 10 µl of lysates using 0.3 mM LH_2 and 2 mM Mg-ATP were performed using a Varioskan Flash plate reader to monitor light emission, as described in Materials and methods.

To fully capture the potential sensitivity advantage of the codon-optimized *P. pyralis* enzymes for typical reporter assay performance, the model reporter assay conditions were optimized for signal intensity and stability. The most favorable result (Fig. 5A) was obtained in an experiment performed with HEK293 lysates of equivalent cell count in which the substrate concentrations were adjusted and CoA was included (compare legends to Figs. 4 and 5A) to maximize light output and significantly combat the rapid signal decay. Under these conditions, the relative intensities of the signals produced by Ppy RE9, Ppy RE8 and CBR were 8.6 x 10^7, 4.8 x 10^7 and 0.1 x 10^7 RLU, respectively. The 60 s integrated activities values correspond to Ppy RE9 providing 1.8- and 97-fold higher activity than Ppy RE8 and CBR, an advantage that would likely be reflected in reporter applications.

Alternatively, reporter assays with the novel codon-optimized *P. Pyralis* variants can be conducted quite conveniently by the addition of a single reagent, as evidenced by a second HEK293 cell lysate trial using LAR (Fig. 5B). Similar to their performance in the first model assay, the Ppy RE8 and Ppy RE9 enzymes emitted strong signals with negligible decay rates (Fig. 5). However, despite a somewhat more prolonged rise time, CBR activity increased in LAR so that Ppy RE9 and Ppy RE8 provided 40- and 21-fold greater signal intensities than the

click beetle enzyme in the commercial reagent.

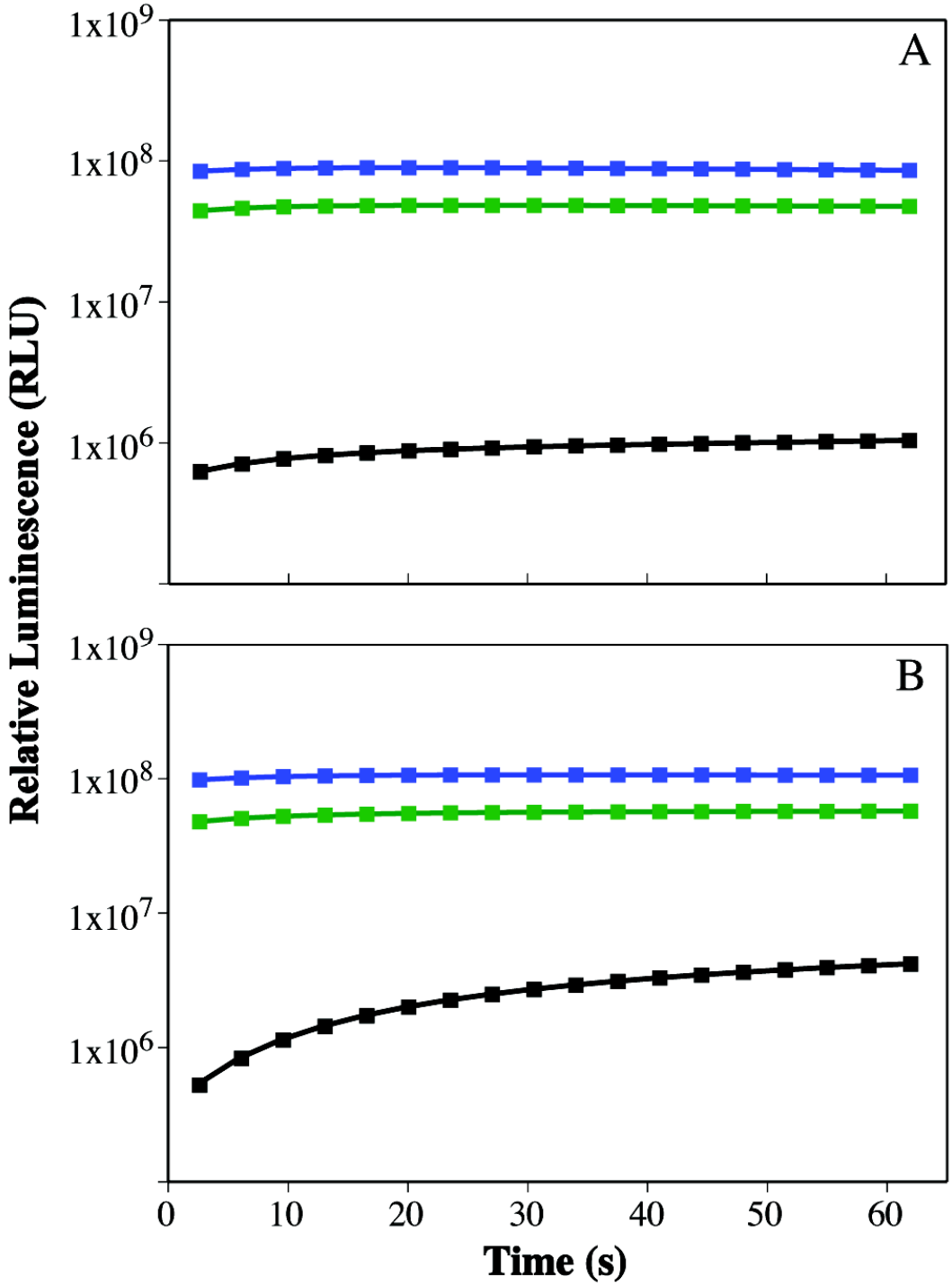

Fig. 5. Relative bioluminescence activities of soluble cell lysates from equivalent numbers of HEK293 cells expressing mammalian codon-optimized Ppy RE8 (green line), Ppy RE9 (blue line), and CBR (black line) at 37 °C. Assays of 10 µl of lysates were carried out in (A) 0.4 mM ATP, 0.2 mM LH_2, 0.1 mM CoA in 25 mM glycylglycine buffer (pH 7.8) or (B) LAR, employing a Varioskan Flash plate reader as described in Materials and methods.

The 53- and 21-fold (Ppy RE8) and 97- and 40-fold (Ppy RE9) greater 60 s integrated activities compared to CBR that were measured with the indicated substrate concentrations (Fig. 5A) and LAR (Fig. 5B), respectively, are positive indicators that the *P. Pyralis* variants are excellent candidates for *in vitro* bioanalytical applications in mammalian cells. As CBR has proven suitable for dual-reporter assays [43] and cell-sensor assays [44], we expect that Ppy RE8 and Ppy RE9 should perform superbly in these types of experiments because compared to CBR they produce brighter signals, have similar bioluminescence color, and appear to express in higher yield in mammalian cells. A highly sensitive system for dual-color assays in mammalian cells could be achieved by pairing either firefly enzyme with a codon-optimized version of Ppy GR-TS [28]. Moreover, Ppy RE9 should be the enzyme of choice for any *in vitro* bioluminescence-based bioanalytical application requiring great sensitivity, red emission and stable expression and activity at 37 °C.

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Major Accomplishment 3.

(3) 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) *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.

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the sources indicated: Mg-ATP from 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 x 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 to 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 developed programs 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% based on 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 (daltons) 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]; and 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 *E. 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, 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 20,000 *x 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, were induced with 0.1 mM IPTG. Following growth for 18-20 hrs 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 - isopropanol 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 13,000 *x 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 *x 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 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 (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 sub-shells each extending an additional 2.00Å with increasing atomic restraints of 100 kJ/Å 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 1.00Å in each Monte Carlo (MC) step. Five thousand MC steps were undertaken during the search and structures within 50kJ/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, 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 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 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 of the single point mutants at Ppy position 250were reduced ~2- to 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 mutations were 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

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

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

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

mutant, 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.

Bioluminescence Emission Spectra. The bioluminescence emission spectra of the luciferase enzymes were measured over the pH range 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 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.

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. Based on 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 (4.5Å) and S1 sulfur (4.1Å) 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 analog 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 3 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 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 if the hydrophobic side chain is influencing the emission maximum.

Luc position 241 and 250 mutagenesis and luciferase sequence comparisons. Ppe2, a luciferase from *Photuris 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 5kJ/mol contained either a new H-bond 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.

