Cloning, Sequence Analysis, and Expression of Active *Phrixothrix* Railroad-Worms Luciferases: Relationship between Bioluminescence Spectra and Primary Structures[†,‡]

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ABSTRACT: *Phrixothrix* railroad-worms emit yellow-green light through 11 pairs of lateral lanterns along the body and red light through two cephalic lanterns. The cDNAs for the lateral lanterns luciferase of *Phrixothrix vivianii*, which emit green light (λ_{max}= 542 nm), and for the head lanterns of *P. hirtus*, which emit the most red-shifted bioluminescence (λ_{max}= 628 nm) among luminescent beetles, were cloned. Positive clones which emitted green (Pv_{GR}: λ_{max}= 549 nm) and red (Ph_{RE}: λ_{max}= 622 nm) bioluminescence were isolated. The lucifereases coded by Pv_{GR} (545 amino acid residues) and Ph_{RE} (546 amino acid residues) cDNAs share 71% identity. Pv_{GR} and Ph_{RE} luciferases showed 50−55% and 46−49% identity with firefly luciferases, respectively, and 47−49% with click-beetle luciferases. Ph_{RE} luciferase has some unique residues which replace invariant residues in other beetle luciferases. The additional residue Arg 352 in Ph_{RE}, which is deleted in Pv_{GR} polypeptide, seems to be another important structural feature associated with red light production. As in the case of other railroad-worms and click-beetle luciferases studied, *Phrixothrix* luciferases do not undergo the typical red shift suffered by firefly luciferases upon decreasing pH, a property which might be related to the many amino acid residues shared in common between railroad-worm and click-beetle luciferase.

Bioluminescence in beetles is characterized by a wide range of colors. Fireflies (Lampyridae) emit in the green-yellow region of the spectrum (*1*, *2*), click-beetles (Elateridae) emit in the green-orange (*2*, *3*), but railroad-worms (Phengodidae) span the widest range of the spectrum, that is, from the green to the red region (*4*, *5*). The emission of green-red light was suggested to be an adaptation to optimize the detection of bioluminescence in distinct photic environments and for different biological functions (*6*). In all cases, such distinct colors arise from structurally homologous luciferases, which catalyze the same ATP-dependent oxidation of D-luciferin (*7*). Most studies about the structure and function of beetle luciferases have focused on a set of luciferases arising mainly from fireflies (*8*−*14*), 2 click-beetle species (*15*, *16*), and recently a North American species of *Phengodes* (*17*). Three main factors at the level of the luciferase active site are believed to govern bioluminescence colors (*7*): (I) the presence of basic residues catalyzing tautomerization between a ketonic (red light emitter) and enolic (yellow-green light emitter) species of excited oxyluciferin (*18*−*20*); (II) the hydrophobicity of the active site (*21*, *22*); and (III) the active site conformation which affects rotation of excited oxyluciferin along the C_2-C_2' bond (*23*). These factors may act together or independently to determine distinct bioluminescence colors in luciferases of different species. The construction of chimeric proteins using click-beetle luciferases (*24*) and firefly luciferases (*25*), along with mutagenesis studies (*26*−*28*), have revealed important regions and key residues for the bioluminescence color determination. The crystallographic structure of firefly luciferase has been recently resolved in the absence of the substrates (*29*), which shows a main N-terminal domain and a smaller C-terminal cleft which supposedly come closer to sandwich the substrates during catalysis. Despite all these studies, no structural investigations had been conducted on naturally occurring red light-emitting luciferases.

The beautiful and rare *Phrixothrix* railroad-worms are probably the most spectacular luminescent beetles, because in addition to their yellow-green bioluminescence (λ_{max}= 542−574 nm), displayed by two sets of 11 dorsal-lateral lanterns along the body, they emit red bioluminescence (λ_{max}= 609−638 nm) through cephalic and postcephalic organs (*4*, *5*), a unique property among terrestrial creatures (Figure 1). The function of the lateral lantern bioluminescence is probably associated with defensive and sexual attraction purposes, whereas in the case of the red lantern bioluminescence was associated with self-illumination (*5*); however experimental evidence is still lacking. Due to their scarcity, only preliminary biochemical studies have been

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[‡] The cDNA sequences of Pv_{GR} and Ph_{RE} were deposited to NCBI gene bank and their access numbers are AF139644 and AF139645, respectively.

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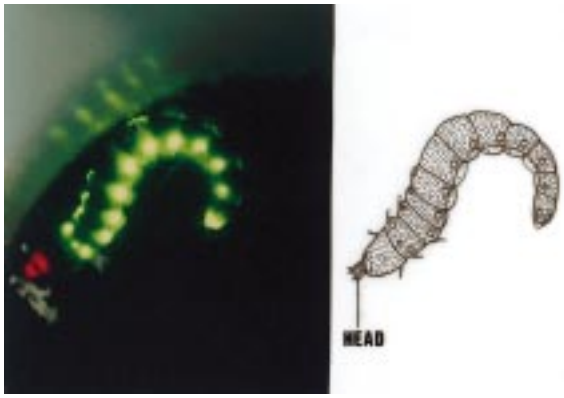


FIGURE 1: *Phrixothrix hirtus* larviform female: (right) by its own bioluminescence and (left) schematic representation showing the head and the lanterns.

conducted about these creature luciferases (*4*, *20*). Railroad-worm and click-beetle luciferases share a common feature: they do not suffer batchromic shift upon decreasing pH as lampyrid luciferases do (*20*). Due to their peculiar spectral properties, *Phrixothrix* luciferases constitute very important models for understanding the mechanism of color modulation in beetle bioluminescence.

