A *Photinus pyralis* and *Luciola italica* Chimeric Firefly Luciferase Produces Enhanced Bioluminescence

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CONTENTS

I. MATERIALS AND METHODS

Materials and general methods. The following materials were obtained from the sources indicated: Mg-ATP (bacterial source) from Sigma-Aldrich (St. Louis, MO); restriction endonucleases and DNA ligase from New England Biolabs (Beverly, MA); mutagenic oligonucleotides from Integrated DNA technologies (Coralville, IA); Glutathione Sepharose 4B media and the pGEX-6P-2 expression vector from GE Healthcare (Piscataway, NJ); QIAquick Gel Extraction kit from Qiagen (Valencia, CA); and the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). Firefly luciferin (LH_2), dehydroluciferin (L), and dehydroluciferyl-AMP (L-AMP) were generous gifts from Promega (Madison, WI), Regis Technologies (Skokie, IL) and Joaquim C.G. Esteves da Silva, respectively. The L-AMP was purified by HPLC prior to use. Luciferyl-adenylate (LH_2-AMP) and oxyluciferin ($OxyLH_2$) were prepared and purified as described previously.[1, 2]

Site-directed mutagenesis was preformed with the QuikChange® Lightning Site-Directed Mutagenesis kit from Agilent (Santa Clara, CA). DNA sequencing to verify all mutations and ligations was performed at the W. M. Keck Biotechnology Laboratory at Yale University.

Construction of expression plasmids encoding LitPpy and PpyLit. The pGEX-6P-2 expression plasmids containing the cDNA encoding LitPpy and PpyLit were generated as follows (Figure S1). The cDNA encoding the peroxisomal targeting signal (^{546}AlaLysMet^{548}) at the carboxyl terminus of *Luciola italica* luciferase[3] was mutated to ^{546}AlaGlyGly^{548} using the following primer and its respective reverse compliment: 5′-AG AAA CCA CAA GCC **GGG GGG** TAA ATC GGT CAA AAT G-3′ (the mutated codons are in bold). The encoded protein is referred to as LitWT in this manuscript. A *Pac*I restriction site was introduced into the LitWT cDNA using the following primer and its respective reverse compliment: 5′-GTA GAT CGT

TTG AAA TCA TTA ATT AAA TAC AAG GGG TAC CAG G-3′ (underline represents the silent change to introduce the *Pac*I site). Next, the pGEX-6P-2 plasmids containing LitWT and PpyWT[4] were digested with *Pac*I and *Xho*I generating two cDNA fragments for each construct encoding the N-terminal and C-terminal domains for each luciferase. The four fragments were purified from an agarose gel with the QIAquick Gel Extraction kit and then ligated to create constructs in pGEX-6P-2 encoding the LitPpy and PpyLit chimeric proteins.

Protein expression and purification. Luciferases in the pGex-6P-2 plasmid were expressed in *E. coli* strain BL21 as GST-fusion proteins and whole-cell extracts were purified as previously described.[5] 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 20 mM Tris-HCl (pH 7.4 at 4 °C) containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT (CB) for 18-20 h at 4 ˚C with gentle mixing. Proteins were eluted in CB with yields of ~5 mg/0.25 L culture, and stored at 4 °C in CB containing 0.8 M ammonium sulfate and 2% glycerol (CBA).[6] Protein concentrations were determined with the Bio-Rad Protein Assay system using BSA as the standard.

Determination of protein masses. Mass spectral analyses were performed by tandem HPLC-electrospray ionization mass spectrometry using a ThermoFinnigan Surveyor HPLC system and a ThermoFinnigan LCQ Advantage mass spectrometer. The conditions for protein mass determinations were: column, Jupiter 5 μm C4 300Å (50 *x* 1.00 mm); mobile phase, 95:5 water (0.1% TFA):acetonitrile (0.1% TFA), gradient after 2 min to 5:95 water (0.1% TFA):acetonitrile (0.1%TFA) over 4 min; flow rate, 0.15 mL/min; MS mode, ES+; scan range, m/z = 200-2000; scan time, 0.2 s. The electrospray source of the MS was operated with a

capillary voltage of 45 V, and source voltage of 3.5 kV. Total mass spectra for protein samples were reconstructed from the ion series using Bioworks Browser 3.0 with BIOMASS deconvolution.[7] The found molecular masses (Da) of the following proteins were within the allowable experimental error (0.01%) of the calculated values (in parenthesis): PpyWT, 61 161 (61 157); LitWT, 60 764 (60 766); LitPpy, 61 094 (61 095); and PpyLit, 60 812 (60 811).

Bioluminescence specific activities. Bioluminescence specific activity assays were performed as described previously[8] with a custom-built luminometer assembly containing a Hamamatsu R928 PMT and a C6271 HV power supply socket assembly. Reactions were initiated by the injection of 0.12 mL of 8.8 mM Mg-ATP into 8 x 50 polypropylene tubes containing 0.4 mL of 0.525 mM LH_2 in 25 mM glycylglycine buffer (pH 7.8) and 0.5-1 μg enzyme in CBA. The final concentrations of LH_2 and Mg-ATP were 0.4 and 2.0 mM, respectively, in a final volume of 0.525 mL. For integrated specific activities, light output was monitored for 15 min. All values were corrected for the spectral response of the detector.