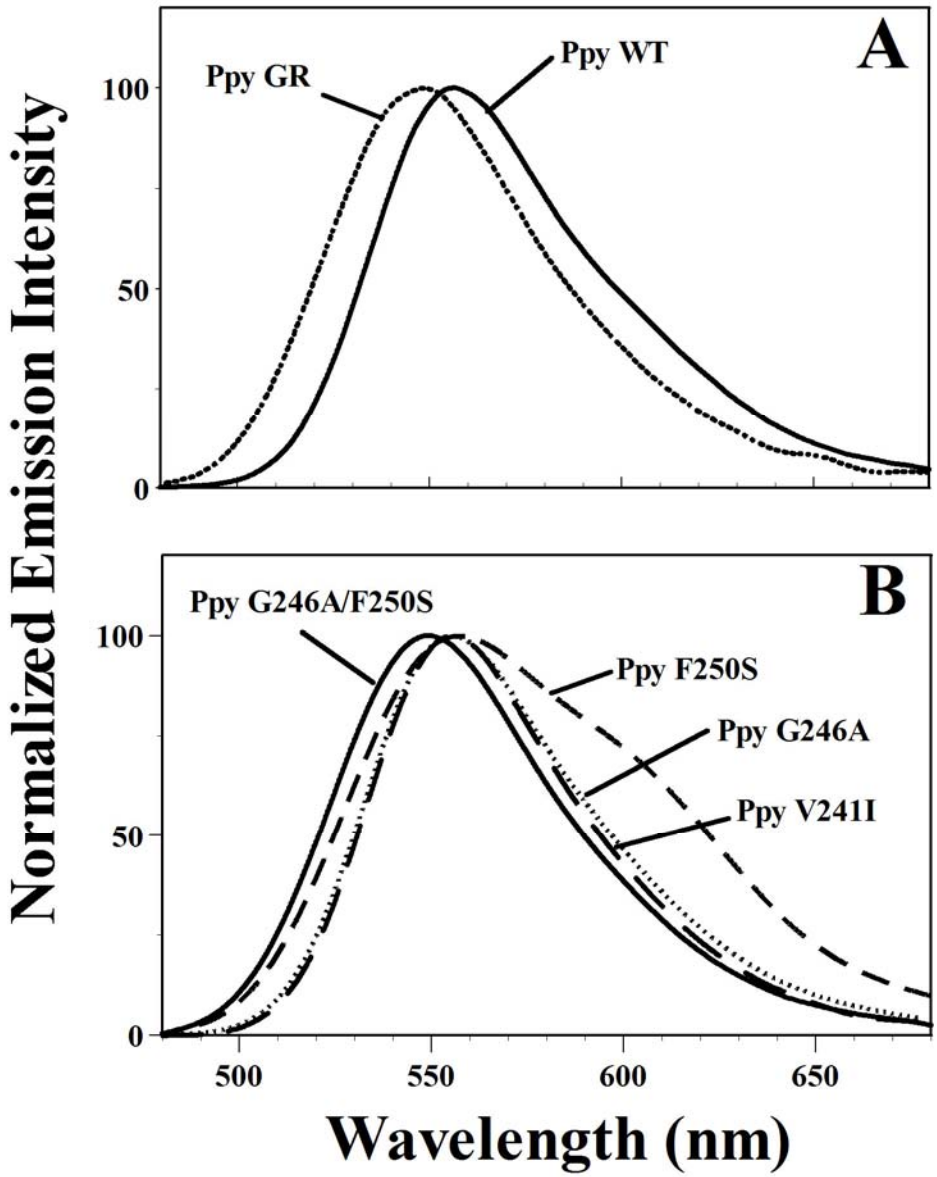

FIGURE 1: Bioluminescence emission spectra at pH 7.8, 25 ºC. The spectra generated by the indicated luciferases were recorded using equipment and conditions previously described (*23*).

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 Viviani (*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*).

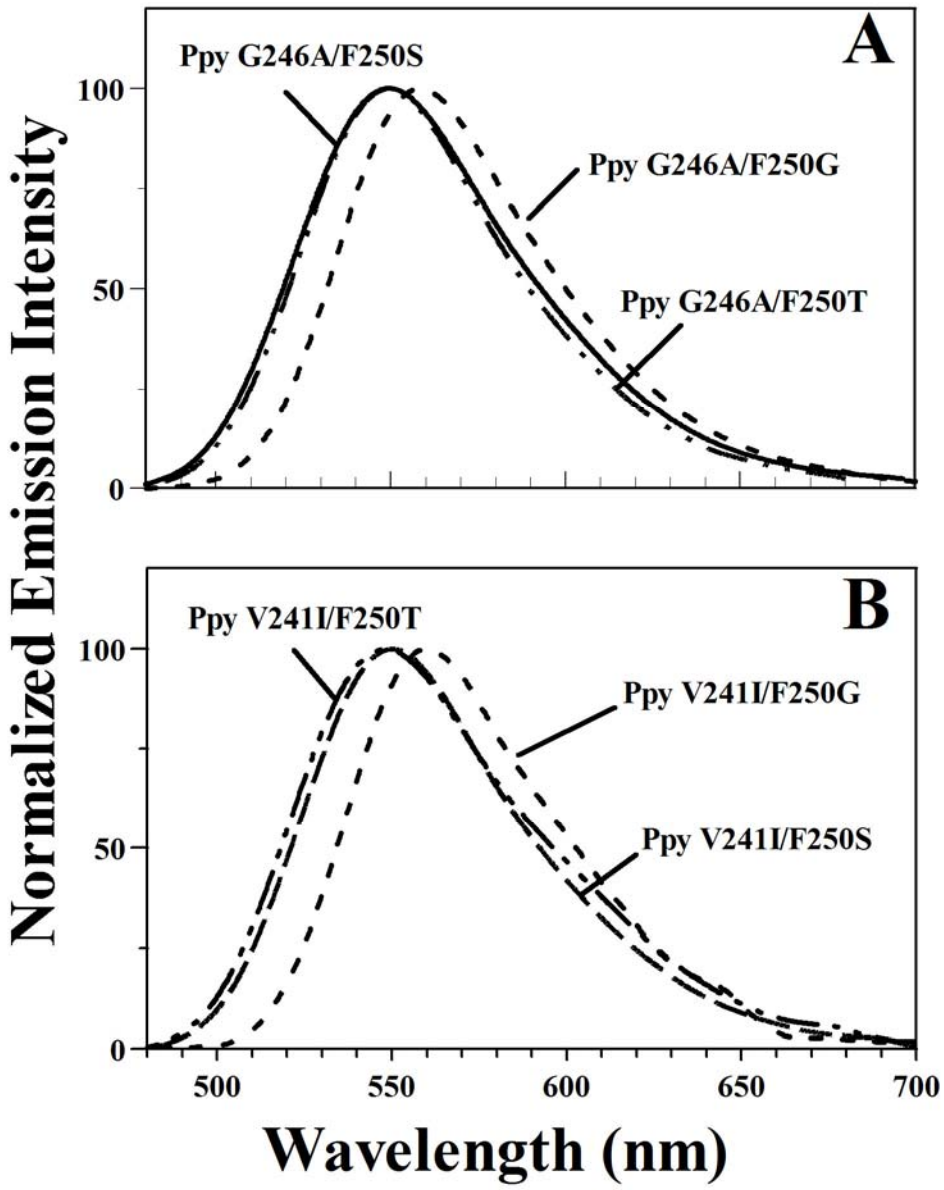

FIGURE 3: Bioluminescence emission spectra at pH 7.8, 25 ºC. The spectra generated by the indicated luciferases were recorded using equipment and conditions previously described (*23*).

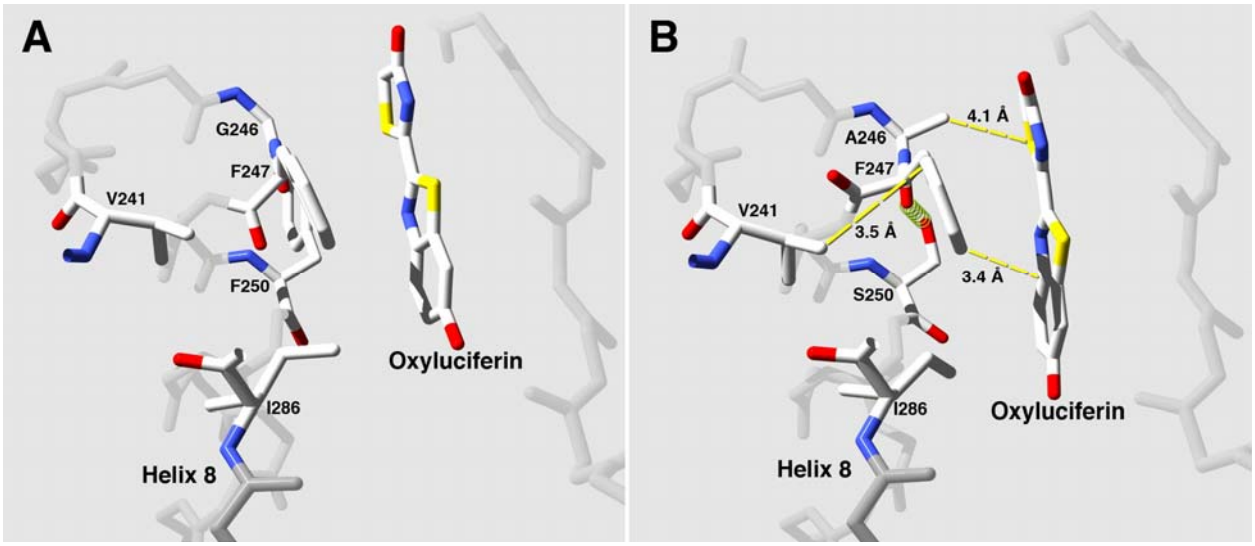

FIGURE 4: Panel A. Diagram created from the Lcr-oxyluciferin-AMP complex 2d1r (*36*) showing oxyluciferin and residues Val241 to Leu256, Val285 to Ile286 and Val336 to Leu342 (numbering according to Ppy sequence[2]). Panel B. Diagram representing lowest energy structure created, as described in Experimental Section, 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 Ppy sequence[2]) are shown.

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Major Accomplishment 4.

(4) A wide variety of biological organisms emit light spanning the visible spectrum. Bioluminescence emission maxima ranging from 450 nm to 625 nm have been recorded (*1*) for species of crustaceans and beetles, respectively. Moreover, the 705 nm peak luminescence emanating from the light organs of several dragonfish (*2*) extends bioluminescence beyond human vision into the near-infrared (nIR[1]) 700 – 1000 nm. In the dragonfish, the emitter is likely a secondary pigment (*2*) excited via bioluminescence resonance energy transfer (BRET), the natural process responsible for the green glow of the aequorin-GFP complex (*3, 4*). BRET-based assays, in which a bioluminescence donor excites a fluorescent acceptor, have been developed for a variety of applications (*5*) including measuring protease activity (*6*) and detecting protein-protein interactions (*7-9*). In the field of *in vivo* imaging, there is heightened interest in the nIR region because it can enable deeper imaging owing to lower background and minimized signal loss from light absorption and scattering (*10*). Promising BRET-based imaging results have been reported with quantum dot (QD)-*Renilla* luciferase (rLuc) conjugates (*11, 12*), including some emitting at 650 nm, and with a *Cypridina* luciferase (cLuc) containing fusion proteins modified with an indocyanine dye emitting at 675 nm (*13*).

