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Red-emitting luciferases for bioluminescence reporter and imaging applications

Bruce R. Branchini [a,*], Danielle M. Ablamsky [a], Audrey L. Davis [a], Tara L. Southworth [a], Braeden Butler [b], Frank Fan [b], Amit P. Jathoul [c], Martin A. Pule [c]

[a] Department of Chemistry, Connecticut College, New London, CT 06320, USA
[b] Promega Corporation, Madison, WI 53711, USA
[c] Department of Haematology, University College London Cancer Institute, University College London, London WC1E 6BT, UK

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ABSTRACT

North American firefly *Photinus pyralis* luciferase, which emits yellow–green light (557 nm), has been adapted for a variety of applications, including gene reporter assays, whole-cell biosensor measurements, and in vivo imaging. Luciferase variants with red-shifted bioluminescence and high specific activity can be paired with green-emitting counterparts for use in dual-color reporter assays or can be used alone for in vivo imaging. Beginning with a previously reported red-emitting thermostable mutant and using mutagenesis techniques, we engineered two luciferases with redder emission maxima while maintaining satisfactory specific activities and thermostability. The novel enzymes were expressed in HEK293 cells, where they performed similarly to Promega's codon-optimized click beetle red luciferase in model reporter assays. When the firefly luciferase variants were codon-optimized and retested using optimized substrate concentrations, they provided 50- to 100-fold greater integrated light intensities than the click beetle enzyme. These results suggest that the novel enzymes should provide superior performance in dual-color reporter and in vivo imaging applications, and they illustrate the importance of codon optimization for assays in mammalian cells.

North American firefly *Photinus pyralis,* known for its flash of yellow–green light, houses a bioluminescent reaction catalyzed by the luciferase enzyme (Luc,[1] EC 1.13.12.7) [1,2]. To initiate this well-characterized reaction, Luc converts the substrate firefly luciferin (LH_2), a heterocyclic carboxylic acid, and Mg–ATP into a luciferyl adenylate (Eq. (1)). This reactive intermediate combines with molecular oxygen at the luciferase active site to produce an electronically excited product (Eq. (2)). Relaxation to the ground state is accompanied by the emission of a photon of visible light (Eq. (3)).

$$\mathrm{Luc} + \mathrm{LH_2} + \mathrm{ATP} \overset{\mathrm{Mg^{2+}}}{\leftrightarrows} \mathrm{Luc \cdot LH_2\text{-}AMP} + \mathrm{PP_i} \quad (1)$$

$$\mathrm{Luc \cdot LH_2\text{-}AMP} + \mathrm{O_2} \rightarrow \mathrm{Luc \cdot AMP \cdot Oxyluciferin^*} + \mathrm{CO_2} \quad (2)$$

$$\mathrm{Luc \cdot AMP \cdot Oxyluciferin^*} \rightarrow \mathrm{Luc} + \mathrm{Oxyluciferin} + \mathrm{AMP} + h\nu. \quad (3)$$

This bioluminescence system has a quantum yield of 41.0 ± 7.4% [3], an impressive efficiency compared with the efficiencies of most chemiluminescent reactions [4]. Also, unlike fluorescence systems, an external light source is not required for bioluminescence, so there is virtually no nonspecific light production induced in the cellular environment. It has been estimated that attomole levels of luciferase can be quantified using photomultiplier tubes (PMTs) or charge-coupled devices (CCDs) [5], making the enzyme an attractive candidate for bioanalytical uses that demand high sensitivity. The vast variety of firefly luciferase applications include probes for detecting bacteria and environmental toxins [6–8], bioluminescence resonance energy transfer-based assays of biomolecular interactions [9–12], whole-cell-based biosensors [13–16], and gene expression assays [17–19]. Firefly luciferase has also been expressed in live mammals to visualize events such as tumor growth and metastasis [20,21], cell trafficking [22,23], and genetic regulation [24].