Bioluminescence emission spectra. Bioluminescence emission spectra were obtained using a Horiba Jobin-Yvon iHR imaging spectrometer equipped with a liquid N_2 cooled charge-coupled device (CCD) detector and the excitation source turned off. Data were collected at 22 ºC in a 0.8 mL quartz cuvette over the wavelength range 450-750 nm with the emission slit width set to 5 nm and were corrected for the spectral response of the CCD using a correction curve provided by the manufacturer. Bioluminescence was initiated by adding 5 μL of enzyme in CBA (0.02-0.03 μM final concentration) to a cuvette containing solutions (0.52 mL) of 100 μM LH_2 and 2 mM Mg-ATP in 25 mM glycylglycine buffer pH 7.8, 25 mM HEPES pH 7.0, or 25 mM MES pH 6.5. The pH values were confirmed before and after spectra were obtained.

Steady-state kinetic constants. Values of K_m and V_{max} for LH_2 and Mg-ATP were determined from bioluminescence activity assays in which measurements of maximal light intensities (bursts) were taken as estimates of initial velocities. Data for LH_2 and Mg-ATP were collected in 0.525 mL reactions in 25 mM glycylglycine buffer, pH 7.8, containing 0.5 – 1 μg of luciferase enzyme in CBA. The concentration of one substrate was maintained at saturation, while the other was varied (2 μM - 0.70 mM for LH_2 and 10 μM- 2 mM for Mg-ATP). Reactions were initiated by injection of solutions of the substrate being maintained at saturating concentration. Kinetic constants were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis–Menten equation to a rectangular hyperbola. The corresponding k_{cat} values were obtained by dividing the V_{max} values by the amount (μmol) of each luciferase in the assay mixtures.

To assess the reversible inhibition of PpyLit by L and oxyluciferin with respect to LH_2, the initial velocities of the enzyme-catalyzed light reactions were determined as a function of LH_2 concentration (5 - 100 μM) in the absence or presence of fixed concentrations of L (0.25 – 3 μM) or oxyluciferin (0.25 – 2 μM) in assays in 25 mM glycylglycine buffer, pH 7.8. All assays contained 0.5 μg enzyme in CBA and were initiated by the injection of a saturating concentration of Mg-ATP (2 mM). The inhibition constants (K_i) were determined by the method of Dixon, (Dixon and Webb, Enzymes, 3rd Ed. 1979 p. 332) using Enzyme Kinetics Pro software (SynexChem, LLC). The K_i for L-AMP was determined as described by Ribeiro and Esteves da Silva[9] in which the adenylate is treated as a tight-binding competitive inhibitor.

Analysis of reaction products of luciferases and LH_2-AMP. Enzyme (1.8 nmol) and D-LH_2-AMP (0.6 nmol) were mixed in 44 μL 0.1 M sodium phosphate buffer pH 7.8 containing 1 mM EDTA (NaPB) and incubated on ice for 2 min. Acetonitrile (2 μL) and caffeine (4 μL of a

50 μM solution in NaPB) were added and an aliquot (25 μL) of the mixture was injected into a Thermo Finnigan Surveyor HPLC system coupled to a Thermo Scientific LCQ Advantage MS. Samples were injected via autosampler and UV-Vis data were collected from a photodiode array (PDA) detector. Water-0.1% trifluoracetic acid (solvent A) and acetonitrile-0.1% trifluoracetic acid (solvent B) were used as eluents. The conditions for the product analysis were: column, Phenomenex Kinetex 2.6 μm 100 Å C18 (100 x 2.1 mm) with guard column; mobile phase, solvent A:solvent B (95:5) for 2.5 min, linear gradient to 65:35 over 30 min, held at 65:35 over 3 min; then 95:5 over 8 min; flow rate, 0.25 mL/min; PDA signal monitored at 350 nm; MS mode, ES+; scan range, m/z = 100-800. The chromatogram produced by 350 nm absorption monitoring is shown in Figure S7.

Under these HPLC conditions, retention times (min) for the following authentic standards were: LH_2-AMP (15.3); L-AMP (16.9); LH_2 (21.5); $OxyLH_2$ (22.5) and L (23.5). Product distributions were calculated by comparing the integrated areas of chromatographic peaks (350 nm absorbance monitoring) to standard curves generated from authentic samples of L, L-AMP, $OxyLH_2$ and LH_2. The sum of the product yields equaled 101% ± 3% of the amount of D-LH_2-AMP consumed. Product yields were expressed as percentages based on the total amount of D-LH_2-AMP consumed in the enzyme-catalyzed reactions. Corrections for buffer hydrolysis of D-LH_2-AMP were based on the amount of LH_2 detected. Since L-AMP can hydrolyze to L and AMP during the course of the experiment, for simplicity we reported the yield of L-AMP as the sum of the yields of L plus L-AMP. Product yields are reported as mean ± SD and are based on 3 trials with each enzyme.

Estimated rates of half-reactions. The estimated rates of the oxidative half-reactions were based on bioluminescence activity assays using synthetic LH_2-AMP as the substrate (Table

S3). Assays (0.510 mL) in 50 mM glycylglycine buffer, pH 7.8, contained aliquots (0.1 mL) of LH_2-AMP solution (final concentration 1.5 - 35 μM) in 10 mM sodium acetate, pH 4.5. Light reactions were initiated by injections of 10 μL of luciferase enzymes (0.4 – 1 μg in CBA). Kinetic constants were determined using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis-Menten equation to a rectangular hyperbola. The corresponding k_{cat} values were obtained by dividing the V_{max} values by the final amounts (μmol) of each luciferase in the assay mixtures.