Our interest is in developing new biomaterials with photonic properties that may be suitable for improved biomarker and biosensor applications focusing on the firefly luciferase system from *Photinus pyralis* (Luc). The firefly enzyme produces yellow-green light (Figure 1a) through a series of reactions that requires substrates firefly (beetle) luciferin (LH_2), Mg-ATP and oxygen. This process is generally considered to have the highest known quantum yield (41 ± 7.4%) (*14*) of any bioluminescence system based on the conversion of substrate (luciferin) into photons. Additional advantages of working with Luc are the superior stability and relatively low cost of LH_2. While notable applications of BRET-based red sources have been achieved (*11-13*), the BRET efficiencies of the materials are modest limiting the sensitivity that could be achieved. The efficiency of BRET is dependent on the spectral overlap, relative orientation and the distance between the bioluminescence donor and fluorescence acceptor. We set out to determine whether the Luc system could be used to produce efficient BRET-based sources of nIR light. Our strategy was to optimize spectral overlap with the long wavelength acceptor absorption spectra typical of nIR fluorescent dyes by starting with a recombinant *P. pyralis* luciferase containing the mutations Thr214Ala, Ala215Leu, Ile232Ala, Ser284Thr, Phe295Leu, Arg330Gly, Ile351Val and Phe465Arg (Ppy RE8), our recently developed (*15*) thermostable Luc variant, which emits red light (617 nm) (Figure 1b) with 63% bioluminescence specific activity compared to Luc. To minimize donor-acceptor distance, we investigated two acceptors, the Alexa Fluor nIR dyes AF680 and AF750 with excitation maxima of 680 nm and 750 nm and emission maxima of 705 nm and 780 nm, respectively. Using versions of these dyes containing maleimide groups, we covalently attached the nIR dyes to Ppy RE10, a variant of Ppy RE8 containing two surface Cys residues at positions 169 and 399, and achieved highly efficiency nIR emission by an intramolecular BRET process. We report here the design, construction and characterization of soluble and immobilized Luc-based sources of nIR light. Additionally, we demonstrate that the novel materials can be applied to the assay of factor Xa activity and we provide information from model studies with blood designed to assess the potential advantage of nIR transmittance.

EXPERIMENTAL PROCEDURES

Materials. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) from Sigma-Aldrich (St. Louis, MO); Glutathione Sepharose 4B media and pGEX-6P-2 expression vector from GE Healthcare (Piscataway, NJ); pQE30 expression vector from Qiagen (Valencia, CA); QuikChange® Lightning Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA); Alexa Fluor® 680 C2-maleimide (AF680) and Alexa Fluor® 750 C5-maleimide (AF750) dyes from Invitrogen (Carlsbad, CA); NanoLinK™ streptavidin magnetic microspheres from SoluLinK Biosciences (San Diego, CA); and Streptavidin agarose resin from Thermo Scientific (Rockford, IL). The recombinant GST-fusion proteins Ppy RE8 (*15*) and Ppy RE10 were expressed and purified as previously reported (*15-19*). LH_2 was a generous gift from Promega (Madison, WI) and the pET-KBPT-Luc plasmid (*20*) was a generous gift from E. S. Yeung, Iowa State University.

General Methods. Concentrations of unlabeled proteins were determined using the Bio-Rad Protein Assay system using BSA as the standard. DNA sequencing to verify all mutations, insertions and ligation sites was performed at the W. M. Keck Biotechnology Laboratory at Yale University. Unless otherwise specified, specific activity measurements, heat inactivation studies and steady-state kinetics constants were determined as previously reported (*16, 17, 19, 21*). Mass spectral analyses were performed by tandem HPLC-electrospray ionization mass spectrometry (LC/ESMS) using a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer. The found molecular masses (kDa) of the newly reported luciferase variants were within the allowable experimental error (0.01%) of the calculated values (in parenthesis): Ppy RE8-Thr169Cys, 61.000 ± 0.006 (60.998); Ppy RE8-Phe368Cys, 60.961 ± 0.006 (60.956); Ppy RE8-Ser399Cys, 61.021 ± 0.006 (61.016) and Ppy RE10, 61.020 ± 0.006 (61.014).

Cloning of Ppy RE8 Variants. Starting with the DNA encoding Ppy RE8 in the pGEX-6P-2 vector (*15*), the QuikChange Lightning Site-Directed Mutagenesis kit was used to introduce the following single mutations using the indicated primers and their respective reverse complements: Thr169Cys 5´- CAG TCG ATG TAC ACG TTC GTC **TGC** TCT CAT CTA CCT CCC GGT TTT AAT G - 3´ [*Ale*I]; Phe368Cys, 5´ - GGT AAA GTT GTT CCA **TGT** TTT GAA GCC AAG GTT GTG GAT CTG - 3´ [*Bst*XI]; and Ser399Cys 5´-T GTC AGA GGG CCC ATG ATT ATG **TGC** GGT TAT GTA AAC AAT CCG - 3´ [*Apa*I] (bold represents the mutated codons, underline represents silent changes to create a unique screening endonuclease site and brackets indicate the screening endonuclease). The gene encoding Ppy RE10 was made by sequential introduction of the Thr169Cys and Ser399Cys changes into the Ppy RE8 DNA.

Cloning of Fusion Protein BXRE10. The vector containing the gene encoding a fusion protein (BXRE10), consisting of an N-terminus hexa-His tagged biotin binding domain joined to Ppy RE10 through the peptide linker –GSGSIEGRGSGS-, was constructed as follows. First the Qiagen pQE30 plasmid was modified to enable the insertion of our gene for Ppy RE10 available in the pGEX-6P-2 vector where it is flanked by *Bam*HI and *Xho*I sites. The existing *Xho*I site in the pQE30 plasmid was removed and a new one was created in the multiple cloning site downstream of the already present *Bam*HI site using the following primers and their respective reverse complements: 5´- ACG AGG CCC TTT CGT CTT CAC CT**G** GAG AAA TCA TAA AAA - 3´and 5´- CAC GGA TCC GCA TGC GAG CTC **GAG** ACC CCG GGT CGA CCT - 3´,

respectively. The gene encoding Ppy RE10 was isolated from the pGEX-6p-2 plasmid by digestion with *Bam*HI and *Xho*I and was then ligated into the modified pQE30 vector, which had been digested with the same restriction enzymes. The modified pQE30 plasmid containing the Ppy RE10 gene was further altered by introducing the *Pml*I and *Spe*I restriction sites upstream of the luciferase gene to enable insertion of the DNA encoding the biotin binding domain (BBD, residues Met12-Val76 in the pET-KPBT-Luc plasmid) using the following primer and its respective reverse complement: 5´ - CAC CAT CAC CAT **CAC GTG AGC ACT AGT** GGA TCC ATG GAA GAC - 3´ (bold represents the inserted codons and the *Pml*I and *Spe*I endonuclease sites are underlined). The BBD was amplified from the pET-KPBT-Luc plasmid by PCR (initial denaturation at 95 °C for 2 min; a 60-cycle amplification of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 1.75 min; and a final extension at 68 °C for 5 min) with the primers 5´-CAT CAT CAC GTG ATG GCT AGA GTC GAC GTC AGC - 3´ and 5´- CCA CCA GT**A CTA GT**A GTA ACG GCC GCC AGT GTG C - 3´ (bold represents the introduced endonuclease sites for insertion of the BBD). The PCR product was cleaned with the Qiagen QIAquick PCR purification kit, digested with *Pml*I and *Spe*I and ligated into the modified pQE30 vector containing the Ppy RE10 DNA, which had been treated with the same restriction endonucleases, yielding pQE30-BBD-RE10. Using the primer 5´ - A CTG GCG GCC GTT ACT ACT **GGA TCC GGA TCC ATC GAA GGT CGT GGA** AGT GGA TCC AT - 3´ (bold represents the inserted codons) and its reverse complement, a 12 amino acid linker containing the factor Xa protease site IleGluGlyArg was inserted between the BBD and Ppy RE10 genes yielding pQE30-BXRE10. This plasmid encodes the fusion protein BBD-GSGSIEGRGSGS-Ppy RE10 containing a hexa-His-tag at the N-terminus (BXRE10).