Here we report the cloning of the cDNAs for luciferases of two Brazilian railroad-worms occurring in the Central-west region, *Phrixothrix vivianii* and *P. hirtus* (Figure 1). *P. vivianii* is a recently discovered species (*30*), which inhabits pastures and termite mound fields and emits green light through the lateral lanterns (λ_{max}= 542 nm) and red through the head lanterns (λ_{max}= 620 nm) (*5*), whereas *P. hirtus* (Figure 1) live in a kind of dense savanna called "cerradão" and emit yellow light (λ_{max}= 568 nm) through lateral lanterns and the most red-shifted bioluminescence among known luminescent beetles (λ_{max}= 636 nm) (*5*, *31*) through the head lanterns. Previously, larvae of *P. hirtus* have been incorrectly identified as *P. heydeni* (*5*, *31*). Although we have isolated two cDNAs for *Phrixothrix* railroad-worm luciferases which emit in the two extremes of the bioluminescence spectrum spanned by luminescent beetles, we still have not isolated cDNAs for luciferases which emit different colors in the same species.

MATERIAL AND METHODS

Reagents. Isopropyl-β-D-thiogalactopyranoside (IPTG[1]), 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-Gal), dithiothreitol (DTT), D-luciferin (sodium salt), ampicillin, tetracyclin, and kanamycin were from Wako Pure Chemicals (Osaka, Japan); coenzyme-A (CoA) and adenosine triphosphate (ATP) from Oriental Yeast Co (Osaka, Japan); Isogen reagent, restriction enzymes, and Taq polymerase from Nippon Gene (Toyama, Japan); Oligo Tex dT30 and DNA ligation kit from Takara Shuzo (Kyoto, Japan); cDNA synthesis kits from Amersham Pharmacia Biotech (Tokyo, Japan); Gigapack III Gold packaging kit from Stratagene (La Jolla, CA); and ABI PRISM Dye terminator Cycle Sequencing kit from Perkin-Elmer (Foster City, CA).

Bacterial Strains and Media. Echerichia coli XL1-Blue MRF′ and SOLR strains were purchased from Stratagene (La Jolla, CA). *E. coli* cells were usually grown in Luria Bertani (LB) medium (1% bacto-triptone, 0.5% yeast extract, 0.5% NaCl). Cell densities were measured by absorbance at 600 nm.

Insects. Railroad-worms were collected at night as described (*5*). Larvae of *P. vivianii* were collected in pastures at Fazenda São Francisco near Parque Nacional das Emas (prefecture of Mineiros, Goías State). Larvae of *P. hirtus* (Figure 1) were collected into cerradão formation at Fazenda Sta Cruz (Costa Rica prefecture, Mato Grosso do Sul State) (*31*) near the former place. Living specimens were cleaned with distilled water, frozen in liquid nitrogen, and stored at −80 °C.

Construction and Screening of cDNA Libraries. cDNA libraries were constructed using methodology similar to *Pyrearinus termitilluminans* luciferase cloning (*16*). For *P. vivianii* larvae, total RNA was extracted from the whole bodies (without head) of 8 specimens, yielding 360 μg, using Isogen reagent according established procedures (*32*). The mRNAs were isolated using Oligo-dT latex in accordance with Kuribayashi et al. (*33*). cDNAs were synthesized from 4 μg of isolated mRNAs using Time Saver cDNA synthesis kit. The first strand reaction was carried out in the presence of oligo-dT_{12-18} primer. After the synthesis of the second cDNA strand, *Eco*RI/*Not*I adaptors were ligated to the blunt ended cDNA. The cDNA (about 50 ng for *P. vivianii* bodies) was ligated to 1 μg of *Eco*RI pre-digested/dephosphorilated λZAP II (Stratagene, La Jolla, CA) vector in a volume of 5 μL of ligation reaction mixture (1 mM ATP, 7 mM $MgCl_2$, 1 mM DTT in 50 mM Tris-HCl, pH 8.0, and 1 Weiss unit of T_4 ligase) overnight at 16 °C. The ligation mixtures were then packaged using Gigapack III Gold packaging extracts. The original library of *P. vivianii* bodies (7.5×10^5 pfu) was then in vivo excised into *E. coli* XL1-Blue cells in the presence of helper phage to obtain pBluescript (pBl) libraries. The excised phagemids were used to transform *E. coli* SOLR cells. The plasmid library was screened by photodetection (*34*) using a cooled-CCD camera system (ATTO; Tokyo, Japan), after spraying 1 mM D-luciferin (0.1 M citrate buffer pH 5.0) onto IPTG induced colonies at 20 °C during 12 h. For *P. hirtus* head lantern library construction, total RNA was extracted from 17 heads and 4 μg of mRNA was used to synthesize cDNA. All other procedures were essentially similar to those described above, except the original library (2.2×10^4 pfu) was further amplified (1×10^9 pfu) before excision of the pBl library.

