BIOCHEMISTRY
including biophysical chemistry & molecular biology

Accelerated Publication

pubs.acs.org/biochemistry

A Route from Darkness to Light: Emergence and Evolution of Luciferase Activity in AMP-CoA-Ligases Inferred from a Mealworm Luciferase-like Enzyme

V. R. Viviani,*[,†,‡] R. A. Prado,[†,‡] D. R. Neves,[†] D. Kato,[§] and J. A. Barbosa[∥]

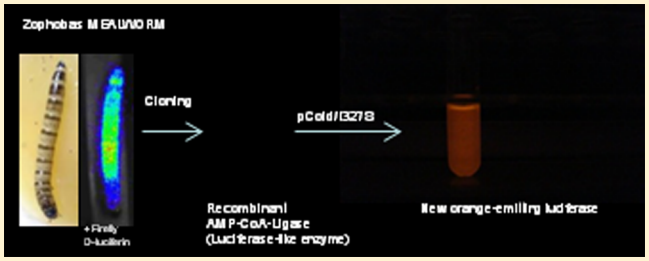
[†]Graduate Program of Biotechnology and Environmental Monitoring, Federal University of São Carlos (UFSCar), Rodovia João Leme dos Santos, km 110, Itinga, Sorocaba, SP, Brazil

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

[§]Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2280, Japan

[∥]University of Brasília, Campus Universitario Darcy Ribeiro, BLE, first FL, Asa Norte, Brasilia-DF 70910-900, Brazil

ABSTRACT: The origin of luciferases and of bioluminescence is enigmatic. In beetles, luciferases seem to have evolved from AMP-CoA-ligases. How the new oxygenase luminogenic function originated from AMP-ligases leading to luciferases is one of the most challenging mysteries of bioluminescence. Comparison of the cloned luciferase-like enzyme from the nonluminescent *Zophobas morio* mealworm and beetle luciferases showed that the oxygenase activity may have emerged as a stereoselective oxidative drift with D-luciferin, a substrate that cannot be easily thioesterified to CoA as in the case of the L-isomer. While the overall k_{cat} displayed by beetle luciferases is orders of magnitude greater than that of the luciferase-like enzyme, the respective oxidation rates and quantum yields of bioluminescence are roughly similar, suggesting that the rate constant of the AMP-ligase activity exerted on the new D-luciferin substrate in beetle protoluciferases was the main enzymatic property that suffered optimization during the evolution of luciferases. The luciferase-like enzyme and luciferases boost the rate of luciferyl-adenylate chemiluminescent oxidation by factors of 10^6 and 10^7, respectively, as compared to the substrate spontaneous oxidation in buffer. A similar enhancement of luciferyl-adenylate chemiluminescence is provided by nucleophilic aprotic solvents, implying that the peptide bonds in the luciferin binding site of beetle luciferase could provide a similar catalytically favorable environment. These data suggest that the luciferase-like enzyme and other similar AMP-ligases are potential alternative oxygenases. Site-directed mutagenesis studies of the luciferase-like enzyme and the red light-producing luciferase of *Phrixotrix hirtus* railroadworm confirm here a critical role for T/S345 in luciferase function. Mutations such as I327T/S in the luciferase-like enzyme, which simultaneously increases luciferase activity and promotes blue shifts in the emission spectrum, could have been critical for evolving functional bioluminescence from red-emitting protoluciferases. Through the combination of I327T/S mutations and N-terminal fusion, the luminescence activity of this enzyme was increased to visible levels, with the development of a totally new orange-emitting luciferase. These results open the possibility of engineering luciferase activity in a set of AMP-CoA-ligases.



Understanding how new catalytic functions arise and evolve in enzymes is a fundamental question in modern biology, providing insights into the origin and evolution of enzymes and of life itself and powerful information for engineering new enzymes for biotechnological and biomedical purposes. Bioluminescence, the production of cold, visible light by living organisms catalyzed by luciferases, offers elegant and powerful models for addressing these questions, because it can be easily detected and quantified by modern sensitive photometers.[1] Despite being functionally classified as oxygenases, most luciferases apparently did not evolve from classical oxygenases, but from a wide repertory of proteins with different functions.[2−4]Beetle luciferases are especially interesting because they are bifunctional enzymes that evolved from AMP-CoA-ligases,[4,5] which make up an ancient and widespread class of enzymes that catalyze the activation of carboxylic acids in a two-step reaction: (I) activation of carboxylic substrates through ATP-dependent adenylation usually followed by (II) product thioesterification by CoA accompanied by AMP release. Like in any other AMP-ligases, the committed step of beetle luciferases is the adenylation of D-luciferin (step 1), a benzothiazolic acid, yielding luciferyl-adenylate. In luciferases, however, the thioesterification reaction (step 2) is replaced by a new oxygenase activity on luciferyl-adenylate (step 2b) that

Received: March 21, 2013
Revised: May 17, 2013
Published: May 24, 2013

   dx.doi.org/10.1021/bi400141u | *Biochemistry* 2013, 52, 3963−3973

ultimately leads to light emission.[4] Nevertheless, how this new oxygenase activity emerged in the ancestral AMP-CoA-ligases and evolved into the efficient luminescence activity of beetle luciferases still defies our understanding of bioluminescence at the molecular level.

Luciferase-like enzymes originally discovered in nonluminescent *Tenebrio molitor* (Tenebrionidae) mealworm larvae[6] provide compelling models for studying the origin of beetle luciferases by virtue of their weak luminescence emission in the presence of D-luciferin and ATP, perhaps acting as ancient beetle protoluciferases, therefore constituting reasonable models for the missing link between "dark" AMP-ligases and bright luciferases. Adequate, fluorescent "luciferin" substrates must have been available as well. Although several luciferase homologues were cloned from *Tenebrio* mealworms[7] in the past, none produced light, and only recently was the first truly luminescent luciferase-like enzyme cloned from the Malpighian tubules of the giant mealworm *Zophobas morio* (Tenebrionidae).[8] This enzyme is a shorter AMP-CoA-ligase (528 residues) with a low level of sequence identity to beetle luciferases (~30%), suggesting that the potential for protobioluminescence in AMP-ligases, provided D-luciferin is offered as a substrate, must have emerged long before the divergence of the main families of bioluminescent beetles. The partially purified enzyme showed poor firefly luciferase properties, with low affinity for luciferin and a small k_{cat} value, but did exhibit stereoselectivity for D-luciferin.[9] A first site-directed mutagenesis survey in the luciferin binding site of this luciferase-like enzyme showed that the replacement of residues by those found in beetle luciferases had a negative effect on luminescence activity, with the exception of I327T, which improved the luminescence activity.[10]

To clarify the origin and evolution of oxygenase/luciferase activity in AMP-CoA-ligases leading to bright luciferases, we overexpressed and purified the luciferase-like enzyme and compared its catalytic constant, oxidative rate, catalytic efficiency, and quantum yield with those of recombinant luciferases from the three main families of bioluminescent beetles (Lampyridae, *Photinus pyralis* firefly; Phengodidae, *Phrixotrix hirtus* railroadworm; Elateridae, *Pyrearinus termitilluminans* larval click beetle). Through site-directed mutagenesis using this enzyme and *P. hirtus* red-emitting luciferase, we demonstrate that the T/S345 residues are critical for luciferase activity. It is noteworthy that, by a combination of these approaches, we succeeded in developing a new orange-emitting luciferase from a luciferase-like enzyme.

MATERIALS AND METHODS

Reagents. Coenzyme A, firefly D-luciferin acid, *N*,*N*′-dicyclohexylcarbodiimide, DMSO, EDTA, and Triton X-100 (Sigma, St. Louis, MO); D-luciferin potassium salt (Promega); dithiothreitol, IPTG, and ATP (Ambresco); and L-luciferin (Resem BV, Lijnden, The Netherlands) were purchased. All the reagents were of the purest available grade.

Alignments. The multisequence structural alignment was done as described,[8,11] using Expresso[12] and the luciferase sequences of the following lampyrids: *Photuris pennsilvanica*, *Ph. pyralis*, *Luciola cruciata*, *Luciola mingrelica*, and *Luciola lateralis* in addition to the luciferase-like enzyme sequence. Expresso was used with the available structural information to improve the alignment.

Homology Modeling. Modeling of the luciferase-like enzyme structure was conducted as described previously[10] using Modeler 9v6[11,12] and the *L. cruciata* luciferase structure determined in complex with oxyluciferin and AMP (Protein Data Bank entry 2D1R) as a template.[13]

Luciferase cDNAs and Subcloning. *Z. morio* AMP-ligase (luciferase-like enzyme) cDNA was subcloned from EcoRI sites of the pTrc-HisC vector (Invitrogen) into the pColdII vector (Takara). *P. hirtus* red-emitting and *Py. termitilluminans* larval click beetle luciferases were previously subcloned into the pCold vector.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using a Stratagene mutagenesis kit (catalog no. 200518). The plasmids containing the luciferase cDNAs were amplified using *Pfu* turbo polymerase and two complementary primers containing the desired mutation, using a thermal cycler (1 cycle at 95 °C and 12 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 12 min). After amplification, mutated plasmids containing staggered nicks were generated. The products were treated with *Dpn*I to digest nonmutated parental plasmids and used directly to transform *Escherichia coli* XL1-Blue cells. The following primers and their respective reverse complements were used (mutation underlined): *Z. morio* protoluciferase (I327T), GGA ATG TCC GAA ACT GGA CTA CTG TC; *Z. morio* protoluciferase (I327S), GGA ATG TCC GAA AGT GGA ACT ACT GTC; *P. hirtus* luciferase (T345I), C CTG ACC GAG ATC TGT AGC CCC.