Expression and Purification of Biotinylated Fusion Protein BXRE10. Cultures (250 mL of Luria-Bertani media supplemented with 100 mg/mL ampicillin, 25 mg/mL kanamycin and 50 μM biotin) of transformed *E. coli* BL21 (pREP4) cells containing the pQE30-BXRE10 plasmid were grown in 1 L flasks at 37 °C to mid log phase ($A_{600\ nm} \sim 0.6$), moved to a 22 °C incubator, allowed to equilibrate for 10 min, induced with 0.1 mM IPTG and incubated at 22 °C for 16-18 h. Cells were harvested by centrifugation at 4 °C and then frozen at -80 °C for 15 min. The cell pellet was resuspended in 25 mL of 150 mM sodium chloride, 100 mM sodium phosphate buffer, pH 7.3 (PBS) containing 0.1 mM phenylmethylsulfonyl fluoride and 5 mM imidazole. After the addition of 2.5 mL lysozyme (10 mg/mL in PBS), the cells were lysed by sonication and treated with DNase (5 mg/mL) and RNase (10 mg/mL) for 5 min on ice. Triton X-100 was added (1% final volume) and the whole-cell extracts were isolated by centrifugation at 20,000 x *g* for 45 min. The biotinylated His-tagged fusion protein was purified using Ni-NTA agarose (Qiagen) affinity chromatography according to the manufacturer's instructions. Fractions eluted with 250-500 mM imidazole were pooled (2 mL) and dialyzed (2 changes, 1 L each) against 20 mM sodium phosphate buffer, pH 7.2 containing 150 mM NaCl, 5 mM EDTA and 0.8 M ammonium sulfate (PBSA buffer). Biotinylation was confirmed by LC/ESMS analysis showing a single eluting peak of 74.592 ± 0.007 kDa corresponding to the predicted mass of BXRE10 plus biotin (74.348 + 0.244 = 74.592 kDa). The protein was stored at 5 °C in PBSA buffer (~2mg/mL) and retained 90% activity for at least 6 weeks.

Covalent Labeling of Luciferase Enzymes with Alexa Fluor Maleimide Dyes. Stock solutions (10 mM, determined by UV-Visible Spectroscopy) of the AF680 (ϵ_{684nm} = 175 000 M^{-1} cm^{-1}) and AF750 (ϵ_{753nm} = 290 000 M^{-1} cm^{-1}) dyes (*22*) were prepared in sterile deionized water, divided into 30 μL aliquots, lyophilized and stored at -20 °C. All labeling reactions were

performed at 10 °C in PBSA buffer. Using Slide-A-Lyzer® 10K MWCO dialysis cassettes (Thermo Scientific, Rockford IL), stock luciferase solutions (~2 mg/mL) in 20 mM Tris-HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT were dialyzed against PBSA buffer (5 changes, 1L each) to remove DTT and introduce 0.8 M ammonium sulfate. The inclusion of ammonium sulfate in PBSA effectively reduced non-specific dye incorporation. Labeling reactions were initiated by addition of 1 mL of 30 μM enzyme in PBSA buffer to a lyophilized aliquot of AF680 or AF750 (final concentration 300 μM) and gently mixed for 15 min. Reactions were quenched by addition of 20 μL of 100 mM glutathione in PBSA buffer, incubated for 15 min and dialyzed against PBSA buffer (8 changes, 1L each). The nIR dye-labeled proteins could be stored in PBSA buffer at 5 °C for at least 6 weeks without loss of more than 10% activity. For long term storage, labeled and unlabeled proteins were dialyzed into 20 mM sodium phosphate buffer, pH 7.2 containing 150 mM NaCl and 5 mM EDTA, flash frozen in liquid N_2 and stored at -80 °C with complete retention of activity.

Determination of Fluorescent Dye Labeling Stoichiometry. The degree of nIR dye labeling was estimated by UV-visible spectroscopy by measuring the μmol of dye (using the molar absorptivity coefficients above) bound to μmol samples of labeled luciferases. A Micro BCA Protein Assay Kit (Thermo Scientific, Rockford IL) was used to determine the quantity of enzyme with readings taken at 540 nm to avoid interference with dye absorption bands.

Additionally, LC/ESMS analysis of the labeled luciferases was performed using a BioBasic-C4 (100 x 1 mm) column eluted at a flow rate of 50 μL/min using an acetonitrile gradient of 10%/min. Elution was monitored by absorbance at 260 nm and 680 nm or 750 nm. With all labeled enzymes, long wavelength absorption associated with the Alexa Fluor dyes was only observed co-eluting with protein indicating the absence of any non-covalently attached label. Dye/protein incorporation ratios were calculated based on the observed masses of each detected species. Mass increases of 0.979 and 1.958 kDa for AF680 and 1.048 and 2.096 kDa for AF750 were taken as 1:1 and 2:1 dye/protein incorporation ratios, respectively. Post run data analysis was performed using ThermoFinnigan BioWorks Browser 3.0 deconvolution software.

Identification of Ppy RE10 Residues Labeled with Fluorescent Dyes. Samples (200 μg) of Ppy RE10 labeled as described above with AF680 or AF750 were digested with thermolysin (protease:protein, 1:10, w/w) in 50 mM ammonium bicarbonate, pH 8.0 for 6 h at 37 °C. Peptides were separated on a Gemini-NX 5μ C18 110Å column (50 x 2.00 mm) at a flow rate of 50 μL/min using 0.1 % aqueous TFA containing linear gradients of acetonitrile: 5 % (v/v) initially for 5 min, 50 % after 90 min, and 95 % after 110 min. Elution was monitored by absorbance at 214 nm, 260 nm, and either 680 nm or 750 nm for AF680 or AF750 labeled peptides, respectively. Each digest of nIR dye labeled enzyme produced two major peaks with long-wavelength absorbance attributable to the dyes. For the AF680-labeled luciferase, masses of 1.199 ± 0.0001 and 1.344 ± 0.0001 kDa were found corresponding to the mass of the dye plus Val168Cys169 and Ile397Met398Cys399, respectively. For the AF750-labeled enzyme, masses of 1268 ± 0.0001 and 1413 ± 0.0001 kDa were observed that were consistent with the mass of the dye plus Val168Cys169 and Ile397Met398Cys399, respectively. While the presence of Val80Cys81 is a potential source of ambiguity, Ppy RE8, which lacks Cys at position 169, did not incorporate the Alexa Fluor dyes. Additionally, the labeled peptide sequences, were confirmed by MS/MS analysis in which masses of 1.100 ± 0.0001 and 1.169 ± 0.0001 kDa, corresponding to Cys covalently bound to AF680 and AF750, respectively, were observed. Upon

prolonged standing at room temperature, proteolysis mixtures produced additional peaks corresponding to mass additions of 18, suggesting hydrolytic ring opening of the maleimide group. Since the dyes are presumably attached through thioether linkages, maleimide ring opening would not release the dyes.

Bioluminescence and BRET Emission Spectra. Bioluminescence emission spectra were obtained using a Horiba Jobin-Yvon iHR imaging spectrometer equipped with a liquid N_2 cooled CCD detector and the excitation source turned off. Data were collected at 25 ºC (in a 0.8 mL quartz cuvette) over the wavelength range 400-935 nm with the emission slit width set to 25 nm and were corrected for the spectral response of the CCD using a correction curve provided by the manufacturer. Reactions (0.525 mL final volume) were initiated by addition of 5 μL aliquots of luciferase in PBSA buffer (0.2 - 0.3 μM final concentration) to solutions containing LH_2 (150 μM) and Mg-ATP (2 mM) in 25 mM glycylglycine buffer, pH 7.8. The pH values of the reaction mixtures were confirmed before and after spectra were obtained. BRET ratios were calculated from emission spectra by dividing the area under the BRET emission peak by the area under the residual bioluminescence peak.

Immobilization of BXRE-680 onto Streptavidin-Supports. Biotinylated BXRE10 was chemically modified with AF680 as described above yielding BXRE-680, the corresponding biotinylated fusion protein with AF680 covalently bound in a 2:1 dye/protein ratio. Streptavidin coated magnetic microspheres and agarose resin were each prepared by aliquoting 100 μL portions of bead slurry, separating and removing the supernatant (via magnet for magnetic beads, quick desktop centrifugation for agarose resin), and washing three times with 100 μL portions of PBSA buffer. The supernatant was removed and the beads were stored on ice. BXRE-680 was bound to the supports by adding 100 μL of an ~1.5 mg/mL protein solution in PBSA to the pre-washed beads, which were then incubated with gentle mixing for 10 min on ice. The supernatant was removed and the immobilized BXRE-680 beads were washed five times with 100 μL portions of PBSA buffer. The materials were resuspended in PBSA buffer to a total volume of 100 μL and stored in PBSA buffer at 5 °C. An equally effective procedure to prepare immobilized BXRE-680 was to add the streptavidin-coated supports to labeling mixtures of nIR dye and BXRE10 (see above), followed by washing with ten 0.1 mL aliquots of PBSA. Excess label and unreated glutathione were effectively removed while avoiding the exhaustive dialysis step. Alternatively, biotinylated BXRE10 can be bound to the insoluble supports and then labeled with AF680 followed by repeated washing with PBSA buffer.

Estimation of Specific Activities of Immobilized Fusion Proteins. Integrated specific activities of bead-bound fusion protein samples were estimated using a Horiba Jobin-Yvon Fluorolog-3 with the excitation source shunted. A “flea”-sized magnetic stir bar was used to suspend the immobilized material throughout the 15 min measurement. A volume of bead-solution equivalent to ~2 μg of protein was mixed with 0.4 mL of 0.6 mM LH_2 in a 0.8 mL quartz cuvette. The reactions were initiated at 25 °C by injection of 120 μL of 18 mM Mg-ATP solution through a modified lid. Signal intensity was measured over 15 min by centering the emission monochromator to the appropriate peak wavelength (617 nm for BXRE10, 706 nm for BXRE-680). The emission slit width was set to the maximum of 29.4 nm and emission intensities were corrected for the spectral response of the R928 photomultiplier tube, and for the relative percentage of the overall emission profile observable through the 29.4 nm window.

Transmittance of Bioluminescence through Blood. Solutions (5 mL) of 2 mM Mg-ATP and 0.60 mM LH_2 in PBS, pH 7.3, were diluted with equal volumes of PBS or citrated human blood and the pH of each was adjusted to 7.3. Aliquots (520 μL) of PBS- or blood-based solutions were added to cuvettes and bioluminescence was initiated by addition of 3 μg of luciferase at 25 °C. After 10 s, bioluminescence emission was recorded with a CCD detector as described above. Percent light transmittance through 50% blood solution was calculated from the ratio of the integrated intensities of the signals transmitted through blood and PBS.