For gene expression assays, it is often useful to employ two luciferase reporters, with one functioning as a control to monitor nonspecific interferences in genetic regulation. A challenge of designing such dual-analyte assays is finding adequate means for

* Corresponding author. Fax: +1 860 439 2477.
E-mail address: brbra@conncoll.edu (B.R. Branchini).

[1] *Abbreviations used:* Luc, *Photinus pyralis* luciferase; LH_2, D-firefly luciferin; PMT, photomultiplier tube; CCD, charge-coupled device; CBR, click beetle red, recombinant *Pyrophorus plagiophthalamus* red-emitting luciferase variant from Promega's Chroma Luc Series; Ppy RE–TS, recombinant Luc containing Thr214Ala/Ala215Leu/Ile232Ala/Ser284Thr/Phe295Leu/Glu354Lys; Ppy RE8, Ppy RE–TS containing Arg330Gly/Ile351-Val/Lys354Glu/Phe465Arg; Ppy RE9, Ppy RE8 containing Glu354Ile; PhRE, recombinant *Phrixothrix hirtus* luciferase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LAR, Luciferase Assay Reagent; HPLC, high-performance liquid chromatography; cDNA, complementary DNA; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline containing 40 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (pH 7.3); CB, cleavage buffer (50 mM Tris–HCl [pH 7.0] containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, and 1 mM dithiothreitol); CBA, CB with 0.8 M ammonium sulfate and 2% glycerol; CoA, coenzyme A.

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separating the two bioluminescent signals while retaining sensitivity. A popular method is Promega's Dual-Luciferase Reporter Assay System, which approaches the problem using two sequential reactions from *Renilla* and firefly luciferases, requiring two different substrates and a stop solution [25].

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. Although 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 because 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 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, femtomolar 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 they 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, USA); restriction endonucleases and Phusion polymerase from New England Biolabs (Ipswich, MA, USA); oligonucleotides from Invitrogen (Carlsbad, CA, USA) and Integrated DNA Technologies (Coralville, IA, USA); Glutathione Sepharose 4B media and pGEX-6P-2 expression vector from GE Healthcare (Piscataway, NJ, USA); QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA); QIAquick polymerase chain reaction (PCR) purification and gel extraction kits from Qiagen (Valencia, CA, USA); Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM I reduced-serum medium from Invitrogen; fetal bovine serum (FBS) from Thermo Scientific HyClone (Logan, UT, USA); and *Trans*IT–LTI transfection reagent from Mirus Bio (Madison, WI, USA). The recombinant Ppy RE–TS protein was expressed and purified as reported previously [28]. The pCBR–Basic Vector, LH_2, Luciferase Assay Reagent (LAR), and Flexi Vector pF9a CMV were obtained from Promega (Madison, WI, USA). The PhRE gene in the pTrc–HisC vector was provided by Ariane Söling (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) and at Agencourt Bioscience Corporation (Beverly, MA, USA), respectively. Mass spectral analyses of purified proteins were performed by tandem high-performance liquid chromatography (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/Lys354-Glu, 5′-GAG ACT ACT AGC GCT ATT CTG **GTA** ACA CCC **GAG** GGG GAT GAT AAA C-3′ [*Spe*I]; and Phe465Arg, 5′-GAA TTG GAA TCC ATA TTG TTA CAA CAC CCC AAC ATC **CGG** GAC GCG GGC-3′ [*Cla*I] (where 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 complementary DNA (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]; and *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] (where 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 *Escherichia 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) and 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 phosphate-buffered saline (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 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*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 cleavage buffer (CB) 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 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 cells (American Type Culture Collection [ATCC], Manassas, VA, USA) were aliquoted into 6-well plates at a concentration of approximately 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 of Opti-MEM I reduced-serum medium was combined with 6 μl of *Trans*IT–LTI transfection reagent. The mixture was vortexed at the highest setting for 1 s, incubated for 20 min at ambient temperature, and 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 approximately 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 min. The cell pellets were then resuspended in 0.1 ml of PBS. Cells were lysed by five freeze–thaw cycles at −80 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 PMT and a C6271 HV power supply socket assembly that was described in detail previously [32]. Reactions were initiated by the injection of 120 ml of 9.0 mM ATP into 8 × 50-mm polypropylene tubes containing 0.4 ml of 25 mM glycylglycine buffer (pH 7.8) with 0.4 mM LH_2 and 0.52 μg of enzyme. The final concentrations of LH_2 and Mg–ATP were 0.3 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 PMT.

