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Review

The origin, diversity, and structure function relationships of insect luciferases

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Abstract. Luciferases are the enzymes that catalyze the reactions that produce light in bioluminescence. Whereas the oxidative mechanism which leads to light emission is similar for most luciferases, these enzymes and their substrates are evolutionarily unrelated. Among all bioluminescent groups, insects constitute one of the most diverse in terms of biochemistry. In the fungus-gnats (Mycetophilidae: Diptera), for example, bioluminescence is generated by two biochemically distinct systems. Despite the diversity, investigations on insect luciferases and biochemistry have been conducted mostly with fireflies. The luciferases from the related phengodid beetles, which can produce green to red bioluminescence using the same chemistry as firefly luciferases, have been recently investigated. Beetle luciferases originated from ancestral acyl-CoA ligases. Present data suggest that conserved motifs among this class of ligases are involved in substrate adenylation. The three-dimensional structure of firefly luciferase was recently solved and mutagenesis studies have been performed identifying putative residues involved in luciferin binding and bioluminescence color determination in several beetle luciferases. The knowledge gained through these studies is helping in the development of useful reporter gene tools for biotechnological and biomedical purposes.

Key words. Luciferases; bioluminescence; Mycetophilidae; Lampyridae; Phengodidae; Elateridae; Staphylinidae; Collembola; Diptera; Coleoptera.

Introduction

Luciferases are the key enzymes that catalyze the light-emitting reactions in bioluminescence. They catalyze the oxygenation of compounds generically known as luciferins, generating energy-rich peroxidic intermediates, whose spontaneous decomposition generates singlet electronically excited products which decay emitting a photon of visible light with high efficiency (scheme 1) [1, 2]. Other enzymes such as peroxidases and oxygenases produce triplet excited states which, due to their intrinsically long lives ($>10^{-6}$ s), may play important metabolic or defensive roles in living cells [3, 4], or decay emitting the ultra-weak chemiluminescence that characterizes any aerobic living cell [5, 6]. However, in contrast to such weakly chemiluminescent enzymes, luciferases are unique because they produce singlet excited states, which are short lived ($<10^{-9}$ s) and preferentially decay emitting light. Luciferases also provide active-site microenvironments favorable to emissive decay rather than other photophysical and photochemical deactivating processes. Consequently, the quantum yields of luminescence are very high (up to ~100%). Thus, functionally, luciferases are classified as special types of oxygenases optimized for light emission. Although all known bioluminescence reactions require oxygen [7] and the intermediacy of peroxides, luciferases constitute a diverse group of unrelated enzymes acting upon chemically different luciferins and employing a variety of cofactors [8, 9]. In bacteria, for ex-

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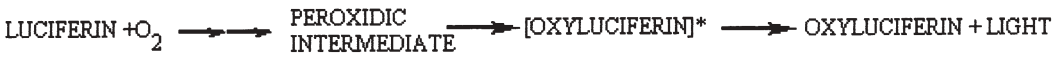


Scheme 1

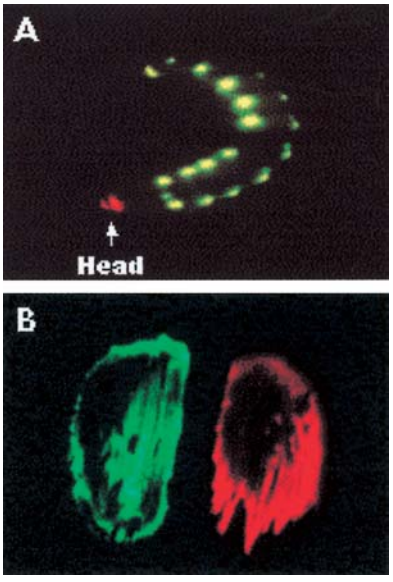


Figure 1. Insect bioluminescence. *Phrixothrix hirtus* railroad worm (Phengodidae) (*A*) and bioluminescence of bacterial colonies (*Escherichia coli*) expressing *Phrixothrix* railroad worm luciferases (*B*) after spraying firefly D-luciferin.