The relative rates of adenylate formation were estimated by fluorescence-based assays of L-AMP formation[10] using a Perkin Elmer LS55 luminescence spectrometer operated in the "time-drive" mode (Table S3). Using an excitation wavelength of 350 nm, the luciferase-catalyzed formation of L-AMP from dehydroluciferin (L), initiated by the addition of Mg-ATP, was assessed by following the decrease in the intensity of the 440 nm fluorescence of the initial enzyme-L complex. The change in fluorescence was used to estimate the rates of L-AMP formation catalyzed by the luciferases. Assays (0.4 mL) in 50 mM Tris buffer, pH 7.4 contained 2.8 μM enzyme and 0.55 μM L. The initial fluorescence at 440 nm was recorded and then the decrease was monitored following the rapid injection of 50 μL solutions of varying concentrations of Mg-ATP in the same buffer. The rates of decrease (slopes) were calculated using the arithmetic function of the UVWinLab software (Perkin Elmer) and used to determine the initial velocities for each Mg-ATP concentration. The V_{max} values were obtained by fitting the data using a nonlinear least squares method of the Enzyme Kinetics Pro software (SynTex), which fits data from the Michaelis-Menten equation to a rectangular hyperbola.

Relative bioluminescence quantum yields. Bioluminescence quantum yields (Table 1) for LH_2 were carried out as previously described.[11] The light output was monitored for 30 s or 5

min until the initial signal intensity decreased by 99% at a sampling rate of 1 kHz (30 s)or 100 Hz (5 min). Additional aliquots of enzyme were added to the spent mixtures and emission intensity was monitored to ensure that the reactions were completed.

Bioluminescence quantum yields with limiting LH_2-AMP (Table 1) were performed in assays (0.5 mL) in 50 mM glycylglycine buffer, pH 7.8 containing 0.1 mL of 0.5 μM LH_2-AMP in 10 mM sodium acetate pH 4.5, and were initiated by the injection of 10 μL of enzyme (16 – 32 μM) in CBA. The final concentrations of enzyme and LH_2-AMP were 310-630 nM and 100 nM, respectively, in a final volume of 0.51 mL. The light output was monitored at a sampling rate of 1 kHz for 15-30 s until the initial signal intensity decreased by 99%. An additional aliquot of enzyme was added to the spent mixtures and emission intensity was monitored to ensure that the reactions were completed.

Relative fluorescence of oxyluciferin bound to PpyWT and PpyLit. Oxyluciferin ($oxyLH_2$) was synthesized according to a literature procedure[1] and resuspended in 50 mM Tris, pH 7.4 that had been purged with argon. Solutions (500 μL) in 50 mM Tris, pH 7.4 were prepared that contained 6 μL of 8.5 μM $oxyLH_2$ and 15 μL of 33 μM enzyme (PpyWT or PpyLit). The final concentrations of $oxyLH_2$ and enzyme were 0.1 μM and 1 μM, respectively. The fluorescence emission spectra of the solutions were obtained on a Horiba Jobin-Yvon iHR imaging spectrometer equipped with a liquid N_2 cooled CCD detector with the excitation source set to 390 nm and the excitation and emission slits set to 2 nm. All spectra were corrected for the spectral response of the CCD using a correction curve provided by the manufacturer. Data were collected at 22 °C in a 0.8 mL quartz cuvette over the wavelength range 400-935 nm. The spectra were integrated using FluorEssence™ software (Horiba). There were no differences in intensity and area between the spectra obtained for $oxyLH_2$ bound to PpyWT versus PpyLit.

Circular dichroism spectroscopy. Circular dichroism (CD) spectra (190-260 nm) and the mean aggregation temperature (T_m) of PpyWT and PpyLit were determined (Table S2) from assays containing 0.2 mg/mL (3.3 μM) luciferase in 0.1 M sodium phosphate buffer (pH 7.8) with 1 mM EDTA, using a Jasco J-810 spectropolarimeter as previously described.[6]

Denaturation of luciferases using guanidine HCl (Gd-HCl). All assays were performed in 0.1 M sodium phosphate buffer (pH 7.8) containing 1 mM EDTA. For monitoring activity and fluorescence (Figure S6), assays containing 0.8 μM of PpyWT or PpyLit and varying concentrations of Gd-HCl (0-1.0 M) were incubated in a 25 °C water bath for 10 min. To determine bioluminescence activity as a function of Gd-HCl, 3 μL aliquots were removed and added to 8 x 50 polypropylene tubes containing 0.4 mL of 0.130 mM LH_2 in 25 mM glycylglycine buffer (pH 7.8). Reactions were initiated by the injection of 0.12 mL of 8.8 mM Mg-ATP. The final concentrations of enzyme, LH_2 and Mg-ATP were 0.5 nM, 0.1 mM and 2.0 mM, respectively, in a final volume of 0.525 mL. Bioluminescence activity assays were performed as described previously[8] with a custom-built luminometer assembly containing a Hamamatsu R928 PMT and a C6271 HV power supply socket assembly. To determine the fluorescence emission of the luciferases as a function of Gd-HCl, 500 μL aliquots were removed and the fluorescence emission was obtained using a PerkinElmer LS55 luminescence spectrometer with excitation set at 295 nm and data collected over the wavelength 310-400 nm. The molar ellipticity of the luciferases as a function of Gd-HCl was determined (Figure S6) as described above for CD spectra except that assays contained 3.3 μM of luciferase and varying concentrations of Gd-HCl (0-1 M) and were incubated in a 25 °C water bath for 10 min before obtaining the spectra.