Sequencing. The mutants were sequenced by a dideoxy chain termination method using a dye-labeled terminator kit specifically developed for the ABI PRISM 377 automatic sequencer (Perkin-Elmer, Foster City, CA). The following primers were used to sequence the mutations: Zop1, CGA TGA TAC CGC AGT CAT GTT C; Z2, GGA CTA CTG TCT ATT TTT CACC; Z3, CAG ACA TCG ATG ATA CCG CAG.

Expression and Purification of Luciferase-like Enzyme and Beetle Luciferases. For luciferase expression, transformed *E. coli* BL21-DE3 cells were grown in 500−1000 mL of LB medium at 37 °C to an OD_{600} of 0.4 and then induced at 18 °C with 0.4 mM IPTG overnight. Cells were harvested by centrifugation at 2500*g* for 15 min and resuspended in extraction buffer consisting of 0.10 M sodium phosphate buffer (pH 7.0) or TRis-HCl buffer (pH 7.5), 1 mM EDTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Roche), lysed by ultrasonication or a French press, and centrifuged at 15000*g* for 15 min at 4 °C. The N-terminal histidine-tagged *Py. termitilluminans* and *P. hirtus* recombinant luciferases and *Z. morio* luciferase-like enzyme were further purified by agarose-nickel affinity chromatography followed by dialysis. Samples were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting using primary polyclonal antibodies raised against *Py. termitilluminans* and *P. hirtus* railroadworm luciferases, and an anti-rabbit secondary antibody employing the ECL Western blotting detection kit (GE Healthcare). According to SDS−PAGE analysis, the enzymes had an estimated purity of ~95%.

Luminescence Activities. Luciferase-like enzyme activities were measured as bioluminescence intensities in counts per second (cps) using a luminometer (AB-2200, ATTO, Tokyo, Japan). Luminescence kinetics and absolute intensities were measured in a TD3000III luminometer (Japan) as described previously.[10] The luminescence assays were conducted by mixing 5−10 μL of purified luciferase-like enzyme (final concentrations of 50−100 μg/mL) with 75−80 μL of assay buffer consisting of 0.10 M Tris-HCl (pH 8.0), 1 mM D-

Table 1. Luminescence Properties of *Z. morio* Luciferase-like Enzyme, *P. hirtus* Railroadworm Red-Emitting Luciferase, and Their Mutants[a]

		K_M (μM)				k_{cat}/K_M				
	pH	luciferin	ATP	k_{cat} ($\times 10^{-9}$ s^{-1})	k_{ox} ($\times 10^{-9}$ s^{-1})	luciferin	ATP	k_D (s^{-1})	Φ_{BL}	λ_{max} (nm) (half-band)
Tenebrionidae (mealworms)										
Zophobas luciferase-like enzyme	8.5	800	260	11	8.4	0.014	0.042	0.005	0.13	616 (92)
I327T	8.0	500	2460	16	24	0.032	0.007	0.005		606 (107)
pCold	8.0	500	260	330	290	0.76	1.3	0.004		610 (72)
I327S		200	120	340	620	1.70	2.833	0.008		592 (80)
Phengodidae (railroadworms)										
P. hirtus (red luciferase)	8.2	7	230	880	785	130	3.8	0.02	0.14	623 (55)
T345I		80	1330	83	130	1	0.06			629 (48)
Phrixotrix vivianii (green luciferase)	8.0	64	330	140		2	0.4	0.0004		546 (70)
Ragophtalmus ohbai luciferase		1300								554 (76)
Elateridae (click beetles)										
Py. termitilluminans (green)	8.0	80	370	1900	470	24	5.1	0.006	0.61	534 (73)
Lampyridae (fireflies)										
Ph. pyralis luciferase	7.8	5	250	6200	5200	1240	25	0.024	0.41	562 (78)

[a]The overall catalytic constant was calculated from the total light intensity in counts per second of the bioluminescence reaction starting from ATP and D-luciferin, whereas the catalytic constant of oxidation was calculated from the luminescence intensity using luciferyl-adenylate as a substrate.

luciferin, 2 mM ATP, and 4 mM $MgSO_4$ in a luminometer tube. The data were obtained from four independent experiments, each measured in triplicate. Assays for luciferyl-adenylate chemiluminescence were conducted by mixing 80 μL of 0.10 M Tris-HCl (pH 8.5), 5−10 μL of luciferase-like enzyme or luciferases, and 5−10 μL of luciferyl-adenylate.

Kinetic Characterization. The K_M assays for luciferin were performed by mixing 5 μL of 40 mM ATP, 80 mM $MgSO_4$ in a solution containing 5−10 μL of luciferase or luciferase-like enzyme (final concentration of 50−100 μg/mL), 75 μL of 0.10 M Tris-HCl (pH 8.0), and luciferin at final concentrations between 0.010 and 1 mM. The K_M assays for ATP were performed by mixing 5 μL of 80 mM $MgSO_4$ in a solution containing 10 μL of luciferase, 75 μL of 0.10 M Tris-HCl (pH 8.0), and ATP at final concentrations in the range of 0.020−2 mM. Both assays were performed in triplicate. The K_M values were calculated using Lineweaver−Burk plots taking the peak intensity (I_0) as a measure of V_0. The catalytic constant (k_{cat}) was calculated using the ratio between the luminescence activity (cps) and the number of luciferase molecules calculated from the protein concentration of purified luciferases, assuming that the purity of luciferases was ∼95%. Commercial *Ph. pyralis* luciferase (PROMEGA) at 14.7 ng/μL was used as the mass standard.

Quantum Yield Measurements. The time-integrated number of photons emitted by luciferases was measured in an ATTO AB-2200 luminometer at room temperature (22 °C). Ten microliters of D-luciferin (10^{-8} to 10^{-9} M in water) and 10 μL of concentrated luciferases (3−5 mg/mL) were mixed in a test tube, which was placed in the luminometer. After the luminometer started collecting data, the bioluminescence reaction was initiated by adding 80 μL of the initiation solution [3 mM ATP and 8 mM $MgSO_4$ in 0.10 M Tris-HCl buffer (pH 8.0)]. The calibration curve was prepared using four luciferases whose bioluminescence quantum yields had been previously determined (PxRE, Pte, Pym-WT, and Pym-N230S) according to Niwa et al.,[14] from linear fitting of the plots of the total number of photons and reported values of quantum yields. The quantum yield of the luciferase-like enzyme was determined by measuring the total number of photons of the bioluminescence reaction under the same conditions of the standard luciferases. Each experiment was repeated three to nine times.

Chemiluminescence and Bioluminescence Spectra. Chemiluminescence and bioluminescence spectra were recorded in a Hitachi F4500 spectrofluorometer. Scans were run between 450 and 700 nm at a speed of 2400 nm/min after 30 s ATP had been added to the assay solution, when there is a negligible decay of emission. The spectra were automatically corrected for the spectral sensitivity of the equipment. For the *in vitro* bioluminescence, 50 μL of crude extracts was mixed with 450 μL of assay solution [0.5 mM luciferin, 2 mM ATP, and 4 mM $MgSO_4$ in 0.10 M Tris-HCl (pH 8.0)]. The effect of pH on bioluminescence spectra was analyzed in 0.10 M phosphate buffer (pH 8.0 and 6.0) and 0.10 M Tris-HCl (pH 8.0). For comparison, some spectra were also measured using a recently acquired AB-18505 luminescence spectrometer (ATTO).

Western Blots. With the aim of analyzing the quantity and purity of the recombinant luciferase-like enzyme and luciferases, SDS−PAGE followed by Western blots was conducted by using polyclonal antibodies raised against firefly or *P. hirtus* railroadworm luciferases using the ECL kit (Amersham, Buckinghamshire, U.K.). As a mass standard, commercial *Ph. pyralis* luciferase was used.

Luciferyl-adenylate Synthesis. The luciferyl-adenylates were prepared from D-luciferin acids and AMP as previously described.[15] Luciferyl-adenylate was analyzed by TLC on silica plates using an ethyl acetate/ethanol/water moving phase (5:3:2), followed by revelation by fluorescence with a UV lamp. Luciferyl-adenylate displayed yellowish fluorescence with an R_f of 0.68 [$R_{f(luciferin)}$ = 0.87 with greenish fluorescence]. The luciferyl-adenylate concentration could not be precisely quantified because of the considerable absorption and fluorescence spectral overlapping with D-luciferin but could be estimated on the basis of stoichiometric amounts of luciferin and ATP used for its synthesis. According to such estimations, the luciferyl-adenylate concentration in a stock solution could be between 5 and 10 mM. All the preparations of luciferyl-

adenylate displayed a spontaneous chemiluminescence activity of ~250000 ± 25000 cps/mL when assayed in 0.10 M Tris-HCl (pH 8.5), showing that the concentration of different preparations was roughly constant.