Factor Xa Assay. Assays of factor Xa activity in a commercial sample of authentic material were performed in PBSA buffer or in citrated human blood. The activity of the sample was confirmed by assay with the chromogenic substrate XXXXX(ref). A sample of BXRE-680 immobilized onto agarose beads (Agarose-SA:BXRE-680) was prepared as described above. The sample was divided into 0.1 mL aliquots that were briefly centrifuged. The supernatants were removed and replaced with 0 to 20 μg/mL of factor Xa in 0.1 mL of either PBSA or blood. The mixtures were resuspended and gently mixed while incubating at 25 °C. At various time intervals, the suspensions were quickly centrifuged, 5 μL aliquots were withdrawn, and incubation was continued after the mixtures were resuspended. Aliquots were assayed for bioluminescence activity as described in reference(s) XXX. To simulate performing the bioluminescence measurements in blood, the aliquots were added to solutions containing 0.25 mL of blood and 0.25 mL of 1mM Mg-ATP and 0.3mM LH_2 in PBS. Following the addition of Luc (X μL of a Y μg/mL solution in Z), light emission was measured using a liquid N_2 cooled CCD as described above.

Table 1. Properties of Firefly Luciferase Variants

Enzyme	Relative Specific Activity [a]	Decay Time [b] (min)	K_m (μM)		Bioluminescence λ_{max} (nm, fwhm [c])	Thermal Inactivation[d]
			LH_2	Mg-ATP		
Ppy WT	100	0.10	15 ± 2	160 ± 20	560 (73)	0.26
Ppy RE8	63	0.26	77 ± 8	391 ± 39	617 (73)	3.50
Ppy RE8/C169	78	3.10	138 ± 9.6	450 ± 45	617 (72)	4.00
Ppy RE8/C368	84	3.90	105 ± 13	523 ± 50	617 (72)	1.10
Ppy RE8/C399	94	2.30	113 ± 13	517 ± 48	617 (71)	3.50
Ppy RE10	97	1.70	175 ± 16	422 ± 41	617 (71)	3.60

[a] Specific activity based on 15 min integration assays performed as previously described. The error of the triplicate measurements was less then 10% of the measured value. [b] Time for maximum signal intensity to decay to 15 % of the initial value. [c] Full width at half maximum intensity. [d] Time for the maximum initial activity to decay to 50 % at 37 °C.

Although Ppy RE8 contained four native Cys residues, molecular modeling based on a crystal structure of Luc in complex with DLSA, an N-acyl sulfamate analog of LH_2-AMP, indicated that the thiols were not in solvent-exposed surface positions. Since it was important to rapidly modify the enzyme to minimize activity loss, we made several Ppy RE8 variants containing one or two additional thiols by mutating surface residues Thr169, Ser185, Ser307, Phe368 and Ser399 to Cys. All enzymes containing additional thiols gave BRET signals that accompanied covalent incorporation of AF-680 or AF-750 when ~30 μM protein was incubated with a 10-fold molar excess of dye for 20-30 min at 10 °C in 20 mM sodium phosphate buffer (pH 7.2) containing 0.8 M ammonium sulfate, 150 mM NaCl and 0.5 mM EDTA. Reactions were quenched with 2 mM glutathione and excess dye was removed by exhaustive dialysis against the reaction buffer. It was necessary to include ammonium sulfate during the chemical modification

reactions and dialysis to minimize non-specific dye incorporation, perhaps because the dyes contain sulfonic acid groups. Control experiments with Ppy RE8 and the nIR dyes produced neither measurable BRET signals nor any covalent dye incorporation as determined by LC/ESMS. Therefore, the control experiments substantiated the necessity for incorporating the additional surface Cys residues into the Ppy RE8 enzyme.

After evaluating our preliminary labeling results with the Cys mutants (see supporting information for data and discussion), we selected Ppy RE8 containing the two additional changes - Thr169Cys and Ser399Cys - as our best template for further study. This luciferase variant, which we named Ppy RE10, provided the best combination of factors including: optimal BRET ratios (integrated BRET emission/integrated residual bioluminescence); retention of enzyme activity; minimal non-specific dye incorporation; long wavelength emission maximum; and extended glow kinetics. Importantly, the additional Cys residues did not alter the 617 nm bioluminescence maximum, nor did they adversely affect specific activity.

Using the procedures described above, we reproducibly and quantitatively labeled mg quantities of Ppy RE10 with AF-680 and AF-750. Thermolysin proteolysis and LC/ESMS analyses verified the covalent attachment of the nIR dyes to the expected Cys169 and Cys399 residues, presumably through thioether linkages. The chemically modified luciferases displayed emission maxima shifted to 705 nm and 783 nm, reflecting BRET ratios of 35.0 and 4.1, respectively (Figure 1c and 1d). Integrated light emission (15 min interval) was 2.8-fold greater for PpyRE10 labeled with AF-680 compared to the AF-750 labeled enzyme, representing a 1.7-fold increase and 1.7-fold decrease with respect to the Ppy RE10 template. Otherwise, the catalytic properties of the labeled Ppy RE10 enzymes were generally quite similar to each other and the template itself.

We considered the basis of the 2.8-fold higher specific activity and 8.5-fold greater BRET ratio obtained with Ppy RE10 labeled with AF-680. Since the dyes are approximately the same size, have similar chemical structures and are attached to the same Ppy RE10 thiols, differences in distance from the oxyluciferin emitter can be ruled out, although spatial orientation could be a contributing factor. The variation in the specific activities can largely be accounted for by the 3-fold higher fluorescence quantum yield of AF-680 (0.36) compared to AF-750 (0.12).[11] That is, even if the energy transfer efficiencies were identical, the AF-680 labeled enzyme would be expected to produce ~3-fold more light than the AF-750 labeled protein. The difference in BRET ratios mainly reflects (1) the 2-fold greater spectral overlap of Ppy RE10 bioluminescence with the AF-680 excitation spectrum and (2) the quantum yield variation of the nIR dyes. To demonstrate that the luciferase BRET sources could be suitable for receptor imaging and enzyme assays in whole blood, we prepared the construct illustrated in Figure 1E.

While the BRET ratio with the attached AF-750 label is very good and is similar to results obtained with Quantum dot- Luc mutant conjugates, the 35.0 value obtained with AF-680 is extraordinary. Moreover, the bioluminescence maximum of Luc has been red-shifted 223 nm by the combined effects of mutagenesis and BRET in the AF-750 labeled enzyme. This shift is ~160 nm greater than has been achieved with mutagenesis alone. The nIR dye-labeled Ppy RE10 enzymes were stable for at least 6 weeks when stored in solution at 5 °C, and they could be flash frozen, stored at -80 °C and thawed without loss of activity. Additionally, the BRET activity was retained at levels greater than 50% for ~2-3 h at 37 °C.

Table 2. Properties of Luciferase Variants and Fusion Protein Labeled with Alexa Fluor® nIR Dyes

Enzyme [a]	Relative Specific Activity [b]	Decay Time [c] (min)	Thermal Inactivation [d]	BRET λ_{max} (nm, fwhm [e])	BRET Ratio [f]	Labeling Stoichiometry [g]
Ppy WT	100	0.10	0.26	-	-	-
Ppy RE8	63	0.26	3.50	-	-	-
Ppy RE10	97	1.7	3.60	-	-	-
BXRE10	60	2.0	3.5	-	-	-
Ppy RE8/C169-AF750	19	5.3	2.3	778 (71)	0.5	0.95 (1:1)
Ppy RE8/C399-AF750	86	2.4	2.8	780 (70)	2.1	1.60 (1:1)
Ppy RE10-AF750	41	4.4	3.0	783 (60)	4.0	1.80 (2:1)
Ppy RE8/C169-AF680	132	2.4	3.3	704 (59)	4.5	1.10 (1:1)
Ppy RE8/C399-AF680	180	1.5	1.3	705 (60)	10.0	1.40 (1:1)
Ppy RE10-AF680	164	7.4	3.5	705 (57)	34.0	2.10 (2:1)
BXRE-680	115	5.7	1.5	706 (49)	14.0	1.98 (2:1)
MagSphere-SA:BXRE-680	34	13.6	-	706 (49)	11.0	-
Agarose-SA:BXRE-680	76	13.0	-	708 (48)	13.7	-

[a] AF750 and AF680 refer to the fluorescent dyes covalently attached to the respective enzymes. [b] Specific activity based on 15 min integration assays performed as previously described . The error of the triplicate measurements was less then 10% of the measured value. [c] Time for maximum signal intensity to decay to 15 % of the initial value. [d] Time for the maximum initial activity to decay to 50 % at 37 °C. [e] Full width at half maximum intensity. [f] BRET ratios were calculated from emission spectra by dividing the area under the BRET emission peak by the area under the residual bioluminescence peak. [g] The number of dye molecules incorporated per molecule of enzyme was calculated by UV/Visible absorption as described in the Experimental Procedures. The error in the UV/Visible based determination is ~20%. The ratios in parenthesis refer to the predominant species detected using LC/ESMS.

To the best of our knowledge, this is the first report of an efficient nIR light source produced by chemical modification of a luciferase primary sequence. The BRET sources described here are available on demand and are activated by the addition of the Luc substrates. Additionally, nIR fluorescence can be generated directly by irradiating the labeled enzymes with nIR light. While chemically modified luciferases like those described here may not be suitable for most reporter applications, it should be possible to use them for in vivo imaging studies if suitable specific delivery mechanisms can be developed. Other potential applications of the reagents and concepts reported here include immunoassays, biosensors, electronics, photography and night vision devices.