ample, luciferase is a heterodimer of 40 and 35 kDa, which catalyzes the mixed-function oxidation of $FMNH_2$-luciferin and a long-chain aldehyde producing the corresponding long-chain carboxylic acid and excited FMN [10]. In some coelenterates, the luciferase aequorin is a monomer of 35 kDa which catalyzes the formation of a stable and tightly bound peroxy intermediate of coelenterazine (an imidazolpyrazine), whose breakdown is triggered by protein conformational changes induced by Ca^{2+} producing excited coelenteramide and CO_2 [11]. Since the product coelenteramide (emitter) often remains tightly associated with the protein after emission, some coelenterate luciferases have been referred to as 'photoproteins' which are defined as proteins that emit light without turnover [12]. In dinoflagellates, a luciferase of 137 kDa, consisting of three identical and catalytically active domains [13], oxidizes a tetrapyrrolic luciferin, which may be found associated to a luciferin-binding protein depending on the species. The three-dimensional structures of bacterial luciferase and the coelenterate photoproteins, aequorin and obelin, were recently determined by X-ray crystallography [14–16].

Table 1. Properties of the bioluminescent system and luciferases of terrestrial arthropods and insects.

Taxonomic group	Photogenic tissue	λ_{max} (nm)	Luciferase molecular weight (kDa)	Luciferin	Substrates/ cofactors
MYRIAPODA (millipedes, centipedes)					
GEOPHILA (millipedes)					
Xystodesmidae					
Luminodesmus		496	104	porphyrin	ATP
HEXAPODA (insects)					
COLLEMBOLA (springtails)					
Poduridae					
Achorutes	fat body	green			
Onychiuridae					
Neanura	fat body	green			
DIPTERA Mycetophilidae					
(fungus-gnats)					
Arachnocampa	Malpighi tubules	485	~36		ATP
Orfelia	black bodies	460	~140		substrate-binding fraction, dithiothreitol
Keroplatus	fat body	blue			
COLEOPTERA (Beetles)					
Elateroidea					
Lampyridae (fireflies)	fat body	538–582	60–61	benzothiazole	ATP, CoA
Phengodidae (railroad worms)	fat body	536–623	60–61	benzothiazole	ATP, CoA
Elateridae (click beetles)	fat body	534–593	60–61	benzothiazole	ATP, CoA
Staphylinoidea					
Staphylinidae		520–568			

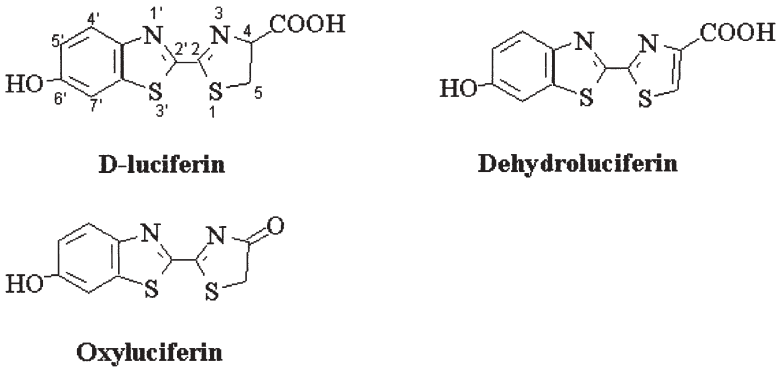


Figure 2. Beetle D-luciferin and its oxidation products.