Luciferyl-adenylate Assays. Luciferyl-adenylate chemiluminescence was assayed by mixing 5−10 μL luciferyl-adenylate with 90−95 μL of 0.10 M Tris-HCl buffer (pH 8.5). Luciferyl-adenylate bioluminescence was assayed by mixing 5−10 μL of luciferyl-adenylate with 80−85 μL of 0.10 M Tris-HCl buffer (pH 8.0) and 5−10 μL of luciferase-like enzyme or beetle luciferases. The assays were conducted in triplicate.

Tryptophan and 2,6-TNS Fluorescence Spectra. Fluorescence spectra were recorded in a Hitachi F4500 spectrofluorometer. Scans were run at 2400 nm/min. The spectra were automatically corrected for the spectral sensitivity of the equipment. For 2,6-TNS fluorescence, purified luciferases at a concentration between 50 and 100 μg/mL were mixed with 1 μM 2,6-TNS in filtered 0.10 M phosphate buffer (pH 8.0). The fluorescence spectra for 2,6-TNS were obtained with excitation at 320 nm and scanning at 350−600 nm (excitation slit width of 2.5, emission slit width of 10.0). The fluorescence spectra of buffers and solvents were subtracted as blanks. Tryptophan fluorescence was measured for 50−100 μg/mL purified luciferases in 0.10 M prefiltered phosphate buffer (pH 8.0). Fluorescence was excited at 280 nm and scanned from 300 to 600 nm.

RESULTS

Overexpression of *Z. morio* Protoluciferase. To amplify luciferase-like enzyme expression in bacteria, its cDNA was subcloned into the EcoRI site of the pColdII vector. The new construct has an additional N-terminal tail with a polyhistidine tail, to facilitate protein purification by nickel agarose affinity. The level of expression of the active enzyme increased about 2 orders of magnitude when compared with that of the former expression system used in the pTHis vector,[9] allowing its purification and the recovery of up to 10 mg of luciferase-like enzyme per liter of cell culture. This is partly explained by the increase in the level of soluble protein expression with the pCold vector at lower temperatures, as seen in SDS−PAGE and Western blots (results not shown).

Kinetic Properties. The properties of the purified enzyme were in general comparable to those previously reported for the partially purified preparations, with similar K_M values for luciferin and ATP (Table 1). However, a much higher k_{cat} and a lower optimal pH (pH 8.0) compared to those estimated for the previous preparations using the pT-His vector-expressed enzyme [pH 8.5 (Table 1)] were found. Although the k_{cat} value of the former preparation could have been underestimated because a partially purified enzyme was used, the overall changes in the kinetics properties suggest that the new construct has indeed enriched its catalytic properties as a luciferase. When compared with beetle luciferases, the purified luciferase-like enzyme expectedly revealed a higher K_M value for D-luciferin (Figure 1 and Table 1) and a K_M for ATP well within the range expected for luciferases and other AMP/CoA-ligases.

Oxygenase Activity. In beetle luciferases, the oxygenase activity is represented by the chemiluminescent oxidation of luciferyl-adenylate.[16] Whereas the measured k_{cat} calculated from luminescence activity starting with D-luciferin and ATP reflects the overall AMP-ligase (k_{lig}) and oxygenase (k_{ox}) activities (Scheme 1), the luminescence activity measured starting with presynthesized luciferyl-adenylate reflects only the oxidative reaction (k_{ox}), once the adenylation step is bypassed. As previously reported, luciferyl-adenylate is spontaneously chemiluminescent in alkaline aqueous buffers.[15,17] Therefore, comparing the spontaneous luciferyl-adenylate chemiluminescence in buffer and in different solvents with the bioluminescence of luciferyl-adenylate with luciferases allows quantitative evaluation of the catalytic effect of the enzymes on the oxidative rate.

The luciferase-like enzyme was found to accelerate the chemiluminescent oxidation rate of luciferyl-adenylate by 6 orders of magnitude above the spontaneous rate in buffer, whereas typical beetle luciferases accelerate the luminogenic reaction more than 7 orders of magnitude (Figure 2). As expected, beetle luciferases displayed higher chemiluminescence and higher k_{ox} values (Figures 1 and 2 and Table 1). Given that the k_{ox} differed much less from the luciferase-like

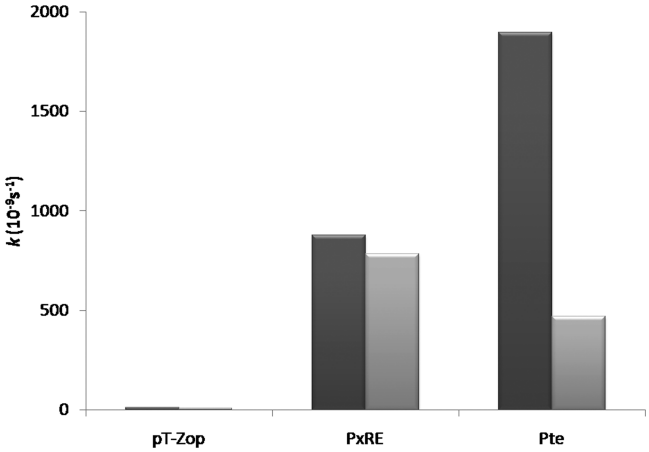


Figure 1. Comparison of the overall relative catalytic constant k_{cat} (dark gray) and oxidation constant k_{ox} (light gray) of luciferase-like enzyme and beetle luciferases: wild-type *Zophobas* luciferase-like enzyme (pT-Zop), *P. hirtus* railroadworm red-emitting luciferase (PxRE), *Py. termitilluminans* larval click beetle (Pte), and *Ph. pyralis* firefly (Ppy). The overall catalytic constant was calculated from the total light intensity in counts per second of the bioluminescence reaction starting from ATP and D-luciferin. The catalytic constant of oxidation was calculated from the luminescence intensity using luciferyl-adenylate as a substrate. The catalytic constants shall be considered only as relative values, once the absolute values of light intensities in counts per second were not estimated in this case.

Scheme 1. Reactions Catalyzed by AMP-Ligases and Beetle Luciferases

1. R-COOH + ATP $\xrightarrow{k_{lig}}$ R-COAMP + PPi + H_2O

2. R-COAMP + HS-CoA $\xrightarrow{k_{thio}}$ R-C-SCoA + AMP

2b. R-COAMP + O_2 $\xrightarrow{k_{ox}}$ R=O + CO_2 + AMP + hν

$$K_{cat\ (AMP\text{-}ligase)} = k_{lig} + k_{thio}$$
$$K_{cat\ (Luciferase)} = k_{lig} + k_{ox}$$

PPi= Pyrophosphate
k_{lig}= rate constant of ligation activity
k_{thio}= rate constant of thioesterification activity
k_{ox}= rate constant of oxidation activity

dx.doi.org/10.1021/bi400141u | *Biochemistry* 2013, 52, 3963−3973

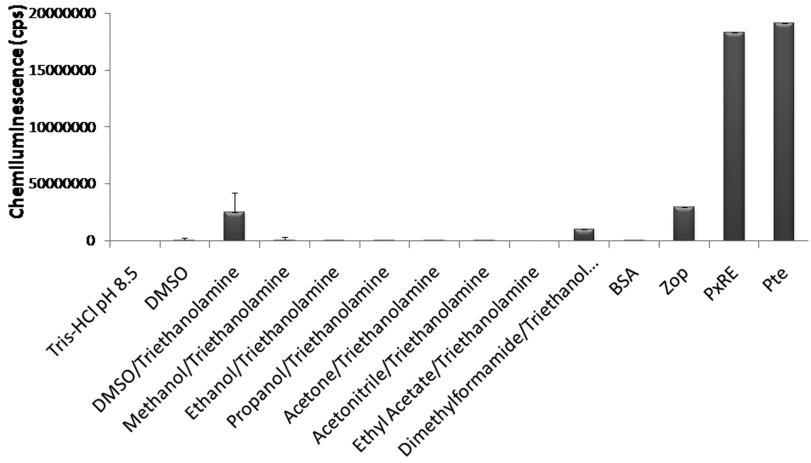


Figure 2. Comparison of chemiluminescence activity of luciferyl-adenylate in 0.10 M Tris-HCl (pH 8.5) in different solvents, with bovine serum albumin (BSA), luciferase-like enzyme, and different beetle luciferases at 1 mg/mL each.

enzyme to typical beetle luciferases than the overall k_{cat}, the catalytic evolutionary gain of beetle luciferases may be attributed mainly to the adenylating activity (k_{lig}). Furthermore, considering that the k_{ox}/k_{cat} ratio is significantly higher for the luciferase-like enzyme, one can conclude that although it is a poor AMP-ligase for D-luciferin, the luciferase-like enzyme is an unexpectedly potent oxygenase for D-luciferin. This finding raises the possibility that the luciferase-like enzyme, and perhaps similar AMP-ligases, may constitute an alternative oxygenase family acting upon substrates incapable of being easily thioesterified by CoASH.