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, Palo Alto, CA, USA) as described previously [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 PMT used to make the measurements, were obtained at 25 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 of CBA to a cuvette containing solutions of LH_2 (0.3 mM final) and Mg–ATP (2.0 mM final) in 25 mM glycylglycine buffer (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, USA) 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. The assays (270 μl) were optimized by including coenzyme A (CoA, 0.1 mM final) and by reducing the concentrations of LH_2 (0.2 mM final) and Mg–ATP (400 mM final). For studies using LAR, each well contained 10 μl of soluble cell lysate and assays were initiated by the injection of 100 μl of LAR. Bioluminescence signals, integrated over 60 s, were measured using a 600-nm longpass filter and PMT voltage was 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 approximately 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 3× 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 *Bam*HI/*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 six 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 to 617 nm (Table 1 and Fig. 1). With respect to the wild-type *P. pyralis* enzyme, the new luciferase, Ppy RE8, contained eight amino acid changes. Although 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.

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 mg/0.25 L culture (Ppy RE8), 10.4 mg/0.25 L culture (Ppy RE9), 2.5 mg/0.25 L culture (CBR), and 2.1 mg/0.25 L culture (PhRE). 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 sequence results, were used to predict the protein masses, all of which were found to be within allowable experimental error (0.01%) of the mass spectral analysis: CBR, 60,596 Da; PhRE, 61,154 Da; Ppy RE8, 61,000 Da; and Ppy RE9, 60,988 Da. However, our sequencing data for PhRE predicted a single amino acid deviation from the GenBank entry (accession no. AF139645.2) at position 6 (Val to Ile) that was confirmed by the difference of +14 Da.

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 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 and 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 with 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 approximately 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 and 6.0 h of incubation, respectively, whereas after 8 h at 37 °C the enzymes retained 28% and 42% of their initial activity, respectively (Table 1 and Fig. 2). Over an 8-h time period, the stability values of the bioluminescence activity of CBR and Ppy RE9 at 37 °C are quite similar and exceed the stability of Ppy RE8 (Fig. 2). Compared with the already impressive

Table 1
Properties of purified luciferases at pH 7.8.

Enzyme	Relative specific activity[a]		Rise time (s)[b]	Decay time (min)[b]	K_m (μM)		Thermal inactivation (h)[c]	Bioluminescence emission maximum (nm)[d]	
	Peak	Integration (60 s)			LH_2	Mg–ATP		25 °C	37 °C
Ppy RE–TS	100	100	0.45	0.23	18 ± 2	68 ± 5	4.5	610 (59)	612 (60)
Ppy RE8	70	78	0.50	0.26	77 ± 8	391 ± 39	3.5	617 (60)	618 (62)
Ppy RE9	77	71	0.34	0.13	84 ± 5	222 ± 24	6.0	617 (57)	617 (59)
CBR	9	41	2.34	>15	22 ± 3	5.1 ± 0.8	5.5	617 (62)	619 (66)
PhRE	52	12	0.56	0.07	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] Bioluminescence rise times (to maximum activity) and decay times (to 20% initial activity) were measured from peak flash heights.
[c] Thermal inactivation is reported as time to 50% initial activity when incubated at 37 °C.
[d] Bioluminescence emission spectra of purified proteins were obtained as described in Materials and methods, with bandwidths at full-width half-maximum indicated in parentheses. Due to the rapid inactivation of PhRE at 37 °C, a bioluminescence spectrum could not be recorded.