Sequence Analysis. The cDNA for green light-emitting luciferase (Pv_{GR}), the 0.75 kb long *Eco*RV/*Bam*HI and *Bam*HI/*Bam*HI fragments were subcloned into pBl and pUC vectors, respectively (*35*). The cDNA for red light-emitting luciferase (Ph_{RE}) was digested with *Eco*RI, and the 3 resulting fragments (0.8; 0.6; 0.3 kbp) were subcloned into *Eco*RI digested/dephosphorilated pUC 18 vector. All of these constructions and the original luciferase cDNA-containing plasmids were sequenced by the dydeoxy chain termination method (*36*) using dye-labeled terminator kit specifically developed for the ABI PRISM 377 automatic sequencer (Perkin-Elmer; Foster City-CA). For extension of pUC vector, M_{13} (−21) and reverse primers were used, whereas for pBl vector T_7 and reverse primers were used. Three

[1] Abbreviations: EDTA, ethylenediamine-*N*,*N*,*N*′-tetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-galactopyranoside; DTT, dithiothreitol; pfu, plaque-forming units; LB, Luria-Bertani medium; PCR, polymerase chain reaction; pBl, pBluescript; Pv_{GR}, *Phrixothrix vivianii* green light emitting luciferase cDNA; Ph_{RE}, *Phrixothrix hirtus* red light emitting luciferase cDNA.

additional primers, VA_1 (5′-ATGTACTTTCAATCTCTTTGC-TAC-3′), VA_3 (5′-AAGTCTAACTATAAGATAAGTTCTTA-3′), and VA_4 (5′-CAAGTTTCAGTTAATCCATAT-3′), were designed from the known partial sequences in order to sequence the internal regions of Ph_{RE} and Pv_{GR}. Sequence comparisons, multi-alignments, and determination of the protein hydropathy profiles, molecular weights and isoeletric points were made using version 7.3 of Genetyx-mac software (Software Development Co., Ltd., Tokyo, Japan).

Expression and Preparation of Luciferase Extracts. Liquid cultures of SOLR cells carrying luciferase insert containing pBl were grown on LB/amp (50 μg/mL) medium at 37 °C with shaking overnight. The preculture (1/100 vol) was then inoculated in LB/amp (50 μg/mL) in the presence of 1 mM IPTG and incubated at 23 °C during 24 h (OD_{600}= 1.8). The cells were harvested by centrifugation at 3000 rpm during 10 min at 4 °C, and the pellet was resuspended in cold extraction buffer (0.1 M sodium phosphate buffer, pH 7.5, containing 2 mM EDTA, 1 mM DTT, and 1% Triton X-100). Lysozyme was added to the final concentration of 1 mg/mL and the suspension incubated 15 min at 0 °C and frozen at −80 °C during 15 min. The lysate was centrifuged at 12000*g* at 4 °C during 15 min. The supernatant was then fractionated with ammonium sulfate. The fraction precipitated between 55% and 70% saturation was dissolved in cold extraction buffer and stored at −20 °C in the presence of glycerol 15%, to maintain the activity.

Luciferase Assays. The activity levels were measured using a Luminescencer AB-2000 luminometer (Atto; Tokyo, Japan) by integration of total light emitted. The assay consisted of the addition of 50 μL of standard solution (2 mM ATP, 0.5 mM D-luciferin, 4 mM $MgSO_4$ in 0.1 M Tris-HCl buffer, pH 8.0) to 10 μL of luciferase-containing extracts at 25 °C. In vivo light intensities were measured after adding 50 μL of 0.5 mM D-luciferin in 0.1 M sodium citrate buffer pH 5.0 to 10 μL of bacterial suspension into a microtiter plate.

Kinetic Measurements. Measurements of light intensities for K_M determinations were made using the luminometer described above. For luciferin K_M estimation, 50 μL of 4 mM ATP solution (0.1 M Tris-HCl, pH 8.0, and 8 mM $MgSO_4$) was injected to 50 μL of crude extract diluted 10 times in 0.1 M Tris-HCl buffer, pH 8.0, containing luciferin (0.03−2 mM). For ATP K_M estimation, 50 μL of 0.5 mM luciferin solution (0.1 M Tris-HCl buffer, pH 8.0, 8 mM $MgSO_4$) was injected to 50 μL of 10 times diluted extract containing ATP (0.1−4 mM). The assays were carried out at 25 °C. Each point of the Michaelis−Menten curve was assayed in quadruplicate. The K_M values were estimated by Lineweaver−Burk plots of the reciprocal of light intensities versus substrate concentration.

Bioluminescence Spectra. Emission spectra were determined using a Hitachi F4500 spectrofluorometer, supplied with a Hamamatsu Photonics R 928 F photomultiplier, with the excitation lamp shut down. The spectra were automatically corrected for the photosensitivity of the equipment. For bacterial in vivo spectra determinations, 500 μL of bacterial suspension and 500 μL of 0.5 mM D-luciferin in 0.1 M sodium citrate buffer, pH 5.0, and 10 mM $MgSO_4$ were mixed into a cuvette in front of the emission window (*16*). In vitro spectra were recorded 3 min after mixing 10−100 μL of luciferase-containing extract to 900 μL of standard reaction mixture (0.5 mM D-luciferin, 2 mM ATP, 4 mM $MgSO_4$, 0.5 mM CoA, and 1% Triton X-100 in 0.1 M Tris-HCl buffer, pH 8.0), to a final volume of 1 mL, into a fluorometer cuvette (*16*) in front of the emission window. The pH effect on in vitro spectra was measured in 0.1 M sodium phosphate buffer (pH 6−8) instead of Tris-HCl buffer as described (*16*). The spectra measured at pH 8.0 in both buffers were essentially identical in shape.