To improve our understanding of the nature of the chemical environment necessary to increase the oxidation rate, we compared the luciferyl-adenylate chemiluminescence in different solvents (Figure 2). In alcohols, almost no luminescence could be detected. Basal luciferyl-adenylate chemiluminescence could be measured only in alkaline Tris-HCl buffer at pH >8.0, and in aprotic polar solvents, especially DMSO, dimethylformamide, and acetone, where the activities were the highest (Figure 2). As previously reported,[15] addition of the weak base triethanolamine to these solvents increases the luminescence intensity by orders of magnitude above that observed in the absence of base. These results strongly suggest that aprotic and nucleophilic environments increase the rate of luciferyl-adenylate spontaneous oxidation and chemiluminescence. In the luciferase-like enzyme and luciferase active sites, environments similar to that of DMSO, dimethylformamide and acetone could be provided by peptide amide bonds.

Quantum Yield. The quantum yields in bioluminescent systems, defined as the number of photons emitted by the number of luciferin molecules oxidized, are the highest among chemiluminescent reactions and have been credited for most of the brightness of luciferases.[1] Firefly luciferase has a very high quantum yield: originally reported as 88%[18] but more recently estimated as 41%.[19] The quantum yield of other beetle luciferases cloned by our group ranges from 0.14 for *P. hirtus* red-emitting luciferase to 0.61 for *Py. termitilluminans* larval click beetle green-emitting luciferase,[14] suggesting that green-emitting luciferases are in general more efficient than red-emitting ones and there is a liner relationship between the emission wavelength and its quantum yield.

The quantum yield for the luciferase-like enzyme studied here reached 0.13, which is surprisingly close to that of *Phrixotrix* red-emitting luciferase and some firefly luciferase red mutants (Figure 3). Therefore, quantum yields of beetle luciferases vary less than 5-fold from the less efficient luciferase-

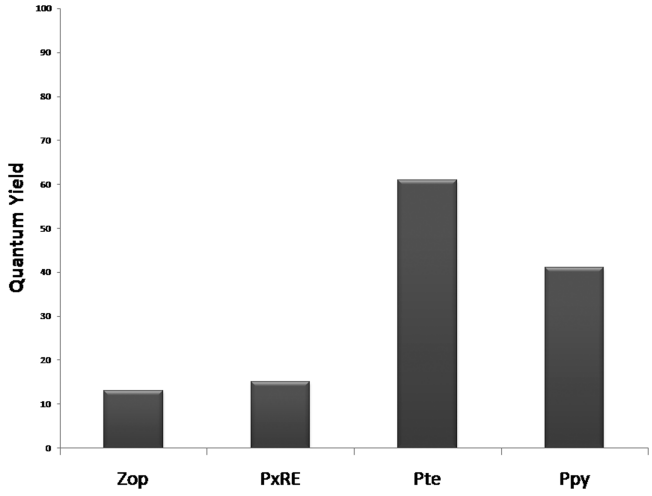


Figure 3. Comparison of the bioluminescence quantum yields, Φ_{BL}, of luciferase-like enzyme and beetle luciferases: *Zophobas* luciferase-like enzyme (Zop), *P. hirtus* railroadworm red-emitting luciferase (PxRE), *Py. termitilluminans* larval click beetle (Pte), and *Ph. pyralis* firefly (Ppy).

like enzyme (0.13) to the most efficient green-emitting *Py. termitilluminans* luciferase (0.61).

Residue T/S345 Plays a Critical Role in Beetle Luciferase Activity. Previously, we showed that, among the mutations replacing the luciferin binding site residues of the luciferase-like enzyme by those found in beetle luciferases, only the I327T substitution (T345 in firefly and railroadworm luciferases) increases the luciferase activity.[10] Similarly, the I327S mutation, which is naturally found in *Py. termitilluminans* click beetle luciferase, also increased luciferase activity (Figure 4) and caused a 20 nm blue shift to the orange region (Figure 5), being the largest blue shift effect ever observed for a single mutation in the luciferase-like enzyme.

Therefore, to check whether T/S345 plays an important function in luminescence activity in beetle luciferases, we made the reverse mutation, T327I, in *P. hirtus* red-emitting luciferase. Indeed, the T345I mutation dramatically decreased the luciferase activity with D-luciferin and ATP by >3 orders of

dx.doi.org/10.1021/bi400141u | *Biochemistry* 2013, 52, 3963−3973

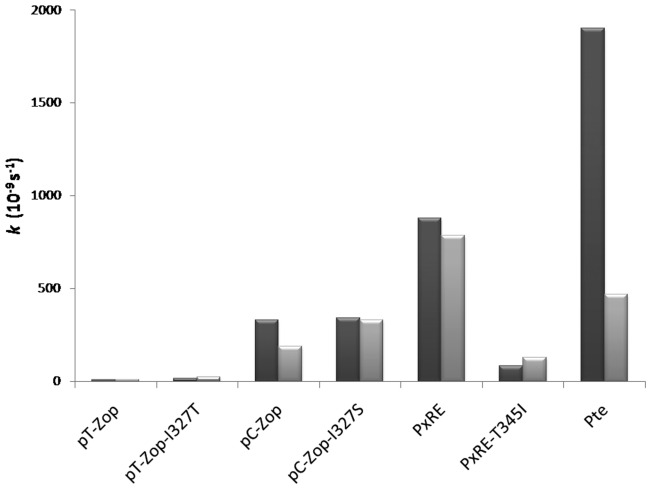


Figure 4. Comparison of k_{cat} (dark gray) and k_{ox} (light gray) of the *Zophobas* luciferase-like enzyme, *P. hirtus* red-emitting luciferase, and their I327T/S (T345I) mutants: pT-Zop, wild-type luciferase-like enzyme expressed in the pTHis vector; pC-Zop, luciferase-like enzyme expressed in the pCold vector; PxRE, beetle luciferases; Pte, *Py. termitilluminans* luciferase.

magnitude (Figure 4). The kinetic parameters of the *P. hirtus* T345I mutant luciferase resemble those of the wild-type *Zophobas* luciferase-like enzyme, with a remarkable decrease in the k_{cat} (Figure 4) and a considerable increase in the K_M for both luciferin and ATP (Table 1), reflecting an overall decrease in luminescence catalytic efficiency. In the luciferase-like enzyme, the I327S mutation increased twice the k_{ox} value, whereas the T345I mutation in *Phrixotrix* luciferase resulted in a much larger decrease in k_{ox} (Figure 4). These results indicate that residues T/S345 are involved mainly with the oxygenase activity, and to a lesser extent with the AMP-ligase activity of *Phrixotrix* luciferase. The luminescence spectrum of the PxRE mutant also underwent a slight red shift in relation to that of the wild-type luciferase (Figure 5).

The N-Terminus Also Influences the Luciferase Activity. As mentioned before, the new fusion protein with the His-tagged N-terminal tail displayed improved luciferase activity compared to the enzyme expressed with the pT-His vector. A larger value of the catalytic constant [k_{cat} (Figure 4 and Table 1)], a lower optimal pH, and a slightly blue-shifted spectrum (Table 1) were observed. Such superior luminescence properties are consistent with an indirect effect of the different fusion N-terminal tails provided by the two vectors on the active site, which increases the catalytic rate.

Luciferin Binding Site Environment. To investigate the luciferin binding site polarity properties of this luciferase-like enzyme and its mutants, we measured the fluorescence spectra of tryptophan, which reflects conformational changes near the active site, and of 2,6-toluidinylnaphthalene-6-sulfonate (2,6-TNS), a fluorescent active site polarity probe that is a competitive inhibitor of firefly luciferase with regard to D-luciferin[20] (Table 2). The fluorescence spectrum of 2,6-TNS peaked at 440 nm, which is quite close to the value measured for *P. hirtus* luciferase, which also elicits red light and the highest degree of structural similarity. These data indicate that the luciferin binding site of the studied red-emitting enzymes should be polar and similar. The effect of polarity index on the fluorescence maxima of 2,6-TNS in different solvents suggests that the active site of the luciferase-like enzyme mimics DMSO. Accordingly, the luciferyl-adenylate chemiluminescence spectrum in DMSO (λ_{max} = 621 nm) is also close to the emission spectrum from the *Zophobas* luciferase-like enzyme (λ_{max} = 615 nm) and *Phrixtrix* railroadworm luciferase (λ_{max} = 623 nm). Altogether, these results support an aprotic polar environment for the luciferase oxygenase activity.

The tryptophan fluorescence data obtained from both the wild-type luciferase-like enzyme and *P. hirtus* red-emitting luciferases and their 327 and 345 mutants, respectively, did not support notable conformational changes linked to the mutations (Table 2). However, although the I327S mutation in the luciferase-like enzyme did not affect the 2,6-TNS fluorescence spectrum, the reverse T345I mutation had a considerable blue shift effect in *Phrixotrix* red-emitting luciferase, indicating an overall mutation-dependent decrease in polarity. Although this result is consistent with the substitution of polar threonine with hydrophobic isoleucine in *P. hirtus* luciferase, it does not explain why the reversal mutation (I327S) in the luciferase-like enzyme has no effect on the 2,6-TNS fluorescence spectrum. Because the close residue S347 interacts with other residues located on the benzothiazolyl side of the luciferin binding site in beetle luciferases, a major change in the conformation of this loop may indirectly affect polarity interactions on the benzothiazolyl side of the luciferin binding site.