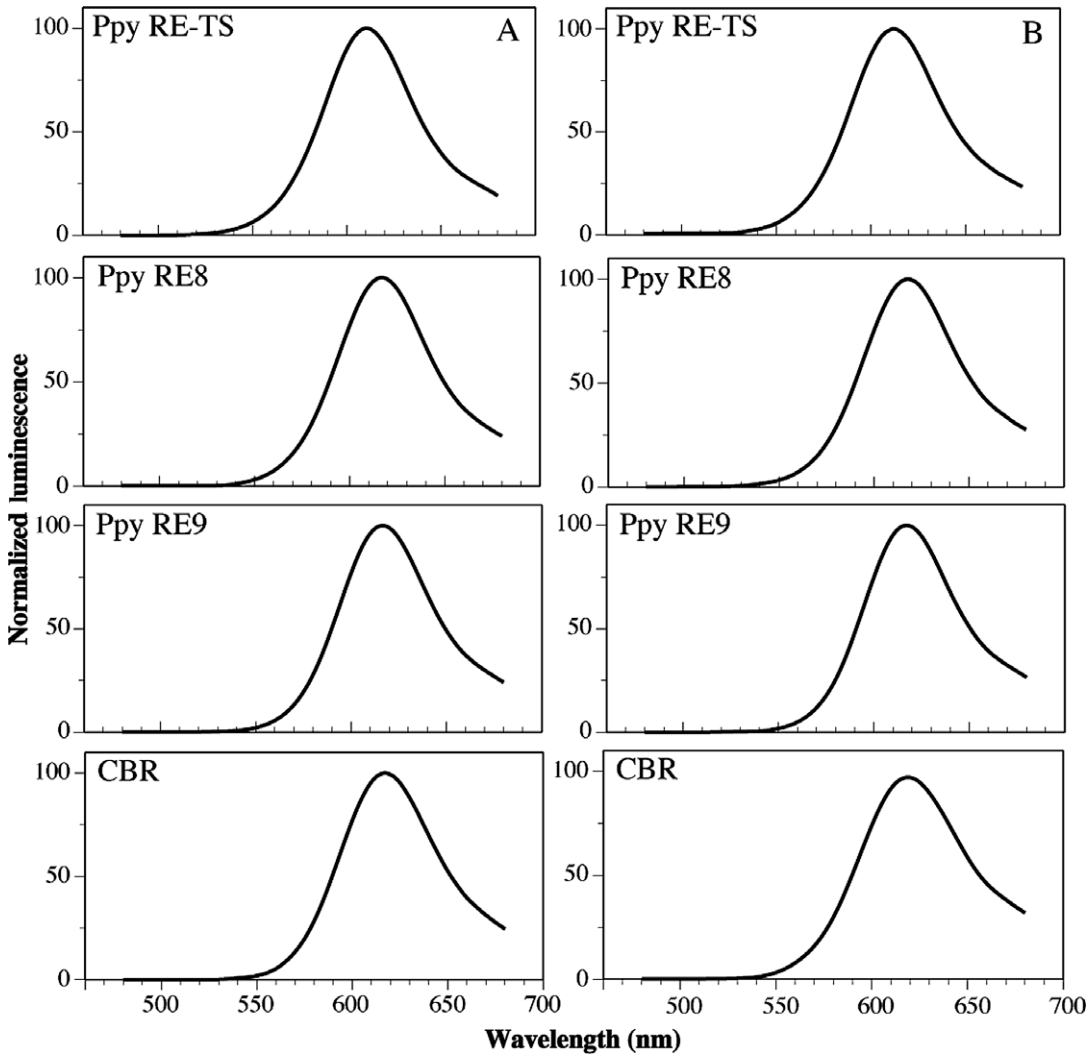

Fig. 1. Normalized bioluminescence emission spectra of purified luciferase enzymes measured at pH 7.8 and 25 °C (A) or 37 °C (B), as described in Materials and methods.