II. ADDITIONAL INTRODUCTORY MATERIAL

While many chimeric proteins containing various luciferases joined to non-bioluminescent proteins have been made and used in a wide variety of bioanalytical applications,[12-14] only a few reports of chimeric luciferases produced by connecting regions of different beetle luciferase sequences have appeared. In an early report,[15] Ohmiya and coworkers made six chimeric luciferases from four segments of the luciferases *Hotaria parvula* and *Pyrocoelia miyako* in an investigation aimed at determining the structural region responsible for bioluminescence color. Their results indicated that, at least for these luciferases, the region containing residues 208-318 was important. In two related studies on luciferase color determinants, Viviani *et al.* reported two chimeric proteins of green light-emitting (552 nm) *Phrixothrix viviani* and red light-emitting (623 nm) *Phrixothrix hirtus* railroad worm luciferases that were constructed[16] by exchanging the regions 1-344. Additional chimeric proteins were made[17] from the railroad worm enzymes by shuffling the regions 1-219, 220-445, and 220-334. The results of the spectral emission studies indicated that emission color was dictated by the source of the 1-244 region. Three chimeric luciferases were constructed from three regions of a thermostable *Luciola cruciata* variant (Thr219Ile) and Luc by the Hirokawa group.[18, 19] One of the proteins featuring *L. cruciata* Thr219Ile residues 1-449 fused to the Luc region 447-550 was subsequently subjected to random mutagenesis. The additional mutation Val239Ile produced a thermostable luciferase with catalytic efficiency approximately equal to Luc and improved resistance to red-shifted emission at low pH. Also, Oba and colleagues made[20] two chimeric proteins with Luc and a fatty acyl-CoA synthetase from *Drosophila melanogaster* by swapping the N-domains (1-437). The investigation highlighted the importance of the Luc C-domain for luminescence efficiency as the chimera containing the Luc N-domain retained only 4% of the

bioluminescence activity of Luc. Additionally, Zako, *et al.* prepared[21] the Luc N-domain (residues 1-437) alone and evaluated it for luminescence production with either firefly luciferin (LH_2) and ATP or with synthetic luciferyl-AMP (LH_2-AMP). These authors demonstrated that while the N-domain could catalyze very weak bioluminescence with a greatly reduced emission rate,[21] the C-domain likely has an important role in coupling the adenylation and oxidation partial reactions.[22] In all of these cited studies, no data were presented that indicated catalytic activity had been enhanced beyond that of wild-type Luc.

III. DISCUSSION OF BIOLUMINESCENCE HALF-REACTION DATA

We further examined the differences in the estimated rates of photon production with saturating concentrations of substrates LH_2 and Mg-ATP. The rate differences were manifested in flash-height based specific activities and k_{cat} values. We were encouraged to find that both the estimated adenylation and oxidation rates (Scheme 1, Table S3) were accelerated by PpyLit ~1.3-fold over that of PpyWT. While the adenylation rates were estimated by changes in fluorescence of enzyme bound L-AMP, the oxidations rates were determined by monitoring photon emission from LH_2-AMP (Scheme 1). We caution that the apparent enhancement in the PpyLit catalyzed oxidation reaction may be a manifestation of the greater Φ_{ES} value. That is, if the oxidation rates of the PpyLit and PpyWT reactions were approximately equal, as we believe they are because of the comparable oxyluciferin yields (Table 1), the oxidation rate of PpyLit would *appear* to be ~1.3-fold enhanced, reflecting a greater efficiency of photon production from the same amount of oxyluciferin produced at approximately the same rate. Unfortunately, we were not able to directly measure the rate of oxyluciferin production by non-luminescent methods.

Table S1: Bioluminescence activity of chimeric and wild type luciferases

Enzyme	Relative Specific Activity [a]		k_{cat} (s^{-1}) [b]	K_m (μM)		k_{cat}/K_m	Bioluminescence Emission λ_{max} [d]		Rise Time	Decay
	Flash Height	Integrated		LH_2	Mg-ATP	($mM^{-1}s^{-1}$) [c]	pH 7.8	pH 6.5	(s) [e]	Time (min) [f]
PpyWT	100 ± 4	100 ± 2	0.18 ± 0.01	15 ± 2	86 ± 7 [g]	2.1 ± 0.1	560 (70)	608 (94)	0.33 ± 0.04	0.21 ± 0.02
LitWT	84 ± 2	163 ± 8	0.14 ± 0.01	90 ± 9	180 ± 14	0.78 ± 0.11	572 (93)	613 (71)	0.21 ± 0.01	3.3 ± 0.07
PpyLit	180 ± 10	200 ± 12	0.32 ± 0.02	25 ± 2	53 ± 5	6.04 ± 0.11	560 (69)	559 (95)	0.29 ± 0.01	0.2 ± 0.01
LitPpy	17 ± 1	73 ± 3	0.033 ± 0.001	54 ± 5	188 ± 10	0.18 ± 0.06	595 (90)	610 (75)	0.43 ± 0.04	8.3 ± 1

[a]Specific activities were obtained at pH 7.8 with LH_2 (400 μM) and Mg-ATP (2 mM) and are expressed relative to PpyWT values, which are defined as 100. Integrated activities are based on total light emission in 15 min. [b]Values of k_{cat} were obtained by dividing the V_{max} values (in units of Einstein × 10^{-6} s^{-1}) obtained from the measurement of the K_m value for LH_2 by the amount (in micromoles) of each luciferase. [c]The k_{cat}/K_m value was determined by dividing the k_{cat} by the Mg-ATP K_m value (in mM). [d]Bioluminescence emission maxima at pH 7.8 and pH 6.5 with the bandwidth at full-width half-maximum shown in parentheses. Emission maxima were obtained from two trials and the standard deviation is ± 1 nm. [e]Bioluminescence rise time to maximum intensity. [f]Time for the bioluminescence signal to decay to 10% of the maximum value. All activity values were obtained from at least three trials and are reported as means ± SD. [g]The Mg-ATP K_m reported for PpyWT differs from the value (160 μM) previously reported.[8] The current value was verified in multiple independent trials with different batches of PpyWT and the difference is likely due to the purity of the Mg-ATP purchased from Sigma.