We made Ppy RE10, a red-emitting (λ_{max} = 617 nm) luciferase variant Ppy RE10 that had optimal properties for BRET to excite near Infrared (nIR) dyes. The point mutations Thr169Cys and Ser399Cys introduced surface exposed thiols that were labeled with AlexaFluor nIR dyes that contained maleimide groups. Covalently labeled Ppy RE10 produced light with maxima at 705 nm or 778 nm via intramolecular BRET processes. A fusion protein consisting of Ppy RE10 and a biotin binding domain linked through a flexible peptide was labeled with a nIR dye. After immobilization on magnetic microspheres containing avidin, this material was a bright source of nIR light that is activated by the addition of substrates. It can be easily detected with commercial CCD detectors and manipulated with a magnet.

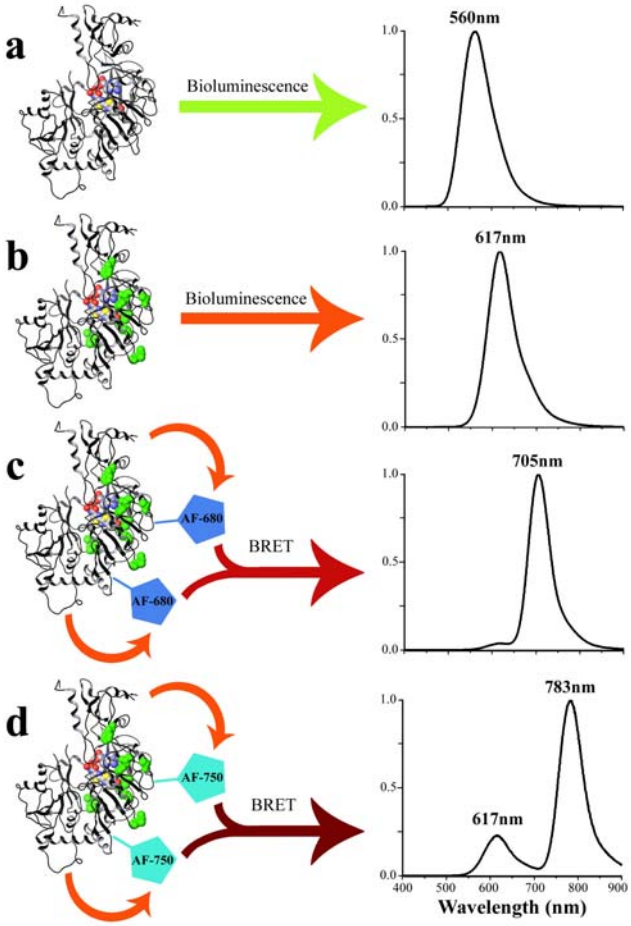

Figure 1. Normalized bioluminescence and BRET emission spectra of (a) Luc, (b) Ppy RE8 and Ppy RE10 labeled with: (c) AF680 or (d) AF750. Reactions were initiated by the addition of LH_2 and Mg-ATP at pH 7.8. Ribbon diagrams are based on a Luc-DLSA crystal structure (*23*, *24*) showing mutated residues (green) and DLSA as CPK models.

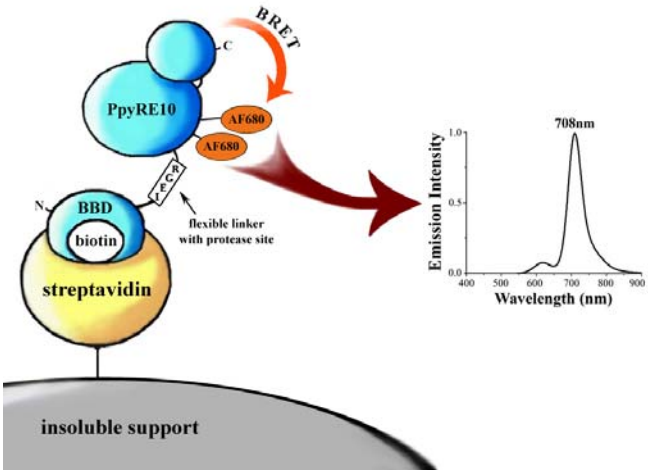

Figure 2. BRET emission spectrum of BXRE-680 immobilized on agarose-SA. The fusion protein contains biotin bound to a biotin binding domain (BBD) that is connected through a 12 amino acid linker (containing a factor Xa protease site) to Ppy RE10 labeled with AF680. The emission spectrum was recorded by suspending an aliquot of the immobilized material in a pH 7.8 buffer containing LH_2 and Mg-ATP.

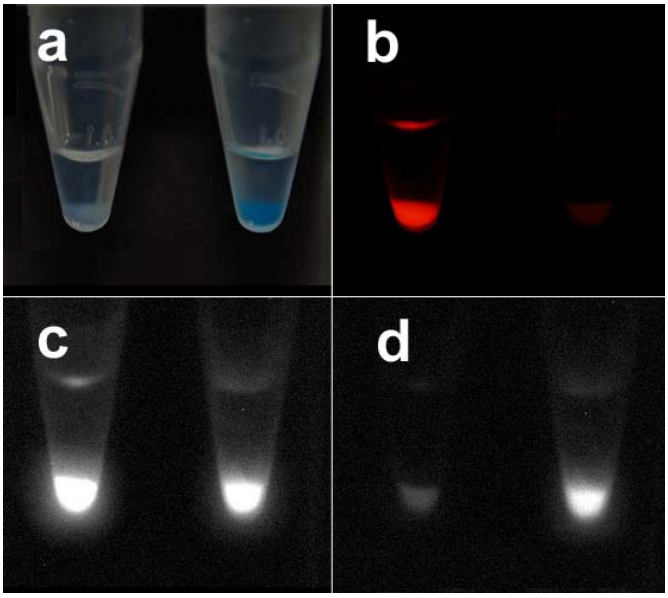

Figure 3. Visible and nIR light emission produced by unlabeled and labeled immobilized fusion proteins. In each panel the tube on the right contains ~20 µL of pelleted agarose-SA bound BXRE-680 in 70 µL pH 7.8 buffer and the tube on the left contains the immobilized fusion protein with no AF680 attached. Photographs were taken: (a) with a Nikon D80 in ambient light prior to addition of substrates; and following addition of LH_2 and Mg-ATP with (b) the Nikon D80 (with stock IR filter) at 15 s exposure showing visible emission; (c) with an iGen NV20/20 camera with night vision (infrared) capability showing total light emission; and (d) with the iGen NV20/20 camera through a Schott 695nm longpass filter showing only nIR emission.

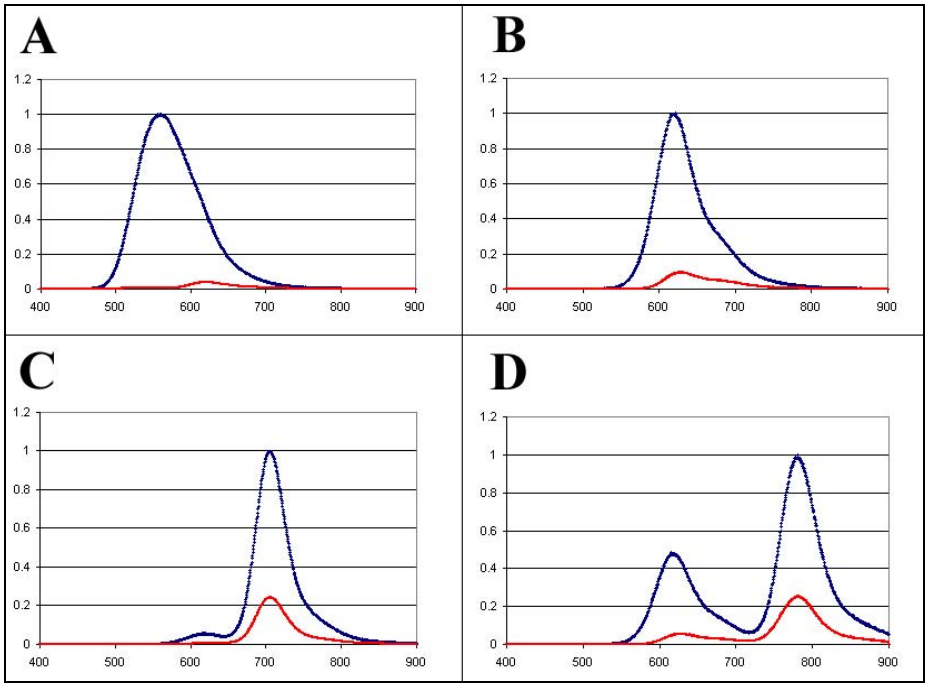

Figure 4. Relative transmittance of bioluminescence through citrated human blood. The emission spectra shown in all panels were initiated by addition of solutions of Mg-ATP (2 mM) and LH_2 (0.60 mM) in PBS to citrated human blood (dashed lines) or PBS (solid lines) containing (a) Ppy GR-TS (*16*); (b) Ppy RE10; (c) Ppy RE10-AF680 and (d) PpyRE10-AF750 as described in detail in the Experimental Procedures. Ppy GR-TS [S2] was used as a source of green light since Ppy WT red-shifted in the assay solution.