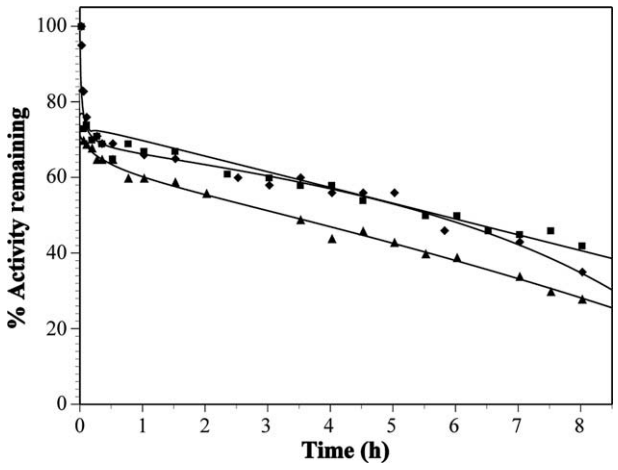

Fig. 2. Heat inactivation of purified luciferases Ppy RE8 (▲), Ppy RE9 (■), and CBR (♦) at 37 °C in 25 mM glycylglycine buffer (pH 7.8). Bioluminescence activities were monitored over 8 h as described in Materials and methods, and data were collected in duplicate.

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 1 min of incubation at 37 °C (Table 1). However, PhRE has been paired with green-emitting luciferases from the same species (*P. 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 and coworkers 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.

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 specific activity, respectively, and 70% and 77% flash height-based specific activity, respectively (Table 1 and Fig. 3). Although CBR had only 9% flash height-based specific activity, the slow signal decay resulted in 60-s integrated activity of 41%, a value approximately 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 and Fig. 3). Because PhRE exhibited relatively low integrated specific activity and poor thermostability at 37 °C (Table 1), we did not perform further studies with this enzyme. However, it is likely that improved PhRE variants can be developed, although this undertaking might compromise the outstanding red bioluminescence color.

Model reporter assays in mammalian cells

The promising results from the characterization of the pure Ppy RE8 and Ppy RE9 proteins suggested that 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, whereas the integrated activity of Ppy RE8 was 1.3-fold greater (Fig. 4). A possible reason why 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 firefly 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

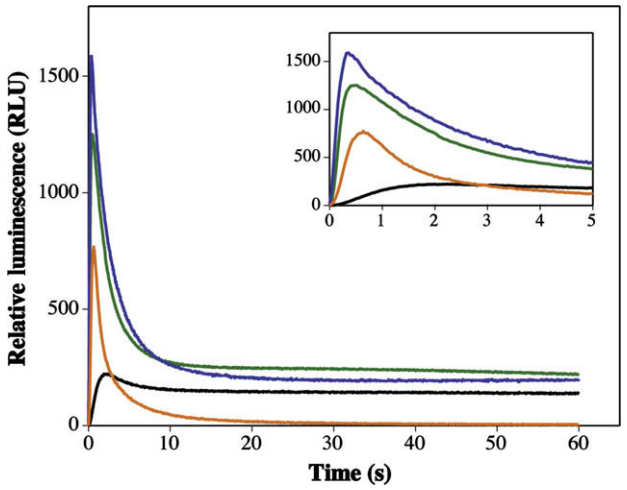

Fig. 3. Relative bioluminescence activities from reactions of equivalent quantities of purified luciferases Ppy RE8 (green line), Ppy RE9 (blue line), CBR (black line), and PhRE (orange line) using 0.3 mM LH_2 and 2 mM Mg–ATP were recorded with a custom-built luminometer as described in Materials and methods. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