Table S2: Structural Properties of Luciferase Enzymes

Enzyme	T_m (°C)	Molar ellipticity [Θ], (deg cm^2/dmol)	
		208 nm	222 nm
PpyWT	43.9 ± 0.02	-7582 ± 634	-7245 ± 531
PpyLit	43.1 ± 0.03	-7610 ± 654	-7198 ± 577

Mean aggregation temperature (T_m) and molar ellipticity were determined by CD spectroscopy as described above in the Materials and Methods.

Table S3: Relative estimated rates of half-reactions

Enzyme	Relative rates (%) [a]		
	Overall	Adenylation	Oxidation
PpyWT	100 ± 4	100 ± 12	100 ± 9
LitWT	84 ± 2	37 ± 5	129 ± 7
PpyLit	180 ± 10	126 ± 11	130 ± 12

[a]Overall and oxidation rates (expressed as % relative to PpyWT) were estimated using flash-height-based activity values obtained with LH_2 and Mg-ATP or LH_2-AMP, respectively. Adenylation rates were estimated from the rates of L-AMP formation. All of the relative rate values in the table were obtained from at least three trials and are reported as mean ± standard deviation. Additional experimental details are included above in the Materials and Methods.

V. FIGURES

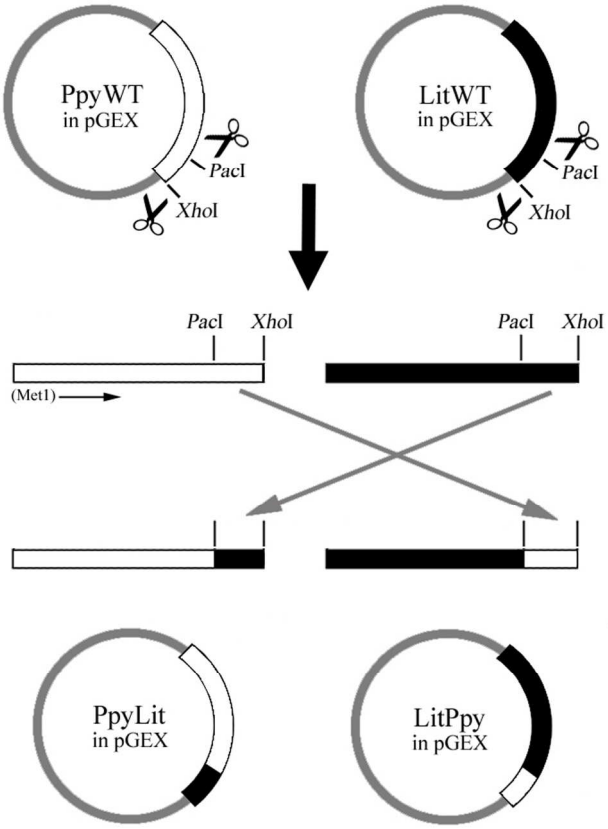

Figure S1. Construction of PpyLit and LitPpy in the pGEX-6P-2 expression plasmid. The pGEX-6P-2 expression plasmids containing the cDNA encoding PpyWT (white) and LitWT (black) were digested with *Pac*I and *Xho*I to cut out the fragments that encode the C-terminal domains. The fragments were purified and swapped by ligation creating the PpyLit and LitPpy chimeric plasmids.