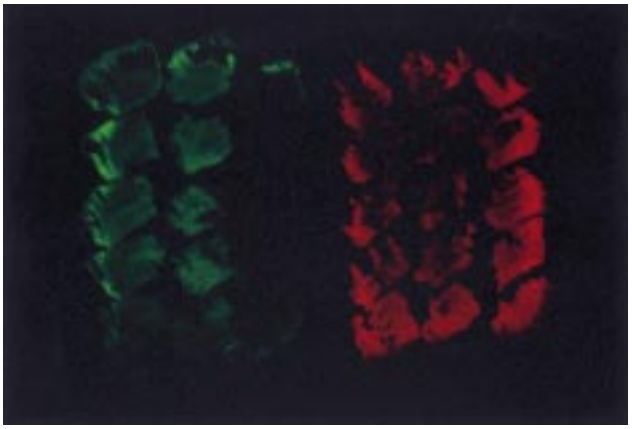


FIGURE 2: Bioluminescence of IPTG-induced SOLR colonies, expressing Pv_{GR} (green light) and Ph_{RE} (red light) luciferases, after spraying D-luciferin. These pictures were taken using Fujichrome 1600 ASA film.

Photographs. Pictures of alive railroad-worms (Figure 1) were taken using a Minolta model XG-1 camera with a 50 mm macrometric lens and Kodachrome 400 ASA films as described (*5*). Pictures of glowing colonies (Figure 2) were taken with the same equipment by long exposure using Fujichrome 1600 ASA film.

RESULTS

Isolation of Positive Clones and Expression of Active Luciferases. The *P. vivianii* body cDNA library yielded 7.5 × 10^5 recombinant plaques. After screening about 10 000 colonies, we isolated 1 positive clone for light emission (Pv_{GR}). The cDNA library for *P. hirtus* head lanterns yielded 2.2 × 10^4 recombinant plaques and was amplified (1 × 10^9 pfu) before excision. Four positive clones were found after screening 3000 colonies of the excised amplified library. The most intense light-emitting clone (Ph_{RE}) was isolated for further analysis. Upon D-luciferin spraying, the IPTG-induced SOLR colonies containing pBl-Pv_{GR} and pBl-Ph_{RE} displayed weak green and red bioluminescence, respectively, visible after dark-room eye adaptation (Figure 2). The luminescence maximum intensity of pBl-Ph_{RE}-containing colonies was reached before and decayed sooner than that of pBl-Pv_{GR}-containing colonies. Luminometer measurement of the total light output of both in in vivo and in vitro bioluminescence assays of IPTG-induced colonies gave nearly the same values for Pv_{GR} and Ph_{RE} luciferases, after correction for the spectral photosensitivity of the equipment.

cDNAs Structures and Sequences. The Pv_{GR} cDNA (NCBI access number: AF139644) is a 1764 bp long fragment. The start codon was found 25 bp downstream from the cDNA 5′ terminus, which follows the *Pst*I restriction site of pBluescript polylinker. An open reading frame of 1635 bp, which codes for a 545 amino acid long polypeptide was found. After the stop codon, a 105 bp long 3′ untranslated region followed by a terminal 26 bp poly-A tail was found.

Table 1: Physical-Chemical Properties of *Phrixothrix* and Other Beetle Luciferases

luciferase[a]	no. of residues	MW (kDa)	pI[b]	pH optimum	K_M (μM) ATP	K_M (μM) luciferin	λ_{max} (nm)	bandwidth (nm)
Phengodidae								
Pv_{GR}	545	59.6	6.26	8.1	330	150	549	70.5
Ph_{RE}	546	60.8	7.0	8.1	240	20	622	55.0
Phe (*17*)	546	60.0			171	64	546	79.0
Lampyridae								
Ppy (*47*)								
pH 8.0	548	60.7	6.42	7.9	250	10	562	78.0
pH 6.0							615	64.0
Elateridae								
Pyt (*16*)	543	60.7	6.75	8.0	280	64	538	73.0

[a] Pv_{GR}, *Phrixothrix vivianii* green light-emitting luciferase; Ph_{RE}, *P. hirtus* red light emitting luciferase; Phe, *Phengodes* luciferase; Ppy, *Photinus pyralis* luciferase; Pyt, *Pyrearinus termitilluminans* luciferase. [b] These values were calculated according to the amino acid sequences using version 7.3 of the Genetyx-mac program.

The Ph_{RE} cDNA (NCBI access number: AF139645) is a 1760 bp long fragment. The cDNA has a 41 bp untranslated region upstream from the first ATG. The sequence of the last 10 bp before the starting codon was essentially identical to that of Pv_{GR}. An open reading frame of 1638 bp, coding for a potential 546 residue long polypeptide was found. After the stop codon, a 61 bp long downstream untranslated region with a terminal 7 bp long poly-A tail was found.