DISCUSSION

Origin of Luciferases from AMP-CoA-ligases. AMP-CoA-ligases activate carboxylic acids at the expense of adenylation usually followed by thioesterification.[21] There is a wide range of AMP-CoA-ligases acting upon different classes of organic acids. Coumarate CoA-ligases adenylate cinamic acids;[22] benzoate CoA-ligases adenylate a range of substituted benzoic acids,[23] and fatty acyl-CoA-ligases may activate different fatty acids. Some AMP-CoA-ligases may also have the ability to adenylate xenobiotic carboxylic substrates.[21] Firefly luciferases are not an exception, because they may also adenylate and thioesterify fatty acids by CoA,[24] leading to the suggestion that beetle luciferases may have evolved from fatty acyl-CoA-ligases. However, it has been shown that several of these AMP-ligases can activate a wider range of carboxylic substrates in addition to their specific substrates, for instance, benzoic acids, several aromatic acids, and fatty acids. Such substrate promiscuity strongly suggests that specific AMP-CoA-ligases may have evolved by gene duplication events from more generalistic ancestors acting upon a broader range of organic acid substrates, followed by subfunctionalization or neo-functionalization of the copied genes leading to more specialized enzymes with narrower substrate requirements.

Beetle luciferases may have evolved from such generalistic AMP-ligases acting upon a class of structurally luciferin-related substrates producing thioesters, and then neofunctionalization upon the appearance of D-luciferin, a new oxidizable substrate that could be adenylated but not further thioesterified to CoA because of steric effects. Once formed, luciferyl-adenylate may potentially undergo three types of reactions: (1) spontaneous hydrolysis, (2) nucleophilic displacement of AMP by CoASH with production of a thioester, and (3) deprotonation of C_4 followed by electrophilic attack by molecular oxygen yielding the chemiluminescent dioxetanone intermediate. Under aerobic conditions, the lack of an efficient thioesterification reaction (k_{thio}) with D-luciferin may have sterically favored the otherwise slower competing oxidative side-reaction (k_{ox}), resulting in the

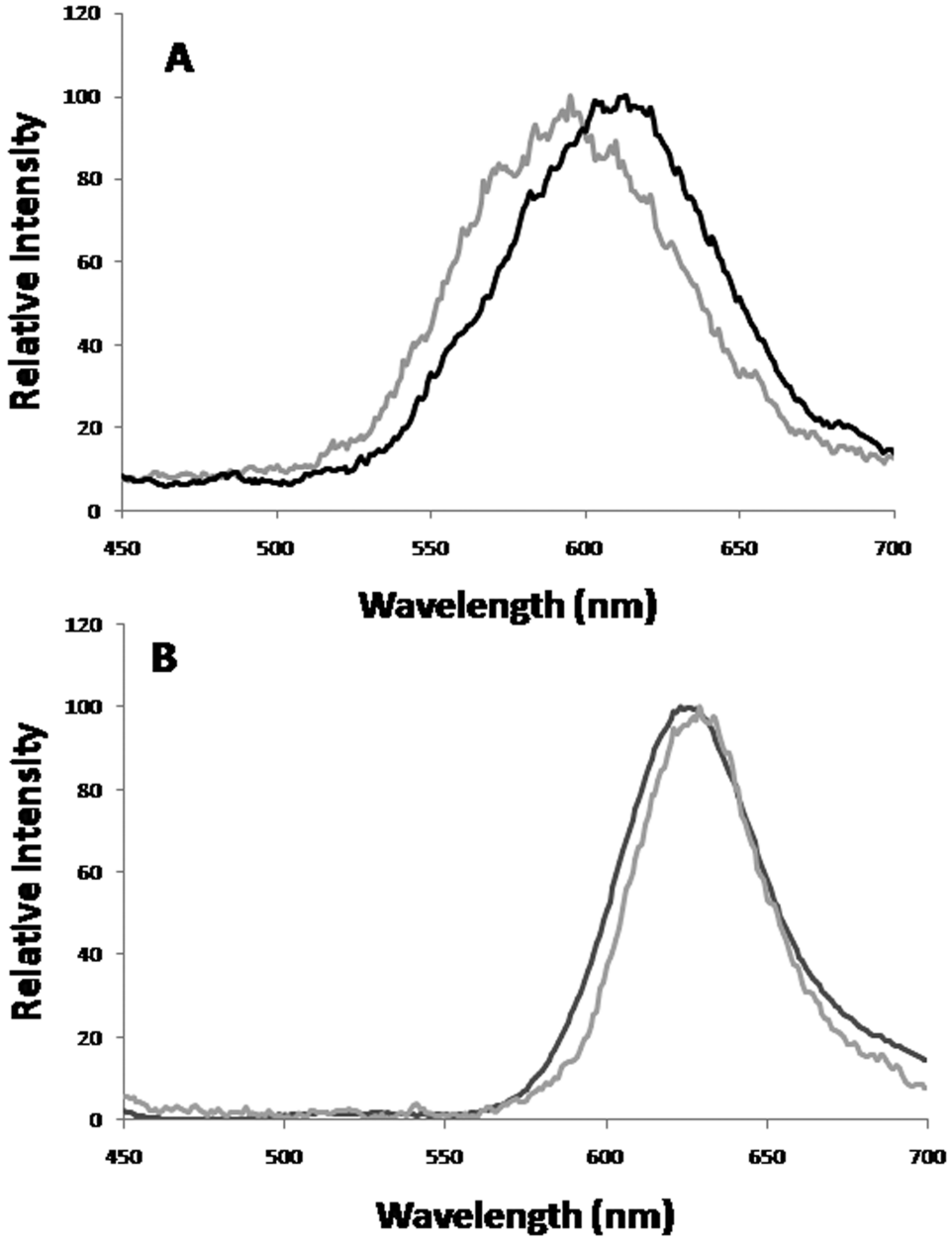


Figure 5. Bioluminescence spectra of *Zophobas* luciferase-like enzyme, *P. hirtus* red-emitting luciferase, and their I327S (T345I) mutants. (A) *Z. morio* luciferase-like enzyme: (gray) wild type and (black) I327S mutant. (B) *P. hirtus* red-emitting luciferase: (black) wild type and (gray) T345I mutant.

Table 2. Tryptophan and 2,6-TNS Fluorescence Spectra of the *Z. morio* Luciferase-like Enzyme, *P. hirtus* Red-Emitting Luciferase, and Their Position 327 and 345 Mutants, Respectively

luciferase	tryptophan λ_{max} (nm)	2,6-TNS λ_{max} (nm)
Z. morio		
wild type	347	440
I327S	348	441
P. hirtus		
wild type	334	437
T345I	335	427

birth of an oxygenase/protoluciferase activity. Thus, this luciferase-like enzyme, beetle luciferases, and perhaps similar AMP-ligases could form a new alternative family of oxygenases in the presence of newly formed endogenous substrates and xenobiotics.

Evolution of Luciferase Activity. Luciferases are enzymes that evolved to efficiently generate light, usually for communicative purposes. Therefore, once a sufficiently selectable luminescent phenotype emerged, these enzymes must have undergone strong selective pressure to increase the visibility of the broadcasted luminescent signal. When considering just the emitter, the visibility of the broadcasted signal relies on two physical parameters: the intensity (number of photons produced over time, which should be maximized) and the spectrum (which should match the visual sensitivity of the receiver) of light emission. The intensity of the bioluminescence depends on the quantum yield of the bioluminescence reaction (Φ_{BL}), as well as on biochemical parameters such as the concentration of the reactants (luciferin,

dx.doi.org/10.1021/bi400141u | *Biochemistry* 2013, 52, 3963−3973

ATP, O_2, and luciferase), and the catalytic constant (k_{cat}) of the luciferase.[1] Our results clearly indicate that the most important enzymatic factor that underwent optimization during the evolution of the new luciferase bioluminescence activity was the catalytic constant, not the quantum yield, although the latter also ultimately contributes to the brightness elicited by luciferases.

Once the visible phenotype appeared, the adenylating activity exerted on the new chemiluminescent substrate and its oxidative activity must have undergone optimization. Our studies with the luciferase-like enzyme and beetle luciferases show that the k_{ox} changed modestly, whereas the overall k_{cat} increased several orders of magnitude. Accordingly, previous studies showed that the ratio of luminescence activities between D- and L-luciferin is orders of magnitude higher than the ratio of the respective adenylates.[9] Overall, these results suggest that the ancestral protoluciferases were poor ligases that displayed a considerable oxidative side reaction with D-luciferin. The bioluminescence activity may have then evolved mainly via an improvement in ligase kinetic properties such as k_{cat} and K_M for the new oxidizable substrate, properties crucial to the increase in the rate of production of the luciferyl-adenylate intermediate for the chemiluminescent oxidative pathway.

Optimization of the quantum yield and selection of the emission spectrum may have happend later, or concomitantly, once a higher catalytic rate approached visibility, thereby optimizing the bioluminescent signal intensity and color toward improved visibility.