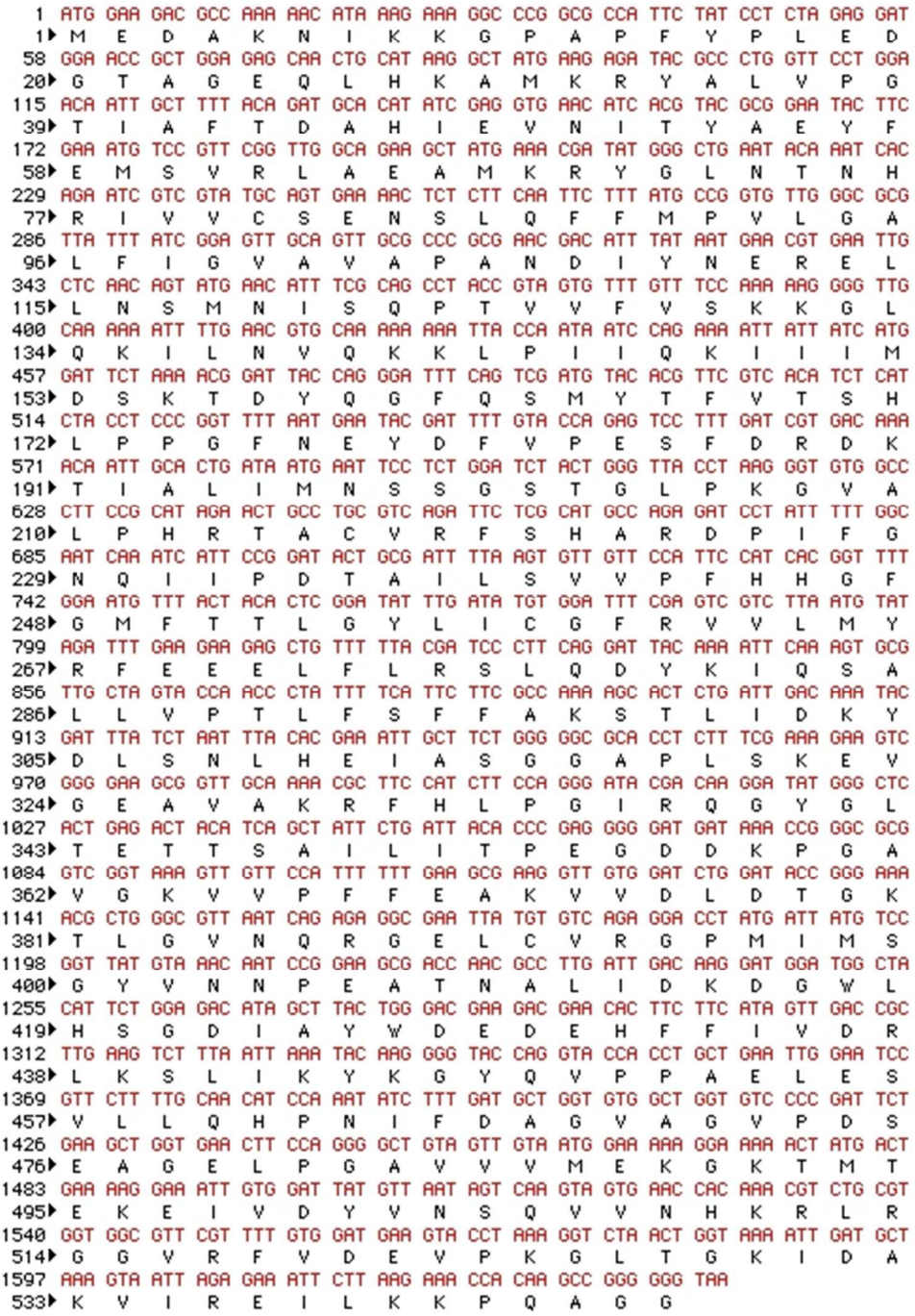

Figure S2. The cDNA and deduced amino acid sequence of PpyLit.

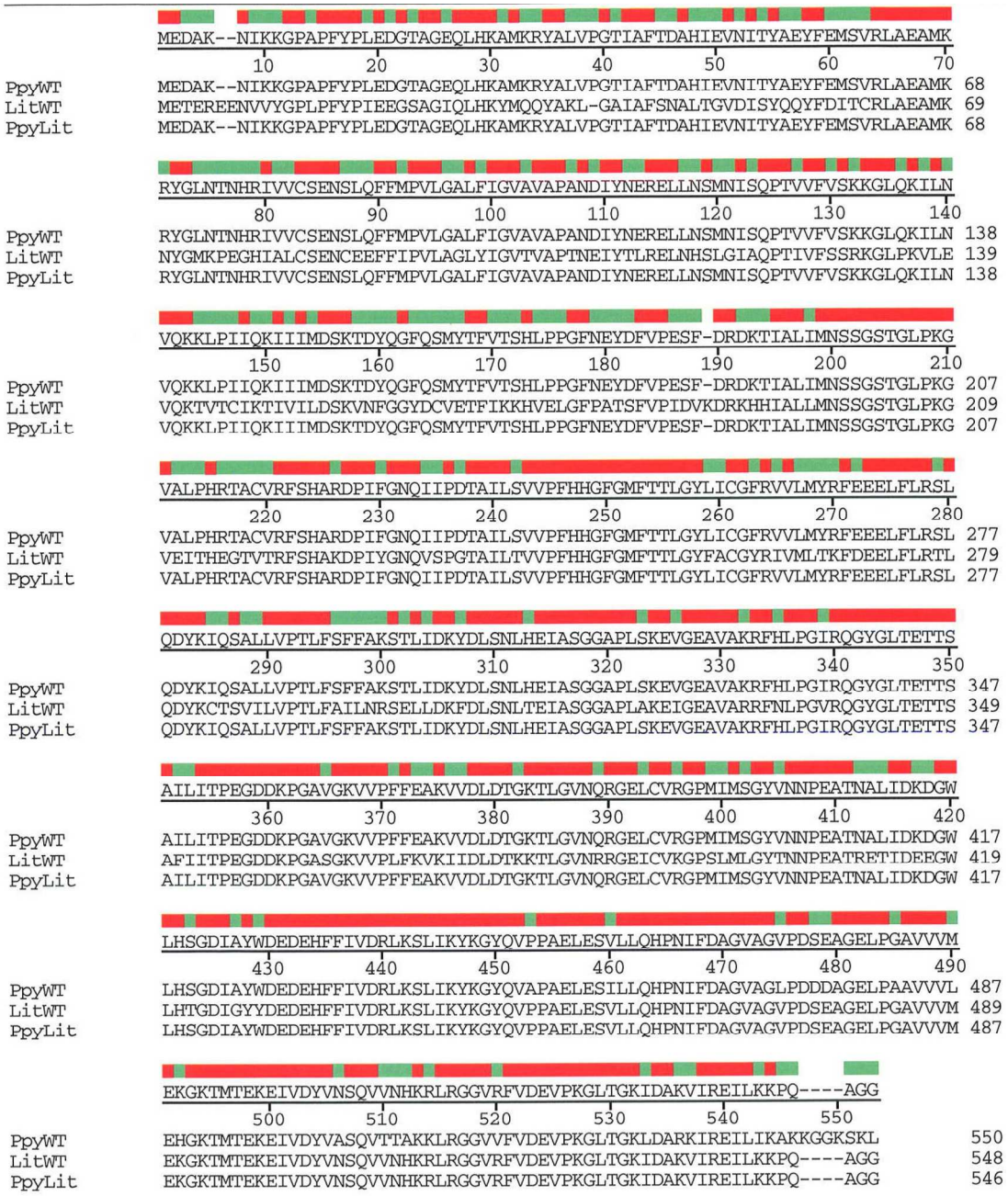

Figure S3. Amino acid sequence alignment of PpyWT, LitWT and PpyLit.
Sequences were aligned using the MegAlign program within the Lasergene suite for sequence analysis (DNA Star, Inc. Madison, WI). The amino acid differences are shown in green and gaps are indicated by a dash.