The insects are the richest and most diverse group of bioluminescent organisms. Luminescence occurs in Collembola (springtails), Diptera (flies), and mainly Coleoptera (beetles) [17] (fig. 1, table 1). About 2500 species are currently described, but many more are probably hidden in the rapidly shrinking tropical forests around the world. In most cases, the identity and properties of their luciferases and luciferins as well as the bioluminescent mechanisms are still unknown or poorly characterized. The exceptions are firefly luciferases, which have been extensively studied during the last 50 years and constitute one of the best characterized bioluminescent models. These luciferases catalyze the ATP-dependent oxidation of a benzothiazole luciferin (fig. 2) producing light in the yellow-green region. The luciferases from the related click beetles and railroad worms (fig. 1), produce light ranging from the green to the orange (λ_{max} = 530–593 nm) and even red (λ_{max} = 623 nm) regions of the spectrum using the same luciferin and probably using the same mechanism as firefly luciferases [18, 19], but have been much less studied. The dependence on ATP has made firefly luciferase one of the most sensitive and practical tools for analytical applications involving ATP measurement [12]. More recently, the genes that code for luciferases have been used as valuable tools as gene reporters in biotechnology and biomedicine [12]. Since the last review on the biochemistry of beetle bioluminescence [20], considerable progress in understanding of the structure/function of firefly luciferases and bioluminescence color determination in other beetle luciferases has been achieved. In this review, I will give an overview of currently known insect bioluminescent systems, including the recent findings in Diptera fungus-gnats, and focus on recent advances in the structure/function relationships and molecular evolution of beetle luciferases.

Diversity of bioluminescence in insects: an overview

Luminescence in terrestrial arthropods is found in Myriapoda and more extensively in insects [21] (table 1). Among the Myriapoda, the millipede *Luminodesmus sequoiae* displays blue-green bioluminescence throughout the body. Its bioluminescent system is activated by MgATP [22], as in the firefly system, and involves a photoprotein of 60 kDa [23] that contains a porphyrin chromophore. In insects, luminescence is definitively confirmed in Coleoptera, Diptera, and Collembola. In the Collembola, except for the observations that bioluminescence is dependent on oxygen and that hydrogen peroxide can stimulate it [7], no studies on their bioluminescence have been carried out. Recently, luminescence was also reported in Amazonian species of Blattodea (Orthoptera), but there are no detailed studies on the luminescence properties [24].

Coleoptera (beetles)

The beetles have the largest number and variety of luminescent species. They are found mainly in the superfamily Elateroidea (former Cantharoidea and Elateroidea) [25], which includes fireflies (Lampyridae), railroad worms (Phengodidae), click beetles (Elateridae), and related families. Luminescence has also been found in a luminescent larva of *Xantholinus* in the Staphylinidae [26] and larvae and adults of an undetermined species [V. R. Viviani, unpublished data]. Fireflies emit green-yellow flashes characterized by duration, interval, and frequency from ventral lanterns for sexual-attraction purposes [27–30]. Click beetles have two dorsal prothoracic lanterns, which usually emit continuous green light, and a ventral abdominal light organ that emits continuous green-orange light when the insect is flying [31]. The function of luminescence in click beetles is related to courtship and defense, but detailed studies are missing [32]. Railroad worms emit the widest range of colors among luminescent beetles, including some of the most spectacular examples with larvae and females having rows of lateral lanterns along the body emitting green-orange light and, in addition, South-American species with cephalic lanterns emitting from yellow-green to red light

depending on the species (fig.1, table 2) [33]. In the larval stage, bioluminescence assumes mainly defensive functions [17, 34], but attraction of prey has also been reported in the larvae of the Brazilian *Pyrearinus termitilluminans* click beetle, which display the phenomenon of luminous termite mounds [31]. The specific patterns and colors of light emission are optimized for maximal visual detection of bioluminescence in different photic environments and for different biological functions [31, 35].