Evolution of Bioluminescence Colors. The bioluminescence spectrum is an important adaptation of bioluminescence to vision in different photic habitats and to different biological functions, ranging from green to red depending on the species, life stage, and lantern.[4,25] Most known extant bioluminescent beetles display green-yellow bioluminescence; thus, it has been assumed that the original bioluminescence color was in the green region.[5] However, such an assumption depends on what stage of bioluminescence evolution is being considered. For example, molecular data suggest that in railroadworms the green lateral and red head lanterns and their luciferases may have evolved from a green-yellow-emitting ancestral luciferase.[26] However, such ancestory assumes an already advanced stage of functional bioluminescence, not protobioluminescence. Physicochemical studies indicate that it is highly unlikely that original protobioluminescence was in the green region. This notion is supported by the production of red chemiluminescence of luciferyl-adenylate in most nonenzymatic assays,[15,27] the red luminescence of the luciferase-like enzyme that is produced in an active site not optimized for D-luciferin and for bioluminescence,[9] the long-known bioluminescence red shift displayed by firefly luciferase under denaturing conditions,[28] and the presence of an ever-present red light shoulder in the bioluminescence spectra of firefly luciferases,[19] constituting clear evidence that red chemiluminescence is more primitive. On the other hand, green chemiluminescence requires more complex chemical conditions such as the presence and positioning of basic groups in aprotic and/or hydrophobic environments around oxyluciferin phenolate and/or the C5 proton,[15,27] which are indicative of derived structured environments that may have evolved later.[13]

Thus, assuming that the original protobioluminescent ancestors of bioluminescent beetles had visual sensitivity in the green region like today's species do, it is hard to rationalize how red protobioluminescence may have been selected and evolved to functional (visually oriented) bioluminescence. The answer to such a dilemma could have been protoluciferases with broad and bimodal spectra with an increasing contribution in the green-yellow region during evolution, like that observed for luciferyl-adenylate chemiluminescence in aprotic media in the presence of a strong base,[17] and in several luciferase mutants. It is very likely that mutations resulting in bimodal and blue-shifted broadbands, resulting in more visible signals, conferred a special selective advantage to protobioluminescence. Bioluminescence color may then have evolved through intermediary broadband stages into narrower bands, in the green region for most cases or toward the red in a few like *Phrixotrix* railroadworm luciferases.

I327T/S: A Critical Substitution for the Structural Evolution of Luciferase Function. Altogether, the effects of the I327T/S mutations in the luciferase-like enzyme and the T345I mutation in *Phrixotrix* red-emitting luciferase are complementary and confirm the critical role of T/S345 for bioluminescence function in beetle luciferases. The corresponding modest improvement caused by I327T/S mutations in the luciferase-like enzyme luminescence activity could be explained by the fact that this enzyme, as an AMP-ligase, lacks other optimizing interactions for luciferase activity. In the case of *P. hirtus* railroadworm luciferase, however, the comparatively much larger impact caused by the reverse T345I mutation on luciferase activity could be explained by the fact that this residue is indeed already optimally positioned to play a specific role in luciferase activity. The residues T/S345 (beetle luciferases; I327 in the luciferase-like enzyme) are located in the β-hairpin motif ^{342}LTETTS^{346}, which is highly conserved among beetle luciferases (Figure 6). In other AMP-CoA-ligases (Figure 6), however, this motif displays different degrees of substitution, suggesting that it may have been critical for the development of luminescence activity in beetle luciferases. It is critically located between the luciferin and AMP binding sites,

Ppy	338	QGYGLTETTS	347
Lcr	339	QGYGLTETTS	348
PxRE	335	QGYGLTETCS	344
Pte	335	CGYGLTESTS	344
DrosCG6178	337	QGYGLSESTL	346
Ten2	335	QGYGLTEATL	344
Agrip	336	YGYGLTECGL	345
Ten3	324	QVYGMTEVSG	333
Zop	320	FGYGMSEIGL	329
At4Cl	345	QGYGMTEAGP	354
PheA	321	NAYGPTETTI	330
Ps.4CBAligase	330	NIYGTTEAMN	339

Figure 6. Multiple-sequence alignment of the primary structures of the functional β-hairpin motif corresponding to residues ^{338}QGYGLTETTS^{347} of *Ph. pyralis* firefly luciferase (Ppy) and ^{320}FGYGMSEIGL^{329} of the *Zophobas* luciferase-like enzyme (Zop), AMP-CoA-ligases, and other beetle luciferases: (gray highlighting) variable position corresponding to I327 of the *Zophobas* luciferase-like enzyme and other AMP-CoA-ligases and (yellow highlighting) the corresponding conserved T/S345 residues of beetle luciferases (red for hydrophobic residues, green for neutral residues, and blue for negatively charged residues). Abbreviations: PheA, phenylalanine synthetase; Ps.4CBA, *Pseudomonas* 4-chlorobenazoate CoA-ligase; At4Cl, *Arabidopsis* coumarate CoA-ligase; Ten2 and Ten3, *Tenebrio molitor* luciferase homologues; Agrip, *Agripynus binodulus* luciferase homologue; DrosCG6178, *Drosophila melanogaster* luciferase homologue; Ppy, *Ph. pyralis* firefly luciferase; Lcr, *L. cruciata* firefly luciferase; Pte, *Py. termitilluminans* larval click beetle luciferase; PxRE, *P. hirtus* railroadworm red-emitting luciferase; Zop, *Z. morio* mealworm luciferase-like enzyme.

dx.doi.org/10.1021/bi400141u | *Biochemistry* 2013, 52, 3963−3973

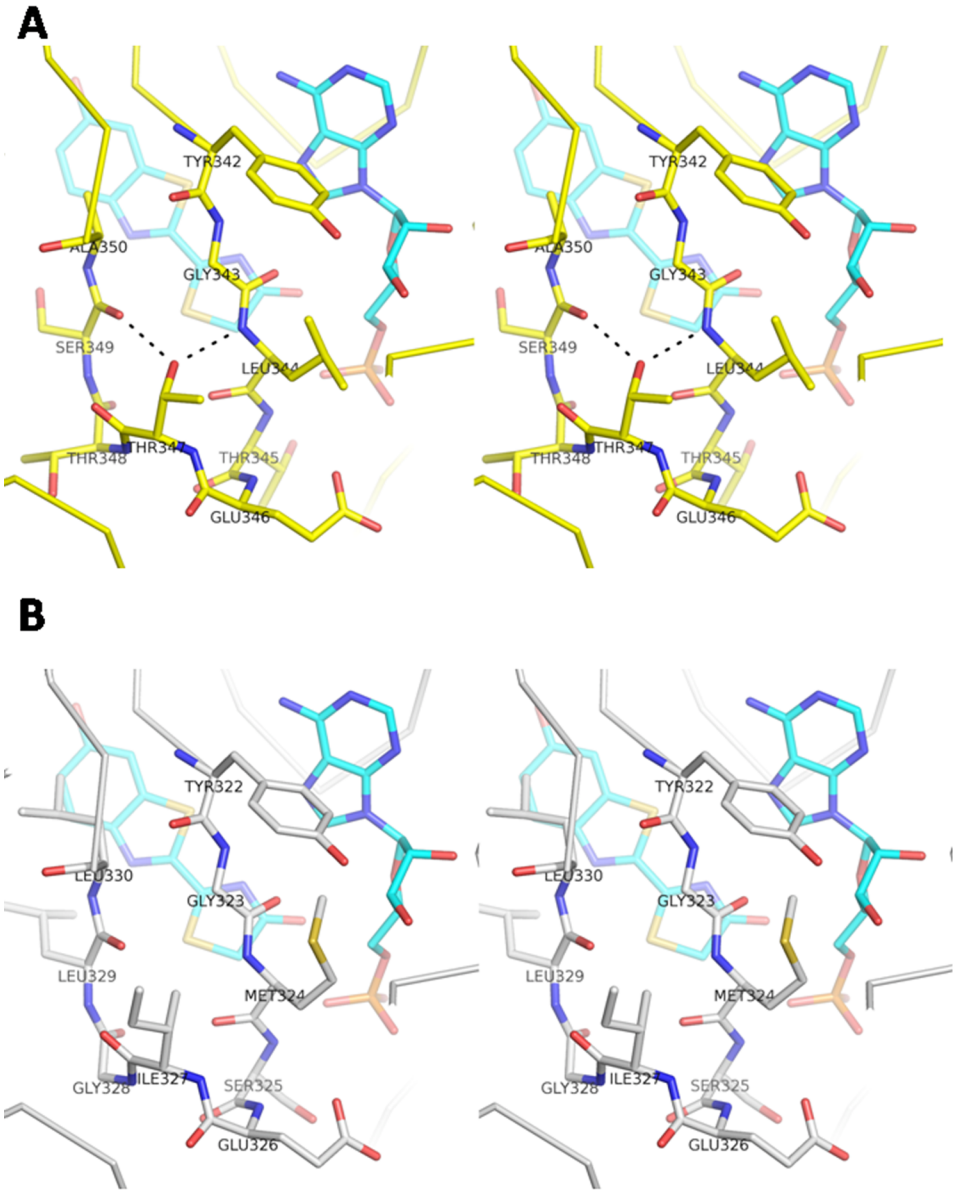


Figure 7. Stereoviews of the active site three-dimensional structure of *Zophobas* luciferase-like enzyme and *L. cruciata* firefly luciferase (template) showing the YGLTET/S β-hairpin motif and the critical T347 residue (*L. cruciata* firefly luciferase) hydrogen bonded with peptide bonds to G343 and S349 and the corresponding I327 residue in *Zophobas* luciferase-like enzyme: (A) *L. cruciata* firefly luciferase (Protein Data Bank entry 2D1R) and (B) *Z. morio* luciferase-like enzyme.

displaying important interactions with both substrates, including the T343 residue that binds the luciferin carboxylate moiety and was formerly associated with stabilization of the pentavalent intermediate.[29] Furthermore, in beetle luciferases, the close S347 residue interacts with S250 and R218, which are critical for bioluminescence colors,[30,31] thus explaining the indirect effect of T/S345I on the bioluminescent spectrum. In beetle luciferases, the conserved T/S345 residues are hydrogen bonded with peptide bonds to G343 and S349, apparently stabilizing the two β-sheets and the loop in a productive conformation for oxygenase activity (Figure 7). Disruption of such interactions with luciferin is expected to affect luciferase activity.