Protein Sequences. The overall identity between Pv_{GR} and Ph_{RE} luciferases was 71%. Pv_{GR} and Ph_{RE} luciferases showed 66.6% and 56% identity, respectively, with the Japanese railroad-worm *Ragophthalmus ohbai* luciferase, recently cloned in our laboratory (*37*). As expected, these values are very close to those observed for *Phengodes* luciferase, with the same set of enzymes (*17*). Pv_{GR} luciferase showed a slightly higher identity with firefly luciferases (50−55%) than Ph_{RE} did with the same set of enzymes (46−49%). Both luciferases showed 47−49% identity with click-beetle luciferases. The overall identity shared with acyl CoA ligases is 25%. Like most beetle luciferases, both luciferases showed the peroxissomal targeting tripeptide SKL before the stop codons (*7*).

Protein Properties. Table 1 shows some physical-chemical properties of *Phrixothrix* and other beetle luciferases. The calculated molecular weights of Pv_{GR} and Ph_{RE} luciferases were 59 626 and 60 951 kDa and were close to those estimated by Western Blotting (data not shown). The isoeletric points, calculated from the deduced primary structures, were 6.26 and 7.0 for Pv_{GR} and Ph_{RE} luciferases, respectively. Pv_{GR} luciferase showed a small increase in the proportion of hydrophobic and neutral residue content in relation to Ph_{RE} luciferase. The hydropathy profiles of these luciferases were similar; however there were some regions which showed major differences (Figure 3). In particular the region from residues 350−360, which includes the additional Arg residue in Ph_{RE} luciferase, showed a major increase of hydrophobic character in Ph_{RE} luciferase in relation to Pv_{GR} luciferase.

Kinetic Parameters. The K_M values for ammonium sulfate fractionated extracts were measured (Table 1). For Ph_{RE} luciferase the K_M for luciferin was 20 μM, whereas for ATP it was 240 μM. However for unknown reasons Pv_{GR} luciferase K_M values were much higher than for Ph_{RE} luciferase (150 μM for luciferin and 350 μM for ATP). The rise time to the peak of intensity and the decay rate of in vitro bioluminescence reaction were faster for Ph_{RE} luciferase than for Pv_{GR} (results not shown). A similar property was observed for the time course of the in vivo bioluminescence after spraying D-luciferin.

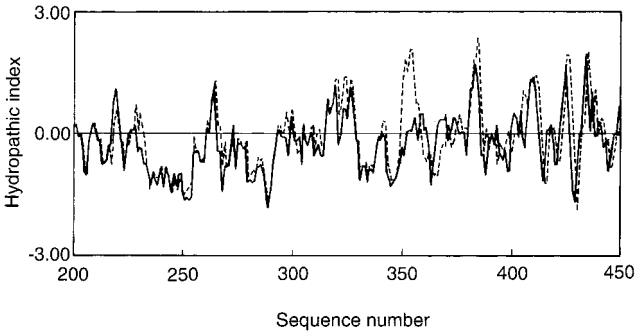


FIGURE 3: Hydropathy profiles of *Phrixothrix* luciferases from residue 200 to 480: (—) Pv_{GR} luciferase and (---) Ph_{RE} luciferase. These profiles were traced using the 7.3 version of Genetyc-max program based on Hoop and Woods parameters (*48*).

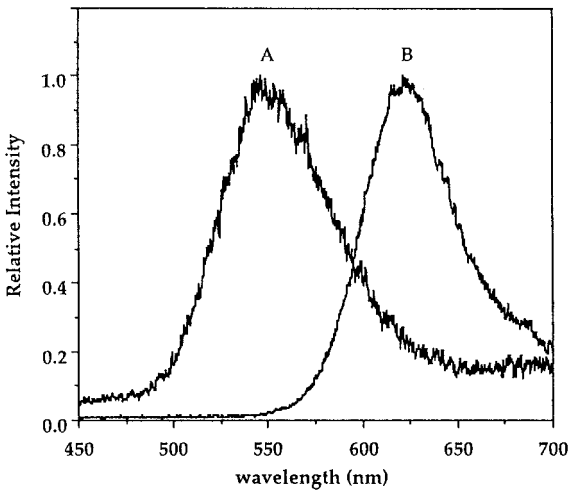


FIGURE 4: In vitro bioluminescence spectra elicited by *Phrixothrix* railroad-worms recombinant luciferases: (A) Pv_{GR} and (B) Ph_{RE}. The BL reactions were conducted on 0.1 M Tris-HCl buffer, pH 8.0. These spectra were corrected for the spectral photosensitivity of the equipment and normalized.

Spectral Properties. The in vivo and in vitro bioluminescence spectra emitted by Pv_{GR} luciferase are centered at 549 nm, thus in the green region, close to that reported for the native enzyme extracted from the larval *P. vivianii* lateral lanterns (λ_{max}= 542 nm; Figure 4) (*5*), and to the spectra emitted by the recently cloned *Phengodes* (λ_{max}= 546 nm)

(*17*) and *R. ohbai* (λ_{max} 556 nm) (*37*) luciferases. Both the in vivo and in vitro bioluminescence spectra of Ph_{RE} luciferase showed a peak at 622 nm (Figure 4), thus 73 nm shifted in relation to Pv_{GR} luciferase. The in vitro spectrum is 6 nm blue-shifted in relation to that emitted by the native enzyme extracted from the larval *P. hirtus* head lanterns (λ_{max}= 628 nm) (*5*), in part due to distinct equipment used for measurements. This spectrum is much narrower (half bandwidth = 55 nm) than that emitted by Pv_{GR} luciferase (half bandwidth = 70.5 nm) and other beetle luciferases. The bioluminescence spectra of both recombinant luciferases did not suffer red shift upon decreasing the pH from 8.0 to 6.0, although they underwent a decrease of intensity.