Bioluminescence in beetles is generated by specialized photogenic organs histologically similar to the fat body [36]. In the simplest case, found in the continuously glowing *Phengodes* larvae, the photogenic organs consist of single giant oenocyte-like photocytes [36, 37]. In the most complex cases, found in the intermittently flashing fireflies such as *Photinus* and *Photuris*, the light organs consist of rosettes of thousands of photocytes, interpenetrated by tracheoles and nerves. The oxygen is supplied by the tracheoles and the bioluminescence is neurally controlled, probably through the admission of oxygen by the tracheolar end-cells [36, 38]. Recently, nitric oxide was found to trigger the firefly flash [39]. Inside the photocytes, luciferin is found both dissolved and concentrated in granules [40], whereas luciferase is associated with the peroxisomes [41]. Bioluminescence is found in almost all life stages of luminescent beetles, being absent only in the adult stage in some species [7]. Luciferase isozymes are expressed during the development of luminescent species [18, 42] and in distinct light organs of the same organism. In addition, in the larval stage of the *Photuris* firefly, the luciferase, as well as luciferin are also found outside the light organs [43], suggesting expression in other tissues.

Diptera (Mycetophilidae)

In Diptera, luminescence is found in the Mycetophilidae. The best known are *Arachnocampa* species from New Zealand caves and the Australasian region [44], whose larvae construct webs on the roof of caves and use their continuous blue-green luminescence to attract the flying insects on which they prey. Luminescence is produced at the terminal end of Malpighi tubules [45]. Other luminescent mycetophilids are found in the genera *Keroplatus* and *Orfelia* [7]. In *Keroplatus*, luminescence has been found associated with the fat body around the digestive tube, but biochemical studies are lacking [46]. *Orfelia fultoni* is another web-building species, which occurs in stream banks of the Appalachian mountains of eastern USA [47] and produces the most blue shifted light among insects. The light organs are located in the five anterior

Table 2. Molecular properties of beetle luciferases.

Luciferase	Residues	Identity with Phtinus pyralis firetly luciferase (%)	pI	λ_{max} (nm)	Genebank assignment
pH-sensitive					
Lampyridae (fireflies)					
Photinus pyralis	550	1	6.4	562	M15077
Pyrocoelia miyako	548	82	6.1	550	L39928
Hotaria parvula	548	68	6.3	568	L39929
Luciola mingrelica	548	67	6.2	570	S61961
Luciola cruciata	548	67	7.1	562	M26194
Luciola lateralis	548	67	6.5	552	X66919
Lampyris noctiluca	547	83	6.1	550	X89479
Photuris pennsilvanica	545	58	8.4	538	U31240
pH-insensitive					
Phengodidae (railroad worms)					
Phengodes	546	54		546	
Phrixothrix viviani	545	55	6.3	548	AF139644
P. hirtus	546	47	7	623	AF139645
Ragophthalmus ohbai	543	53		555	
Elateridae (click beetles)					
Pyrophorus plagiophthalamus					
GR	543	47	6.5	546	
YG	543	46	6.5	560	
YE	543	47	6.5	578	
OR	542	46	6.4	593	
Pyrearinus termitilluminans	543	46	6.75	536	AF116843

The pI was calculated from protein sequences.

segments and on the tail, and consist of black bodies that are giant cells full of dark granules secreted by mitochondria [48].

Until recently, knowledge of the biochemistry of the mycetophilid bioluminescence system was very scant and limited to *Arachnocampa* [49, 50]. As in the case of beetles, luminescence in *Arachnocampa* is dependent on ATP. A luciferin-luciferase reaction has been demonstrated [51]. However, cross-reaction between luciferin-luciferase of *Arachnocampa* and beetles was negative, indicating the systems are different. A phylogenetic relationship with the luminescent system of beetles could be speculated, based on the involvement of ATP in both systems [51]. Recently, we have been able to isolate a 36-kDa luciferase and a fluorescent luciferin with properties different from that of the beetles [52]. Of note, the bioluminescent system of the closely related North-American *O. fultoni* is biochemically different, involving a luciferase of 140 kDa, possibly a dimer, a still uncharacterized luciferin, and a storage form of luciferin that is activated by reductants such as dithiothreitol (DTT) and ascorbic acid, and tentatively identified as a luciferin-binding protein (table 1) [52]. This is the first case of two species in a single family using two different bioluminescent systems.