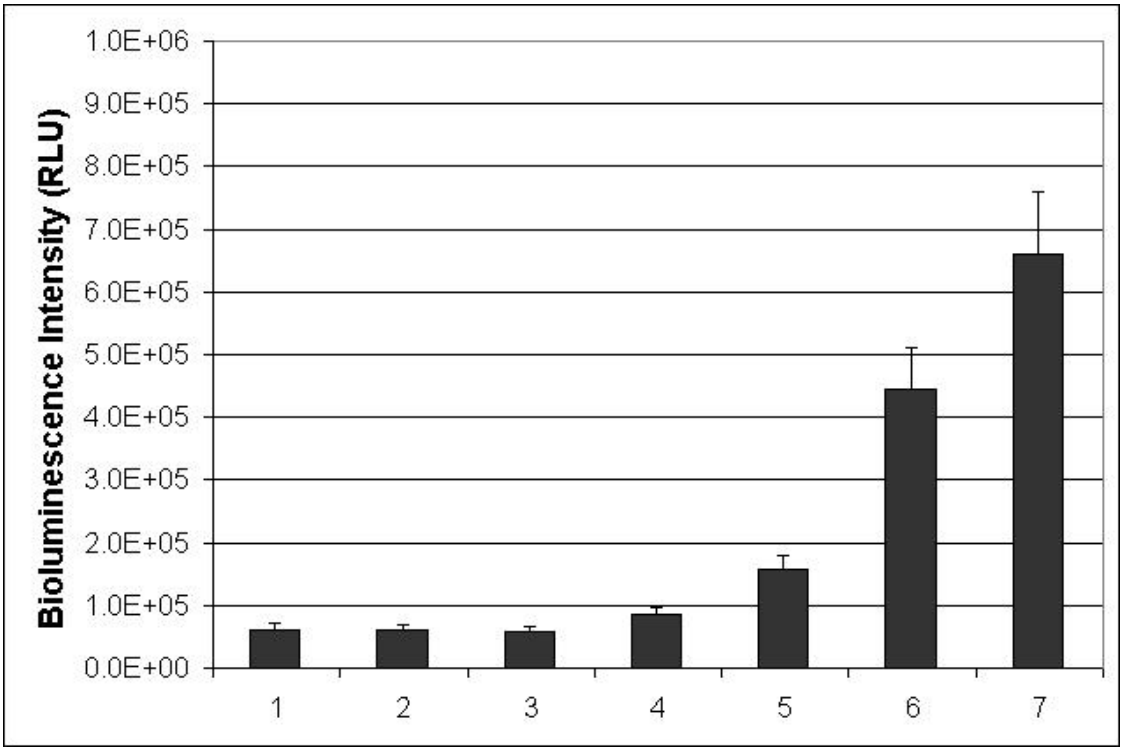

Figure 5. Bioluminescence endpoint assays (30 min) of factor Xa activity in whole blood using Agarose-SA:BXRE-680. Stirred substrate suspensions were mixed with: 1, 10 μg/mL BSA; 2, 10 μg/mL heat-denatured factor Xa; 3, nothing added; and factor Xa; 4, 1 μg/mL; 5, 2.5 μg/mL; 6, 5 μg/mL; and 7, 10 μg/mL.

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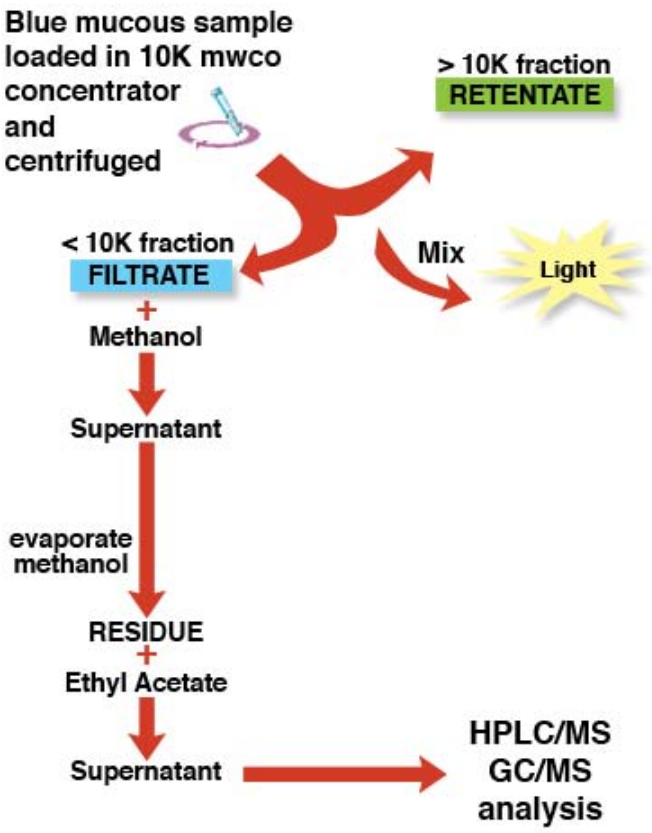
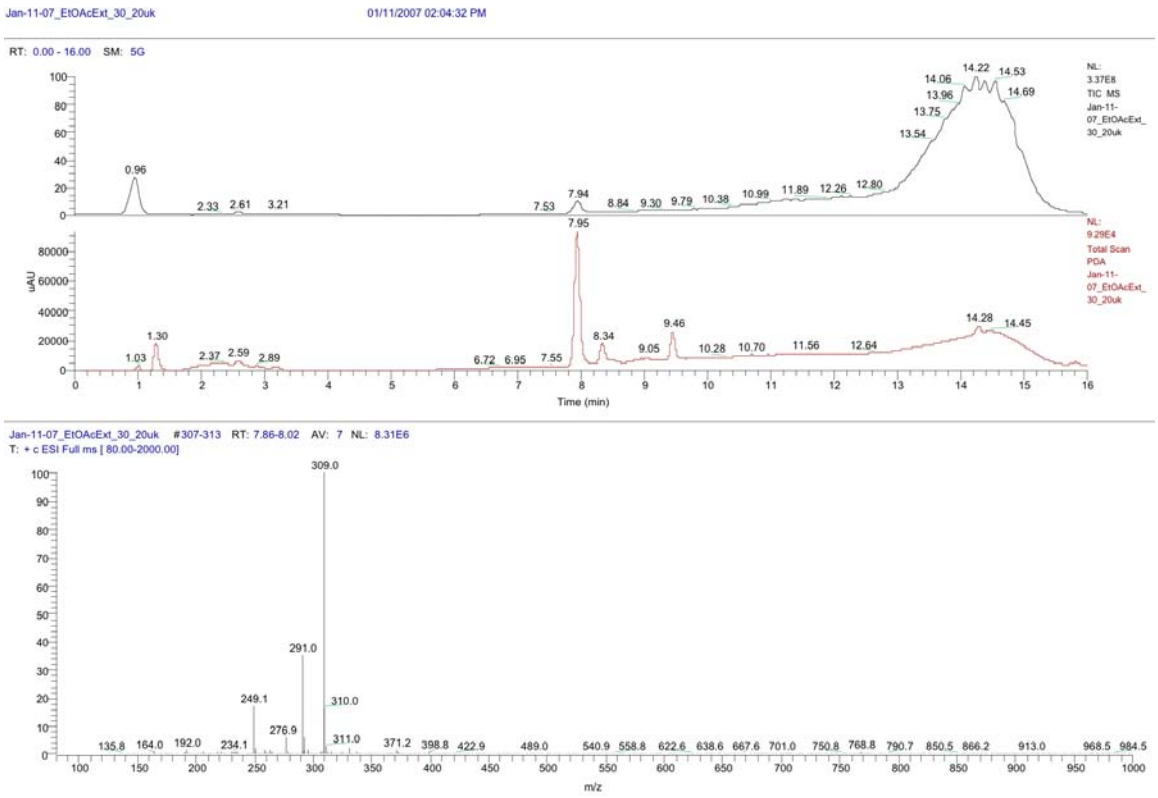
Major Accomplishment 5.

(5) A method was developed to extract *O. phosphorea* mucous to attempt to find metabolites related to the bioluminescence process. The method is outlined in the diagram below.



An analysis of the small molecule fraction produced the result shown below. The 2 isolated substances were readily water-soluble and were also methanol soluble but ethyl acetate insoluble. One had mass = 252 and the other mass = 284. The latter, but not the M=252, has a UV peak at 360nm and fluoresces at 425nm. Using hrms analysis we determined the molecular formulas to be $C_{12}H_{16}N_2O_4$ and $C_{13}H_{20}N_2O_5$, respectively for M=252 and M=284. Based on our MS/MS analysis, it doesn't appear that these substances are related to each other or to the $C_{14}H_{16}N_2O_6$ previously characterized and discussed above. This M=252 species has an unsaturation index = 6H. Even though this value is relatively high, the UV λ_{max} is ~ 230 nm with a shoulder at ~ 250-260 nm, so it is not a highly conjugated molecule. The MS/MS analysis indicates that the M=252 substance is a stable species that arises from loss of two CO_2 (44) from an unstable M=340 species. Possibly it is a stable monomer. M=252 loses H_2O (18) to form M=234, which then loses 57 to give M=177. The fluorescent M=284 loses 15 (CH_3) to M=269, then loses CO_2 (44) to form 225 and then 29 (CH_3CH_2?) to form M=195. The number of known organic compounds corresponding to the formulae of the mass=252 and mass = 284 species is 2,080 and 481, respectively.



We sent isolated HPLC fractions containing each species to the Deheyn lab and they arranged for 1H-NMR (600 MHz with cryoprobe) analyses of each. Both samples gave similar NMR spectra (see below) that were consistent with those expected of mixtures. Unfortunately, the major peaks likely represent trace contaminants whose signals dwarf those of the samples. The weak signals more likely associated with substance extracted from the mucous appear to be mixtures of carbohydrates (signals in ~ 2 -6 ppm region) from the mucous probably not related to the masses we identified, although this is speculation. Again, the problem of working with too little starting sample proved to be serious.