DISCUSSION

Comparison of Phrixothrix Luciferases. Although a *Phengodes* luciferase had already been cloned, the sequences of *Phrixothrix* are the first ones of Phengodidae to be reported. The identity between *Phrixothrix* luciferases (71%) is lower than that expected for proteins of different species of the same genus, usually above 80% in the case of click-beetle (*16*) and of firefly (*11*, *13*) luciferases. Some dissimilarity must result from the divergence of these luciferases toward the emission of distinct bioluminescence colors for the distinct biological functions played by the lateral and head lanterns. Indeed, the luciferase of *R. ohbai* railroad-worm, which arises from lateral lanterns with function and bioluminescence colors similar to those of *P. vivianii* railroad-worm lateral lanterns (*37*), showed higher identity with Pv_{GR} luciferase (66%) than with Ph_{RE} luciferase (56%) that emits a color very different from that of a distinct lantern.

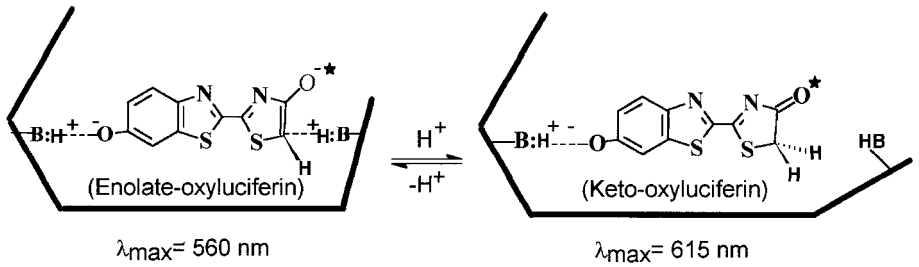
Comparison of *Phrixothrix* luciferases with a set of 12 other beetle luciferases showed 118 invariant residues, most of them located in the C-terminal region (Figure 5). Beside these invariant residues, *Phrixothrix* luciferases showed 21 additional residues in common with click-beetle luciferases and 31 residues with firefly luciferases (Figure 5). Most of the residues in common with click-beetle luciferases are located in the region between residues 242 and 333, whereas those in common with firefly luciferases are preferentially located in the region between residues 310 and 350. The peroxisomal targeting tripeptide SKL was found in all railroad-worm and click-beetle luciferases and in most firefly luciferases.

Role of Basic Residues in Excited Oxyluciferin Tautomerization. It was long suggested that red light emission among firefly luciferases results from ketonic oxyluciferin and yellow-green light emission, resulting in turn from enolic oxyluciferin in the presence of a basic residue assisting enolization (Scheme 1) (*18*, *19*). This was supported by some lines of evidence. (I) spontaneous oxidation of adenyl-luciferin in aqueous media (*38*) and of 5 dimethyl analogue of luciferin, in which enolization is blocked by two methyl groups, gives rise to red chemiluminescence (*18*, *38*). Early studies showed that in excess of strong bases the chemiluminescence spectrum of oxyluciferin shifted toward yellow-green. Recent studies, however, have failed to reproduce such results (*39*). (II) Chemical modification of an hypothetical basic residue in the firefly luciferase active site by alkylating agents resulted in an enzyme which produced only red light (*19*), and the same result was obtained by selective modification of cysteinyl residues by the modifying agent methyl

FIGURE 5: Comparison of primary structures of *Phrixothrix* railroad-worm green (Pv_{GR}) and red (Ph_{RE}) bioluminescence-emitting luciferases: (light shadow) invariant residues among beetle luciferases; (dark shadow) residues unique to Ph_{RE} luciferase at locations conserved among all beetles luciferases including PvGR; (box) residues conserved among railroad-worm luciferases; (∗) residues in common with firefly luciferases; (●) residues in common with click-beetle luciferases and (italic) active site residues.

Scheme 1

A. Basic residue (B:) catalyzed enolization mechanism



B. Oxyluciferin rotation mechanism

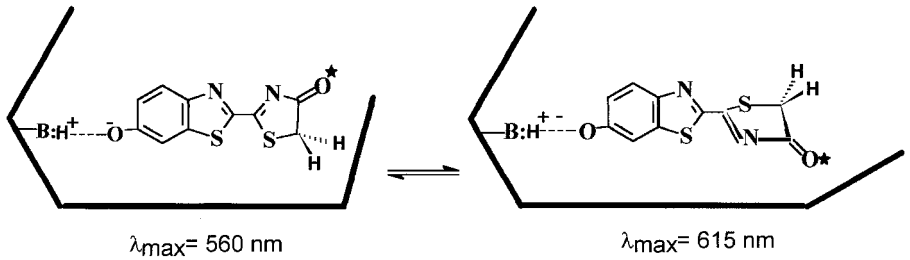


Table 2: Comparison of Firefly Luciferase Red Mutant-Substituted Residues with the Respective Locations in Other Beetle Luciferases