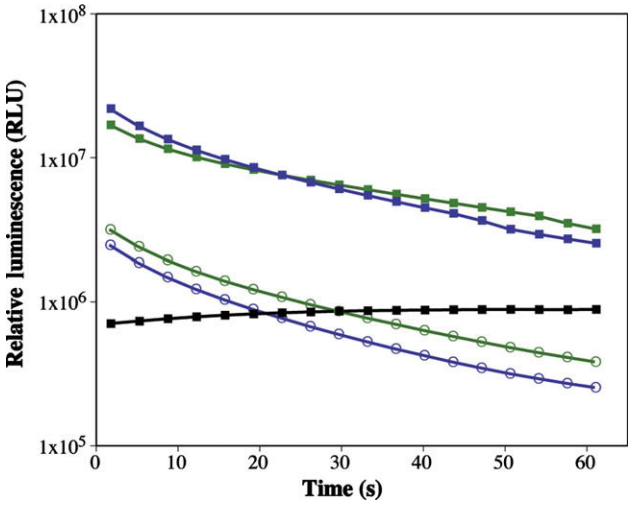

Fig. 4. Relative bioluminescence activities of soluble cell lysates from equivalent numbers of HEK293 cells expressing Ppy RE8 (green ○), Ppy RE9 (blue ○), human codon-optimized Ppy RE8 (green ■), human codon-optimized Ppy RE9 (blue ■), and mammalian codon-optimized CBR (black ■) at 37 °C. Assays of 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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[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 given that the 60-s integration-based activities were enhanced 6.7- and 9.5-fold, respectively (Fig. 4). Because 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. However, we did not verify this by direct measurement of the individual enzyme concentrations in the lysates.

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 (cf. 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 8.6×10^7, 4.8×10^7, and 0.1×10^7 RLU, respectively. The 60-s integrated activity values correspond to Ppy RE9 providing 1.8- and 97-fold higher activity than Ppy RE8 and CBR, respectively, 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, respectively, than the click beetle enzyme in the commercial reagent.

The 53- and 21-fold (Ppy RE8) and 97- and 40-fold (Ppy RE9) greater 60-s integrated activities compared with 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

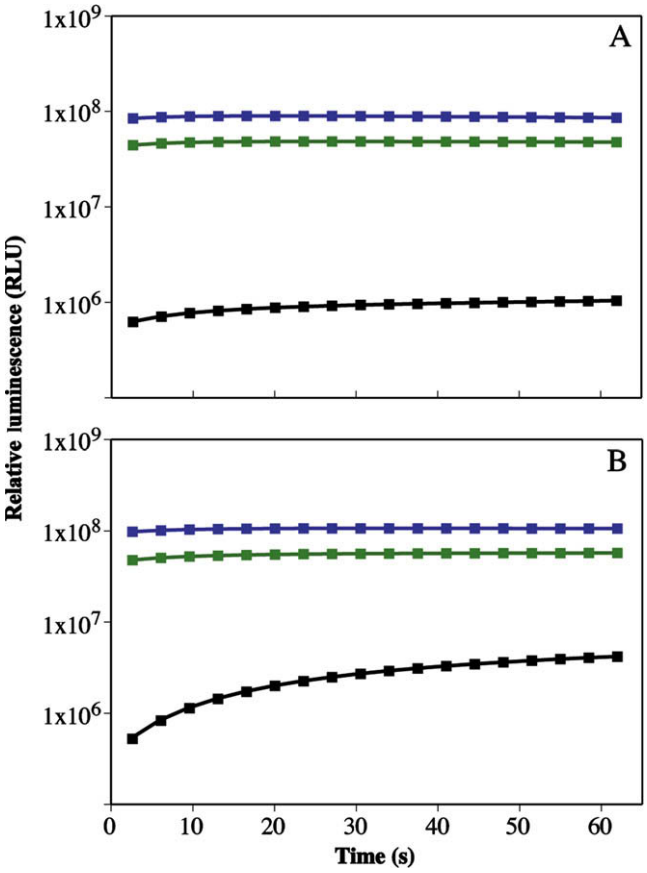

Fig. 5. Relative bioluminescence activities of soluble cell lysates from equivalent numbers of HEK293 cells expressing mammalian codon-optimized Ppy RE8 (green line), Ppy RE9 (blue line), and CBR (black line) at 37 °C. Assays of 10 µl of lysates were carried out in 0.4 mM ATP, 0.2 mM LH_2, 0.1 mM CoA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

applications in mammalian cells. Because CBR has proven to be 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 given that, compared with 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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