Therefore, mutations such as I327T/S, which simultaneously increased luminescence activity and blue-shifted the spectra in protoluciferases, may have been one of those especially advantageous mutations for the evolution of visually oriented bioluminescence.

Influence of the N-Terminus on the Evolution of Luciferase Activity. The unexpected improved catalytic properties of the new pCold construct suggest that the luciferase N-terminus may have considerable influence on the active site. It has been shown that substitutions in the N-terminus in firefly luciferases alter some functional properties such as the bioluminescence activity,[32] spectrum, and pH sensitivity.[33] Wang et al.[32] showed that removal of the first six residues from firefly luciferase diminishes the activity, whereas removal of the seventh residue abolishes it. Furthermore, known beetle luciferase primary structures vary in length from 528 residues in the luciferase-like enzymes to 541−546 residues for click beetles, railroadworms, and larval fireflies to 550 residues in some adult firefly luciferases.[4,34] The additional residues in longer beetle luciferases are usually accommodated

dx.doi.org/10.1021/bi400141u | *Biochemistry* 2013, 52, 3963−3973

in the N-terminal region and in internal loop regions. Altogether, these results suggest that beetle luciferases may have evolved from shorter AMP-ligases via an increase in the length of the N-terminal polypeptide chain and support the influence of the N-terminus in strengthening interactions on the active site.

Turning an AMP-CoA-ligase (luciferase-like enzyme) into a New Luciferase. Noteworthy is the fact that the new luciferase-like enzyme construct with the I327S mutation and the His-tagged N-terminal tail showed properties comparable to those of some typical luciferases with higher k_{cat} values, lower K_M values for luciferin, and visible orange bioluminescence (Figure 8). The k_{cat} of this mutant approaches that of *P. vivianii*

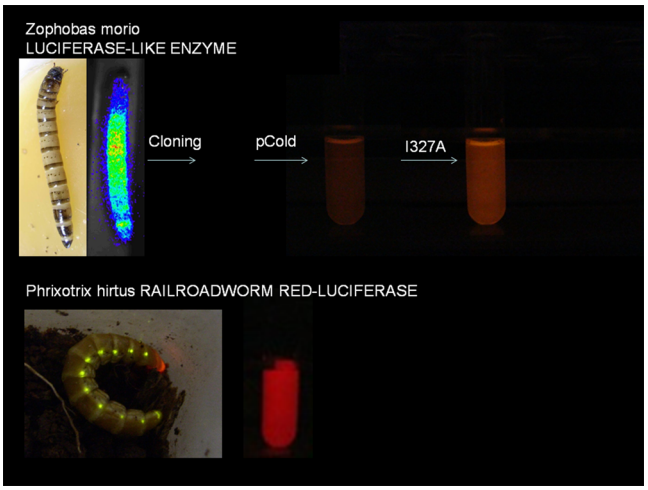


Figure 8. Scheme showing the development of an orange-emitting luciferase from *Zophobas* mealworm luciferase-like enzyme. From left to right (top): *Zophobas* mealworm (daylight), *in vivo* bioluminescence CCD imaging of *Zophobas* mealworm after injection of D-luciferin, and an *in vitro* assay showing pCold-expressed luciferase-like enzyme and orange-emitting pC-I327S mutant (new luciferase), respectively. The bottom panels show *P. hirtus* railroadworm and an *in vitro* assay of its recombinant red-emitting luciferase for comparative purposes.

green-emitting luciferase,[35] whereas the K_M for luciferin is within the range found in some green-emitting pH-insensitive luciferases such as *P. vivianii* and *Py. termitilluminans*,[35,36] being lower than that of *R. ohbai* luciferase.[37] Therefore, we report here the first orange-emitting luciferase developed by genetic engineering departing from an AMP-CoA-ligase with proto-luciferase activity, as can be clearly seen in Figure 8. Previously, Oba et al.[38] reported the conversion of a fatty acyl-CoA synthetase from the nonluminescent click beetle *Agrypinus* into a luciferase upon introduction of L345S, C344S, and G345T mutations. However, the reported activities (0.001%) were orders of magnitude below that of typical luciferases, despite starting from a much more similar enzyme (55% identical with beetle luciferases), and also lower than that of the *Zophobas* luciferase-like enzyme. Furthermore, the corresponding residue of L344 is L328 (and not serine) in *Zophobas* luciferase-like enzyme, which already displays substantial luciferase activity,[9] and the L328S mutation was shown to severely impact luciferase activity instead of increasing it.[10] These findings rule out the S344 residue as being essential for luciferase activity. Additionally, the C344S mutation in the AMP-CoA-ligase of the nonluminescent click beetle, corresponding to the I327T/S mutation in *Zophobas* luciferase-like enzyme, did not show any effect on luminescence activity.

CONCLUDING REMARKS

Using the recently cloned luciferase-like enzyme from *Z. morio* giant mealworm to simulate a protoluciferase and recombinant beetle luciferases, we provide evidence that luciferase activity in protoluciferases may have evolved with an increase in its catalytic constant, especially that of adenylation of the nascent D-luciferin substrate, whereas the oxidative rate and quantum yield apparently changed much less during the course of evolution. The luciferase-like enzyme displays considerable oxygenase activity, suggesting that this enzyme and similar AMP-ligases (including beetle luciferases) could form an alternative new family of oxygenases acting upon new carboxylic substrates. Site-directed mutagenesis of the luciferase-like enzyme (I327S/T) and *Phrixotrix* red-emitting luciferase (T345I) demonstrates a critical role of residues T/S345 in the ^{340}YGLTET/S^{345} motif for luciferase activity. Substitutions like I327S/T that display simultaneous increases in luminescence activity and blue shifts in the spectrum may have been critical for the evolution of luciferase activity in protoluciferases. Accordingly, site-directed mutagenesis and N-terminal chimerization of a luciferase-like enzyme (AMP-CoA-ligase) led for the first time to the development of a new orange-emitting luciferase comparable to some beetle luciferases, which can be harnessed to engineer luciferase activity in other AMP-ligases.

AUTHOR INFORMATION

Corresponding Author

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

Funding

This work was supported by grants of FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil) and CNPq (Conselho Nac. Desenv. Científico e Tecnológico, Brazil). D.K. is grateful for financial support from the Japan Society for the Promotion of Science (JSPS) for the Institutional Program for Young Researcher Overseas Visits.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are deeply grateful to Therese Wilson and John W. Hastings (Harvard University, Cambridge, MA) and Etelvino Bechara (IQ-USP) for their discussions, continuous encouragement, and inspiration through their pioneering work with chemiluminescence and bioluminescence and love for science.

ABBREVIATIONS

Ppy, *Ph. pyralis* luciferase; Pte, *Py. termitilluminans* larval click beetle luciferase; PxGR, *P. vivianii* railroadworm green-emitting luciferase; PxRE, *P. hirtus* railroadworm red-emitting luciferase; Mac, *Macrolampis* sp2 firefly luciferase; Zop, *Z. morio* luciferase-like enzyme; LH_2, D-luciferin; CAPS, *N*-cyclohexyl-3-amino-propanesulfonic acid; DLSA, 5′-*O*-[*N*-(dehydroluciferyl)-sulfamoyl]adenosine; AMP, adenosine monophosphate.

REFERENCES

(1) Wilson, T., and Hastings, J. W. (1998) Bioluminescence. *Annu. Rev. Cell Dev. Biol. 14*, 197−230.

(2) Rees, J. F., de Wergifosse, B., Noiset, O., Dubuisson, M., Janssens, B., and Thompson, E. M. (1998) The origins of marine bioluminescence: Turning oxygen defense mechanisms into deep-sea communication tools. *J. Exp. Biol. 201*, 1211−1221.

(3) Hastings, J. W. (2001) Bioluminescence. In *Cell Cell Physiology* (Sperelakis, N., Ed.) 3rd ed., pp 1115−1131, Academic Press, New York.