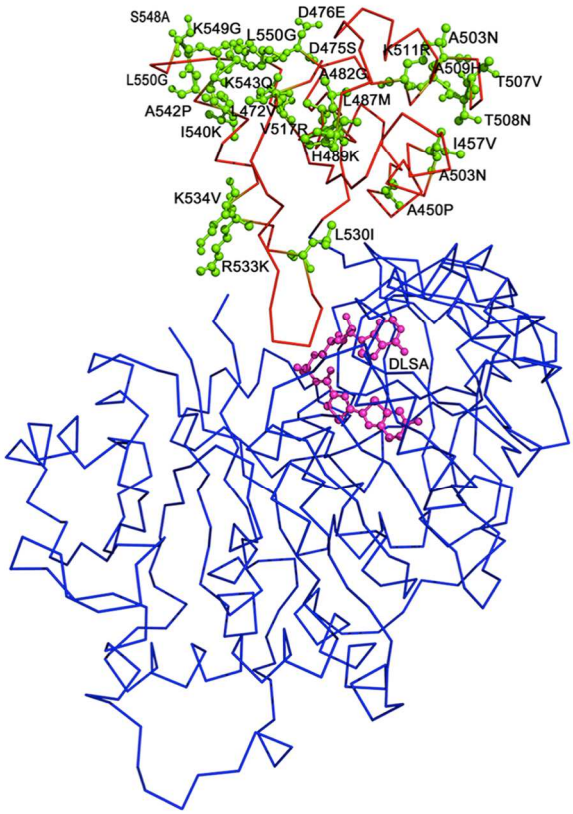

Figure S4. Backbone trace diagram of PpyWT complexed with the stable adenylate analog 5-O-[(N-dehydroluciferyl)-sulfamoyl]- adenosine (DLSA) in the adenylation (4G36) conformation. The N-domain (blue), C-domain (red) and DLSA (purple) are shown along with the 23 PpyLit side chain groups (green) of the residues that are different than those of PpyWT. See Figure S2 an S3 for the full amino acid sequence of PpyLit and the sequence alignment with PpyWT. This figure was made using the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

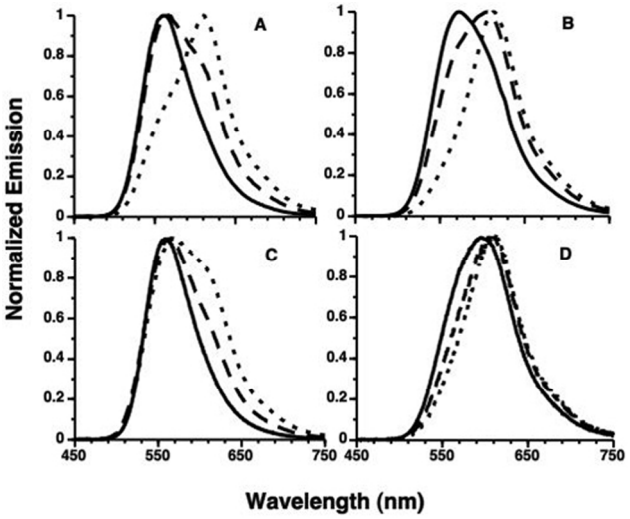

Figure S5. Bioluminescence emission spectra as a function of pH. The normalized emission spectra for (A) PpyWT, (B) LitWT, (C) PpyLit and (D) LitPpy are shown at pH 7.8 (—), pH 7.0 (– –) and pH 6.5 (- - -). Spectra were obtained as described above in the Materials and Methods.

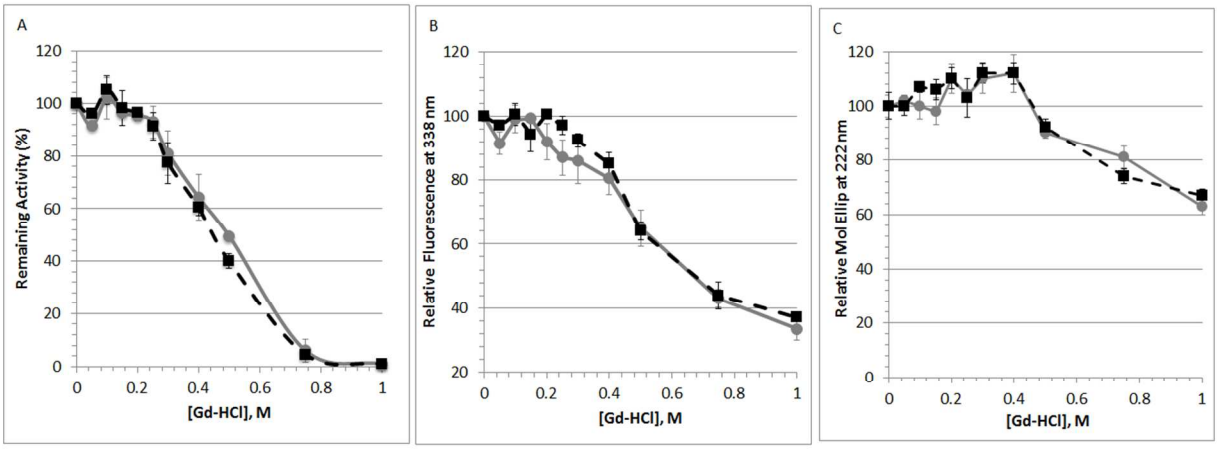

Figure S6. Stability of PpyWT and PpyLit to chemical denaturation with guanidine HCl (Gd-HCl). The denaturation induced by Gd-HCl was monitored by (**A**) bioluminescence activity, (**B**) fluorescence at 338 nm, and (**C**) molar ellipticity for PpyWT (− −■− −□ and PpyLit (—●—).