The results really serve to illustrate the difficulty of natural product identification. While we have the necessary tools of spectroscopy available to us, we lack material of sufficient quantity and purity to make further progress. If sufficient material does become available, it may be possible to conduct mass spectral and NMR-based database searches of known compounds and to successfully perform the spectroscopic approach (that would be necessary if the substrate and related compounds are novel).

I.

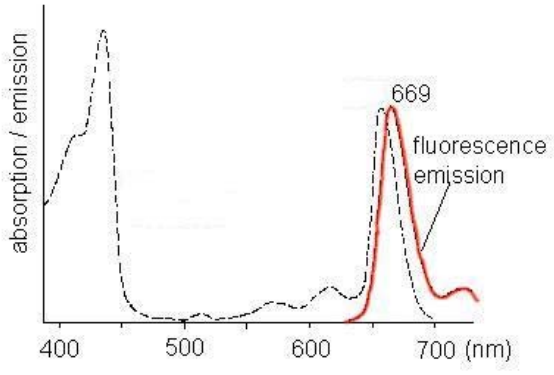
309 to 291 (loss of H_2O (18); alcohol OH present)
291 to 259 (loss of CH_3OH (32); methyl ester present)
259 to 220 (loss of 39 either C_2HN or C_3H_3)
220 to 202 (loss of H_2O (18); alcohol OH present)

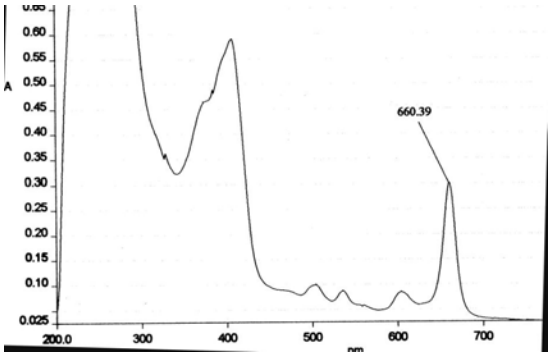
II.

309 to 277 (loss of CH_3OH (32); methyl ester present)
277 to 249 (loss of CO (28); methyl ester present)
249 to 192 (loss of C_2H_3NO (57)
192 to 164 (loss of CO (28)
164 to 107 (loss of C_2H_3NO (57)
107 C_7H_7O

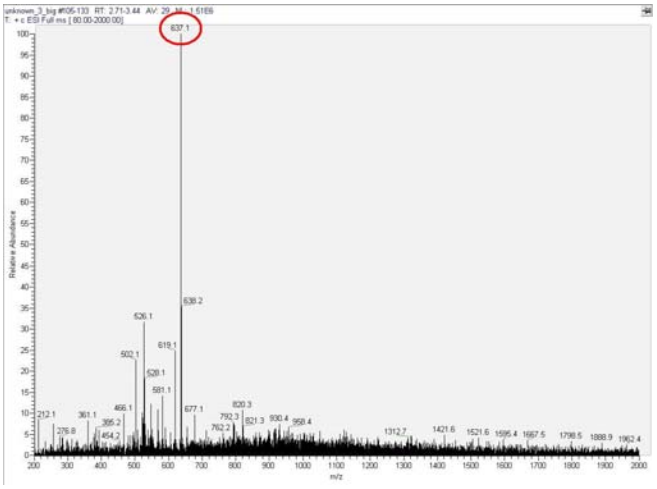
Nominal mass resolution LC/ESIMS and GC/MS along with preliminary high resolution LC/ESIMS data have been used to identify fragments of a MW=308 species that participates in the worm bioluminescence process. Perhaps prohibitively large amounts of mucous are needed to further identify the MW=308 to further study the role of the substance in the worm bioluminescence process.

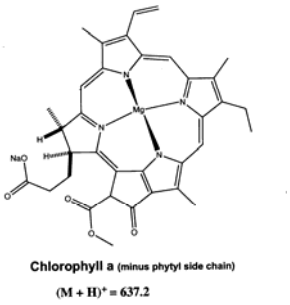
In a separate project, a fluorescent substance was isolated from *Chaetopterus variopedatus* body parts provided by the Deheyn lab. It was partially characterized by mass spectrometry, UV-Vis and fluorescence spectroscopy. The isolated substance (right) was highly fluorescent, emitting in the far red. UV-vis and fluorescence spectroscopy, combined with mass spectrometry, suggested the compound was chlorophyll a (left).





LC/ESMS studies the confirmed that the structure of the isolated substance is a Chlorophyll *a* derivative.

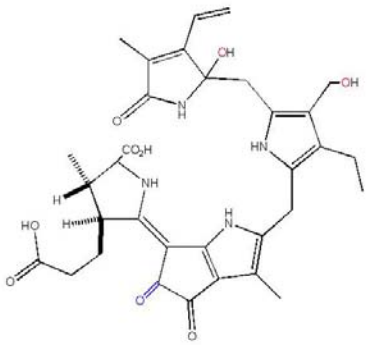




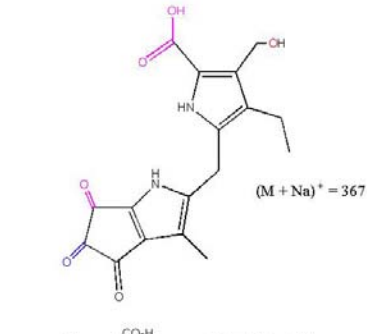
Chlorophyll a (minus phytyl side chain)

$(M + H)^+ = 637.2$

Previously observed masses from LC/MS analysis of *Odontosyllis* mucous may be metabolites of dinoflagellate/krill luciferins. The two worms may contain similar luciferins (lmax~510 nm for *Odontosyllis* & ~460 nm for *Chaetopterus*) that are related to the dinoflagellate/krill luciferin with λmax~473 nm



Dinoflagellate/Krill OXYluciferin



$(M + Na)^+ = 367$

$(M + H)^+ = 391$

Odontosyllis Metabolites ??

Funding Profile

March 1, 2007 to November 30, 2009 [$220,750 + $193,237 (equipment supplement)] = **$413,987**. FY 09 [$75,000 + $50,000 (equipment supplement)] = **$125,000**.

FY07	**FY08**	**FY09**
$164,392	$124,595	$125,000

Personnel Involved in this Grant

Bruce Branchini, Principle Investigator

Martha Murtiashaw, Research Scientist (part-time)
Tara Southworth, Senior Research Technician (part-time)
Danielle Ablamsky, Research Technician

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Justin Rosenberg, Research Technician

Lerna Uzasi, Undergraduate Student
Jen DeAngelis, Undergraduate Student
Sloan Devlin, Undergraduate Student
Julie Rosenman, Undergraduate Student
Audrey Davis, Undergraduate Student
Kelsey Taylor, Undergraduate Student
Samantha Linder, Undergraduate Student

Publications Acknowledging Financial Support from this Grant

1. "Thermostable red- and green-light producing firefly luciferase mutants for bioluminescent reporter applications", Branchini, B.R., Ablamsky, D. A., Murtiashaw, M. H., Uzasci, L., Fraga, H. and Southworth, T. L., *Analytical Biochemistry*, 361 (2): 253-262 (2007).

2. "Synergistic Mutations Produce Blue-Shifted Bioluminescence in Firefly Luciferase," B. R. Branchini, D. M. Ablamsky, J. M. Rosenman, L. Uzasci, T. L. Southworth and M. Zimmer, *Biochemistry* 46 (48): 13847-13855 (2007).

3. "Development of a Multiplexed Bioluminescent Cell-based Assay wit the *Luc* Gene from *Luciola italica* for High Throughput Screening of Cholesterol-Lowering Drugs." E. Michelini, T. L. Southworth, D. Ablamsky, B. R. Branchini and A. Roda, in Proceedings of the 14th International Symposium on Bioluminescence and Chemiluminescence: Chemistry Biology and

Applications, San Diego, CA, October 15-19, 2006, edited by A. A. Szalay, P. J. Hill, L. J. Kricka and P. E. Stanley, World Scientific Publishing Co. Pte. Ltd., Singapore, pp. 119-122, 2007.

4. "Spectral-Resolved Gene Technology for Multiplexed Bioluminescence and High-Content Screening," E. Michelini, L. Cevenini, L. Mezzanotte, D. Ablamsky, T. Southworth, B. Branchini and A. Roda, *Analytical Chemistry* 80: 260-267 (2008).
5. "Combining Intracellular and Secreted Bioluminescent Reporter Proteins for Multicolor Cell-Based Assays," E. Michelini, L. Cevenini, L. Mezzanotte, D. Ablamsky, T. Southworth, B. R. Branchini and A. Roda, *Photochem Photobiol Sci.* 7: 212-217 (2008).

6. "Redshifted Codon-Optimized Firefly Luciferase is a Sensitive Reporter for Bioluminescence Imaging," H. Caysa, R. Jacob, N. Müther, B. Branchini, M. Messerle and A. Söling, *Photochem. Photobiol. Sci.* **8**: 52-26 (2009).

7. "Red-Emitting Luciferases for Bioluminescence Reporter and Imaging Applications," B. R. Branchini, D. M. Ablamsky, A. L. Davis, T. L. Southworth, B. Butler, F. Fan, A. P. Jathoul and M. A. Pule, *Analytical Biochemistry* **396** (2): 290-297 (2010).