luciferase	luciferase residues[a] 286	326	433	λ_{max} (nm)
Phengodidae				
Pv_{GR}	Thr	Ala	Asn	549
Ph_{RE}	Ser	Ala	Phe	622
Lampyridae (*8*−*14*)				
Photinus pyralis	Ser	Gly	His	562
Pyrocoelia miyako	Ser	Gly	His	550
Luciola cruciata (26)	Ser	Gly	His	562
CM-1	**Asn**	Gly	His	607
CM-2	Ser	**Ser**	His	609
CM-3	Ser	Gly	**Tyr**	612
Luciola lateralis	Ser	Gly	His	552
Luciola mingrelica	Ser	Gly	His	570
Hotaria parvula	Ser	Gly	His	568
H433−Y (28)	Ser	Gly	**Tyr**	612
Photuris pennsilvanica	Ser	Gly	His	538
Lampyris noctiluca	Ser	Gly	His	550
Elateridae				
Pyrophorus plagiophthalamus (*15*)				
GR	Ser	Ala	His	546
YG	Ser	Ala	His	560
YE	Ser	Ala	His	578
OR	Ser	Ala	His	593
Pyrearinus termitilluminans (*16*)	Ser	Ala	Tyr	538

[a] Bold residues indicate substitutions which give rise to red bioluminescence in the firefly luciferase red mutants.

methanetiosulfonate (*40*). No invariant His and Cys residues among beetle luciferases, which are more likely to play the basic residue role at physiological pHs, were found to be replaced by a nonbasic residue only in *Phrixothrix* red light-emitting luciferase. The highly conserved His 433 (*14*), whose substitution by Tyr in firefly luciferases resulted in a dramatic red shift (*26*, *28*), was replaced by Asn in Pv_{GR} luciferase, by Phe in Ph_{RE} enzyme (Table 2), and by Tyr in the recently cloned *P. termitilluminans* luciferase (*16*), which emits very blue-shifted bioluminescence (λ_{max}= 538 nm). Thus it seems unlikely that such His residue is directly involved in enolization and thus color determination. The invariant residue His245 was found to be conserved also in *Phrixothrix* luciferases (*14*). The photo-oxidation and mutagenesis studies of this residue in *Photinus pyralis* luciferase suggest that such a residue is not directly involved in tautomerization of excited oxyluciferin (*41*, *42*). At position 351, Pv_{GR} and *R. ohbai* luciferases, which emit green light, have an His residue replacing an Asn in Ph_{RE}. However, since in firefly luciferases these residues were replaced by Asp and Glx, which are strongly acidic residues, and in click-beetle luciferases the corresponding position was replaced by residues as different as Arg, His, and Gly, it is also unlikely that such a position plays a direct role in tautomerization.

Active Site Residues. Recently the crystallographic structure of *P. pyralis* firefly luciferase has been resolved (*29*). This structure was determined without bound substrates. Nevertheless, some insights were obtained about the structure of the catalytic site mostly on the basis of conserved residues among known beetle luciferases and the family of acyl-adenylating enzymes. More recently, hypothetical models of the catalytic site were constructed on the basis of this structure and that of phenylalanin-activating enzyme (*42*, *43*). In addition, mutagenic studies have revealed some key residues involved in luciferase catalysis (*42*). In accordance with these models on firefly luciferase, the invariant residues Arg218, His245, Phe247, Ala348, and Lys529 appear to interact with luciferin in its binding site. All of these residues appeared to be conserved in *Phrixothrix* luciferases (Figure 5). However, other invariant residues (Figure 5) which possibly play important catalytic roles in beetle luciferases remain to be identified.

The narrow spectrum emitted by Ph_{RE} luciferase (Figure 4) suggests a close fitting of excited oxyluciferin into the active site, resulting in the restriction of vibrational modes of the emitter. Comparison of K_M values (Table 1), which are higher for Ph_{RE} luciferase than those of Pv_{GR} one, seems

to support this hypothesis. On the other hand, the broader red bioluminescence spectrum emitted by *P. pyralis* luciferase under acidic and other denaturing conditions (λ_{max}= 615 nm; half bandwidth = 64 nm) (*44*) suggests a distinct microenvironment for the red light emitter, indicative of a loosening active site. Thus, whereas not conclusive, our experimental results with Ph_{RE} luciferase argue against a higher degree of rotational freedom of excited oxyluciferin in the active site.

Residues Typical of Ph_{RE} Luciferase. The region from residue 300 to about 480 appears to be more homologous with Pv_{GR} and *R. ohbai* green light-emitting luciferases than with Ph_{RE} luciferase. In Ph_{RE} luciferase, the occurrence of many unique substitutions in this otherwise conserved region (Figure 5) suggests that this region plays some role in the bioluminescence color determination. The substitution of Ala314 by Ser in Ph_{RE} luciferase is located in a fragment which supposedly interacts with luciferin according to a recently proposed model (*42*). Whereas substitution of Ala 314 by Ser is associated with considerable chemical changes, the substitution of Ile410 by Leu (Ph_{RE} numbering), except for sterical hindrance effects, does not seem to have considerable influence since these residues have very similar chemical properties. Other substitutions lie in the region 430−480, mainly in the fragment 469−479 (Figure 5) which displays a quite hydrophilic character in relation to other luciferases (Figure 3). In click-beetle isoenzymes, which emit such different colors as green and orange, this fragment is invariable and thus does not account for color determination. If such substitutions influence bioluminescence spectra through a cumulative effect, it is more likely that they do so through influencing the conformation of active site (Scheme 1), rather than through a solvent effect, since no trend indicative of a relationship between hydropathy profiles and bioluminescence colors was found. Furthermore, the solvent effect created by many substitutions on the active site, although influencing short-range spectral shifts (<40 nm), is not enough only by itself to explain green-red shift, since fluorescence studies on oxyluciferin, dehydroluciferin, and analogues, in solvents with distinct dielectric constants, failed to get such a large spectral shift as that observed in *Phrixothrix* luciferases (>70 nm) (*45*, *46*).