(4) Viviani, V. R. (2002) The origin, diversity and structure function relationships of insects luciferases. *Cell. Mol. Life Sci. 59*, 1833−1850.

(5) Wood, K. V. (1995) The chemical mechanism and evolutionary development of beetle bioluminescence. *Photochem. Photobiol. 62*, 662−673.

(6) Viviani, V. R., and Bechara, J. H. (1996) Larval *Tenebrio molitor* (Coleoptera: Tenebrionidae) fat body extracts catalyze D-luciferin and ATP-dependent chemiluminescence. A luciferase-like enzyme. *Photochem. Photobiol. 63*, 713−718.

(7) Oba, Y., Sato, M., and Inoye, S. (2006) Cloning and characterization of the homologous genes of firefly luciferase in the mealworm beetle, *Tenebrio molitor*. *Insect Mol. Biol. 15*, 293−299.

(8) Viviani, V. R., Prado, R. A., Arnoldi, F. G. C., and Abdalla, F. C. (2009) An ancestral luciferase in the Malpighian tubules of a non-bioluminescent beetle. *Photochem. Photobiol. Sci. 8*, 57−61.

(9) Viviani, V. R., Scorsato, V., Prado, R. A., Pereira, J. G. C. P., Niwa, K., Ohmiya, Y., and Barbosa, J. A. R. G. (2010) The origin of luciferase activity in *Zophobas* mealworm AMP/CoA-ligase (protoluciferase): Luciferin stereoselectivity as a switch for the oxygenase activity. *Photochem. Photobiol. Sci. 9*, 1111−1119.

(10) Prado, R. A., Barbosa, J. A., Ohmiya, Y., and Viviani, V. R. (2011) Structural evolution of luciferase activity in *Zophobas* mealworm AMP/CoA-ligase (protoluciferase) through site-directed mutagenesis of the luciferin binding site. *Photochem. Photobiol. Sci. 10*, 1226−1232.

(11) Sali, A., and Blundell, T. L. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J. Mol. Biol. 234*, 779−815.

(12) DeLano, W. L. (2002) *The Pymol Molecular Graphics System*, DeLano Scientific, San Carlos, CA.

(13) Nakatsu, T., Ichiyama, S., Hiratake, J., Saldanha, A., Kobashi, N., Sakata, K., and Kato, H. (2006) Structural basis for the spectral difference in luciferase bioluminescence. *Nature 440*, 372−376.

(14) Niwa, K., Ichino, Y., Kumata, S., Nakajima, Y., Hiraishi, Y., Kato, D., Viviani, V. R., and Ohmiya, Y. (2010) Quantum yields and kinetics of the firefly bioluminescence reaction of beetle luciferases. *Photochem. Photobiol. 86*, 1046−1049.

(15) Viviani, V. R., and Ohmiya, Y. (2006) Bovine serum albumin displays luciferase-like activity in presence of luciferyl-adenylate: Insights on the origin of protoluciferase activity and bioluminescence colours. *Luminescence 21*, 262−267.

(16) McElroy, W. D., and DeLuca, M. (1978) Chemistry of firefly bioluminescence. In *Bioluminescence in Action* (Herring, P. J., Ed.) pp 109−127, Academic Press, New York.

(17) Seliger, H. H., and McElroy, W. D. (1962) Chemiluminescence of firefly luciferin without enzyme. *Science 138*, 683−685.

(18) Seliger, H. H., and McElroy, W. D. (1960) Spectral emission and quantum yield of firefly bioluminescence. *Arch. Biochem. Biophys. 88*, 136−141.

(19) Ando, Y., Niwa, K., Yamada, N., Irie, T., Enomoto, T., Kubota, H., Ohmiya, Y., and Akiyama, H. (2007) Development of a quantitative bio/chemiluminescence spectrometer determining quantum yields: Re-examination of the aqueous luminol chemiluminescence standard. *Photochem. Photobiol. 83*, 1205−1210.

(20) DeLuca, M. (1969) Hydrophobic nature of the active site of firefly luciferase. *Biochemistry 8*, 160−166.

(21) Knights, M., and Drogemuller, C. J. (2000) Xenobiotic-CoA ligases: Kinetic and molecular characterization. *Curr. Drug Metab. 1*, 49−66.

(22) Stuible, H. P., and Kombrink, E. (2001) Identification of the substrate specificity-conferring amino-acid residues of 4-coumarate:-coenzyme A ligase allows the rational design of mutant enzymes with new catalytic properties. *J. Biol. Chem. 276*, 26893−26897.

(23) Beuerle, T., and Pichersky, E. (2002) Purification and characterization of benzoate:coenzyme A ligase from *Clarkia breweri*. *Arch. Biochem. Biophys. 400*, 258−264.

(24) Oba, Y., Ojika, M., and Inouye, S. (2004) Firefly luciferase is a bifunctional enzyme: ATP-dependent monooxygenase and a long chain fatty acyl-CoA sunthetase. *FEBS Lett. 540*, 251−254.

(25) Lall, A. B., Cronin, T. W., Carvalho, A. A., de Souza, J. M., Barros, M. P., Stevani, C. V., Bechara, E. J., Ventura, D. F., Viviani, V. R., and Hill, A. A. (2010) Vision in click beetles (Coleoptera: Elateridae): Pigments and spectral correspondence between visual sensitivity and species bioluminescence emission. *J. Comp. Physiol., A 196*, 629−638.

(26) Arnoldi, F. G., Silva Neto, A. J., and Viviani, V. R. (2010) Molecular insights on the evolution of the lateral and head lantern luciferases and bioluminescence colors in Mastinocerini railroad-worms (Coleoptera: Phengodidae). *Photochem. Photobiol. Sci. 9*, 87−92.

(27) White, E. H., Rapaport, E., Hopkins, T. A., and Seliger, H. H. (1969) Chemi- and bioluminescence of firefly luciferin. *J. Am. Chem. Soc. 91*, 2178−2180.

(28) Seliger, H. H., and McElroy, W. D. (1964) The colors of firefly bioluminescence: Enzyme configuration and species specificity. *Proc. Natl. Acad. Sci. U.S.A. 52*, 75−81.

(29) Branchini, B. R., Southworth, T. L., Murtishaw, M. H., Bojie, H., and Fleet, S. E. (2003) A mutagenesis study of the putative luciferin binding site residues of firefly luciferase. *Biochemistry 42*, 10429−10436.

(30) Viviani, V. R., Amaral, D., Prado, R. A., and Arnoldi, F. G. C. (2011) A new blue-shifted luciferase from the Brazilian *Amydetes fanestratus* (Coleoptera: Lampyridae) firefly: Molecular evolution and structural/functional properties. *Photochem. Photobiol. Sci. 10*, 1879−1886.

(31) Viviani, V. R., and Ohmiya, Y. (2000) Bioluminescence color determinants of *Phrixothrix* railroadworm luciferases: Chimeric luciferases, site-directed mutagenesis of Arg215 and guanidine effect. *Photochem. Photobiol. 72*, 267−271.

(32) Wang, X. C., Yang, J., Huang, W., He, L., Yu, J. T., Lin, Q. S., Li, W., and Zhou, H. M. (2002) Effects of removal of the N-terminal amino acid residues on the activity and conformation of firefly luciferase. *Int. J. Biochem. Cell Biol. 34*, 983−991.

(33) Ugarova, N. N., Maloshenok, L. G., Uporov, I. V., and Koksharov, M. I. (2005) Bioluminescence spectra of native and mutant firefly luciferases as a function of pH. *Biochemistry (Moscow) 70*, 1262−1267.

(34) Viviani, V. R., Bechara, E. J. H., and Ohmiya, Y. (1999) Cloning, sequence analysis and expression of cDNAs for *Phrixothrix* railroad worm luciferases: Relationship between bioluminescence spectra and primary structures. *Biochemistry 38*, 8271−8279.

(35) Viviani, V. R., Arnoldi, F. G. C., Venkatesh, B., Neto, A. J. S., Ogawa, F. G. T., Oehlmeyer, A. T. I., and Ohmiya, Y. (2006) Active-site properties of *Phrixotrix* railroad worm green and red bioluminescence-eliciting luciferases. *J. Biochem. 140*, 467−474.

(36) Silva-Neto, A. J., Scorsato, V., Arnoldi, F. G., and Viviani, V. R. (2009) *Pyrearinus termitilluminans* larval click beetle luciferase: Active site properties, structure and function relationships and comparison with other beetle luciferases. *Photochem. Photobiol. Sci. 8*, 1748−1754.

(37) Ohmiya, Y., Sumiya, M., Viviani, V. R., and Ohba, N. (2000) Comparative aspects of a luciferase molecule from the Japanese luminous beetle *Ragophtalmus ohbai*. *Yokosuka-shi Hakubutsukan Kenkyu Hokoku, Shizen Kagaku 47*, 31−38.

(38) Oba, Y., Iida, K., and Inouye, S. (2009) Functional conversion of fatty acyl-CoA synthetase to firefly luciferase by site-directed mutagenesis: A key substitution responsible for luminescence activity. *FEBS Lett. 583*, 2004−2008.