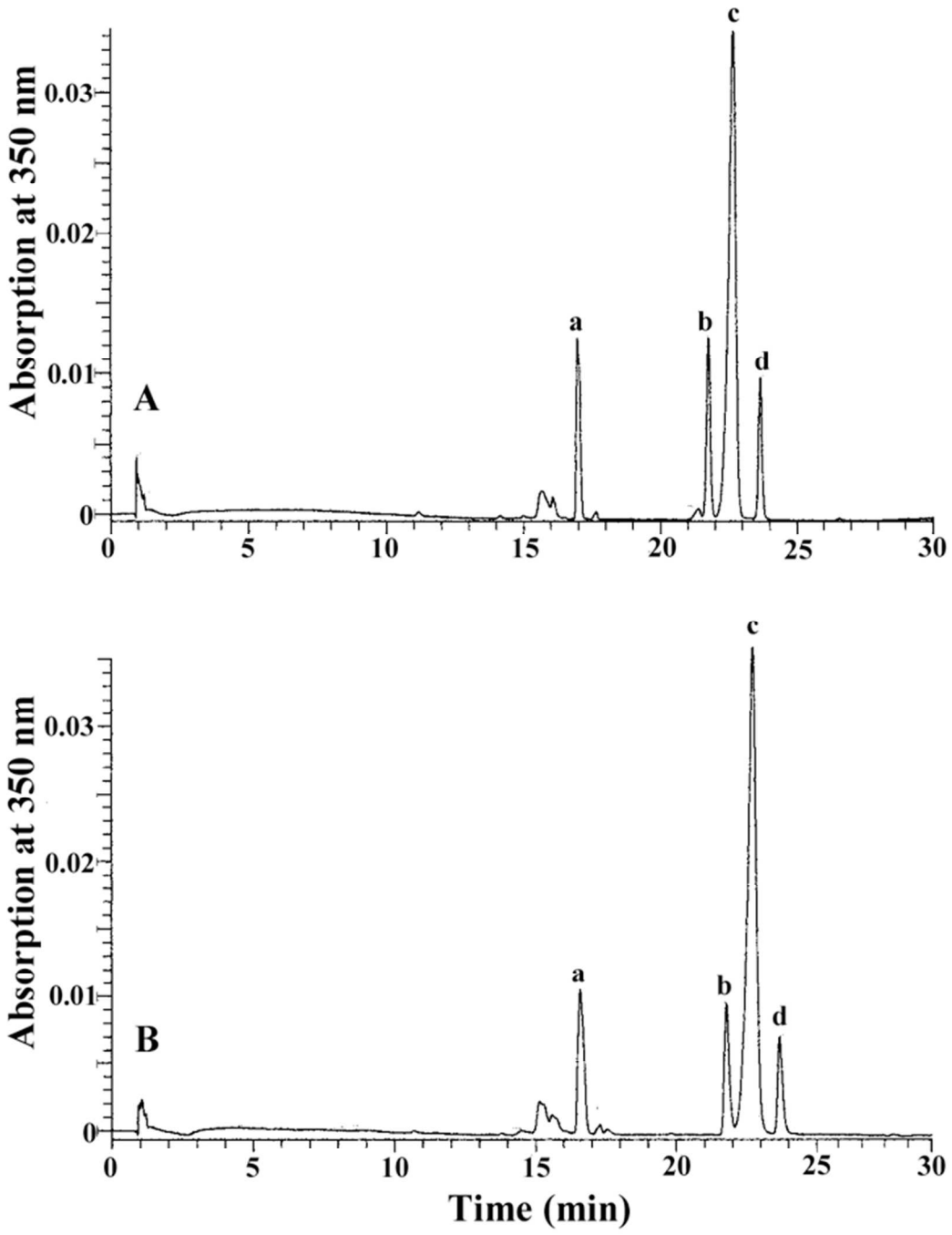

Figure S7. HPLC Chromatograms comparing product distributions for reactions of LH_2-AMP with PpyWT (A) and PpyLit (B). Retention times and peak areas are the same (within error) for L-AMP (a), LH_2 (b), oxyluciferin (c), and L (d).

VI. ABBREVIATIONS AND REFERENCES

Abbreviations used: CB, 20 mM Tris-HCl (pH 7.4 at 4 °C) containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT; CBA, CB containing 0.8 M ammonium sulfate and 2% glycerol; CCD, charge-coupled device; CD, circular dichroism; GdCl, guanidine hydrochloride; L, dehydroluciferin; L-AMP, dehydroluciferyl-AMP; LH_2, D-firefly luciferin; LitWT, recombinant *Luciola italica* luciferase (UniProtKB: Q1AG35) containing the changes Lys547Gly/Met548Gly and the additional N-terminal peptide GPLGS-; LH_2-AMP, luciferyl-AMP; LitPpy, chimeric protein comprised of LitWT residues 1-441 and PpyWT residues 440-550 containing the additional N-terminal peptide GPLGS-; Luc, firefly luciferase; NaPB, 0.1 M sodium phosphate buffer pH 7.8 containing 1 mM EDTA; $OxyLH_2$, oxyluciferin; PpyWT, recombinant *Photinus pyralis* luciferase (UniProtKB: P08659) containing the additional N-terminal peptide GPLGS-; and PpyLit, chimeric protein comprised of PpyWT residues 1-439 and LitWT residues 442-548 containing the additional N-terminal peptide GPLGS-.

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