The presence of Arg352 in Ph_{RE} luciferase, which corresponds to the deleted residue in the shorter Pv_{GR} luciferase (Figure 5), is another important distinctive structural feature between these proteins. In Ph_{RE} this region (residues 350−362) is associated with a large increase of hydrophobicity in relation to Pv_{GR} luciferase (Figure 3). Such a feature could be potentially involved with considerable conformational changes among these luciferases. Such changes could affect the bioluminescence colors through proper positioning a basic residue in the neighbors of C-5 of excited oxyluciferin in the case of yellow-green-emitting luciferases (*20*), or by influencing the active site geometry according to hypothesis III (*23*).

Comparison of Phrixothrix Luciferases with Firefly Luciferase Red Mutants. Other residues whose mutation in firefly luciferases results in red light (*26*−*28*) were compared with Ph_{RE} and Pv_{GR} luciferases (Table 2). Ser286, whose substitution by Asn in the firefly luciferase results in a red shift (*26*), was found to be conserved in Ph_{RE} luciferase, but replaced by Thr in Pv_{GR} and *R. ohbai* luciferases. Because Ser and Thr have very similar properties, these distinct residues do not seem to be related with determination of bioluminescence color in *Phrixothrix* luciferases. Gly 326, which is replaced by Ser in the firefly red mutant CM-2 (*26*), was found to be conserved among firefly luciferases, but it was replaced by Ala in all railroad-worm and click-beetle luciferases studied. Thus these substitutions cannot account for red bioluminescence in Ph_{RE} such as in the case of firefly luciferase mutants, probably because changes at these positions affect differently the tridimensional structure of these luciferases. Indeed, the substitutions in the red mutants always involved replacements of charged or polar groups, which can potentially interact with other residues in the tertiary structure of the firefly luciferase through electrostatic interactions or by hydrogen bonding, to keep a conformation necessary for yellow-green light emission. Also, no particular similarities were found between Ph_{RE} luciferase and *P. plagiophthalamus* orange light-emitting isoenzyme, which emits closer bioluminescence color (λ_{max}= 593 nm). Ph_{RE} and Pv_{GR} luciferases showed independently the same degree of homology with both *P. plagiophthalamus* green and orange bioluminescence-emitting isoenzymes.

pH Effect. As previously reported for the native phengodid enzymes (*18*), the bioluminescence spectra of both recombinant luciferases did not undergo the typical batchromic shift which firefly luciferases undergo by decreasing the pH from 8 to 6. This same feature was also reported for all click-beetle luciferases studied up to now (*15*, *16*, *20*). This probably means that there is some common structural feature among railroad-worm and click-beetle luciferases not shared with firefly luciferases. In fact, although railroad-worm luciferases show a slightly higher overall identity with firefly luciferases (48−55%) than with click-beetle enzymes (46−49%), they share many residues in common only with click-beetle luciferases, whereas many others are shared with firefly luciferases but not click-beetle enzymes (Figure 5). Most of these residues in common with click-beetle luciferases are located in the region between residues 242 and 333. Furthermore, firefly luciferases typically exhibit a considerable increase of hydrophilic character in the region between 262 and 268, which is absent in railroad-worm and click-beetle luciferases. Thus it would not be surprising if such a region was involved in pH effect. The red shift which firefly luciferases undergo upon decreasing pH, increasing divalent heavy metal cation concentration, or increasing temperature (*44*) was thought to be related to the presence of the same basic residue in the active site of luciferase involved with the abstraction of the proton from C-5 of excited oxyluciferin (*19*) (Scheme 1). According with this hypothesis (*19*, *20*), the absence of pH effect on the red light-emitting luciferase could be related to the absence of a basic residue removing C-5 proton from excited oxyluciferin and thus eliminating the enolization step. In the case of green-yellow light-emitting luciferases, the lack of pH effect could result from the presence of a stable basic residue in the neighbors of excited oxyluciferin which is not affected by such changes (*20*). Alternatively, the absence of pH effect in railroad-worm and click-beetle luciferases may be related to the lack of some basic residue which in the case of firefly luciferases is indirectly involved in keeping the active site conformation necessary for yellow-green light emission.

Concluding Remarks. Comparison of the unique Ph_{RE} luciferase with Pv_{GR} and other known beetle luciferase sequences revealed some unique structural features which are potentially associated with red light emission. The presence of an additional residue, Arg 352, in the longer Ph_{RE} polypeptide (546 residues) in relation to Pv_{GR} (545 residues), along with other unique residues in the C-terminal region could be potentially involved in color determination, probably through optimization of active site conformation, although the specific effect such residues play is still not understood. Also, the presence of residues shared only between click-beetle and railroad-worm luciferases may be related to the lack of pH effect on the bioluminescence spectra emitted by these luciferases, contrary to those emitted by firefly luciferases, which undergo a batochromic shift at acidic pHs. Mutagenesis studies are in progress to elucidate the function of these residues in bioluminescence color determination.

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