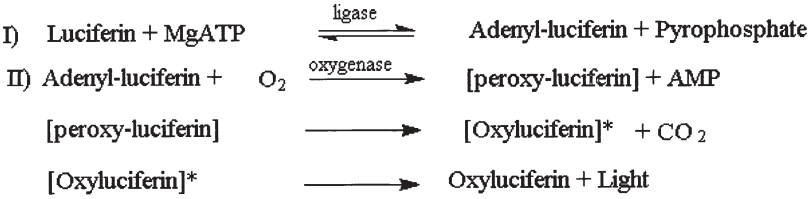
Beetle luciferases

During the past 50 years, firefly luciferases have been intensively studied. Most studies have focused on the North-American *Photinus pyralis* luciferase [53]. The luciferases from the Caucasian *Luciola mingrelica* and the Japanese *Luciola cruciata* and *Luciola lateralis* fireflies have also been characterized [54, 55]. Firefly luciferase is a euglobulin catalytically active as a ~60-kDa monomer that can dimerize at high concentrations [54, 56]. It has some properties of membrane proteins such as the association with phospholipids [54]. According to stoichiometric studies, there are two independent sites for the substrates luciferin and ATP and one for luciferyl-adenylate [54]. In addition, ATP is also an allosteric modulator of luciferase, with two putative allosteric sites.

Chemistry and enzymatic mechanisms of bioluminescence

Luciferases catalyze two essential enzymatic steps for bioluminescence (scheme 2, fig. 3): (i) adenylation of D-luciferin and (ii) oxygenation of adenyl-luciferin. During the first step, luciferase catalyzes the activation of luciferin through the adenylation of the carboxyl group at the expense of MgATP, in a reaction analogous to the activation of amino acids catalyzed by tRNA synthetases and of fatty acids by acyl-CoA:ligases [20, 57]. In the second step, luciferase acts as an oxygenase, catalyzing oxygenation of adenyl-luciferin and forming an energy-rich-dioxetanone intermediate. The cleavage of the dioxetanone ring yields carbon dioxide and singlet excited oxyluciferin, which then decays emitting a photon in the green-red region of the spectrum with very high efficiency (0.88) [58]. To oxidize luciferin, luciferase must abstract the C4 proton producing a carbanion that undergoes electrophilic attack by molecular oxygen [59]. This process is greatly facilitated by the activating effect of AMP which increases the acidity of C4 and is a good leaving group, allowing the formation of the dioxetanone ring. The abstraction of the proton from C4 was suggested to be the rate-limiting step of the oxygenase reaction [59]. Proton exchange studies and circular dichroism (CD) spectra showed that after the adenylation, luciferase undergoes considerable conformational changes [53, 59]. To achieve an efficient production of singlet excited species, the breakdown of the dioxetanone ring intermediate is suggested to be activated by an intramolecular chemically initiated electron exchange luminescence (CIEEL) process [60]. In this process, the electron transfer from the benzothiazolyl moiety of the luciferin in the phenolate form destabilizes the dioxetanone ring yielding a radical ion pair luciferin and CO_2 followed by the return of the electron to generate a singlet excited state of the oxyluciferin moiety [60, 61].

The kinetics of the *L. mingrelica* firefly luciferase reaction have been studied in some detail [54]. Although the reaction is non-steady state, the maximum intensity (V_{max}) dependence on substrate concentrations obeys the Michaelis-Menten equation. Despite the fact that oxyluciferin was suggested to be highly inhibitory causing the rapid decay of luminescence [62], studies with *L. mingrelica* luciferase showed that after emission, the enzyme-bound product is catalytically active, suggesting that oxyluciferin may migrate to another site [54].



Scheme 2

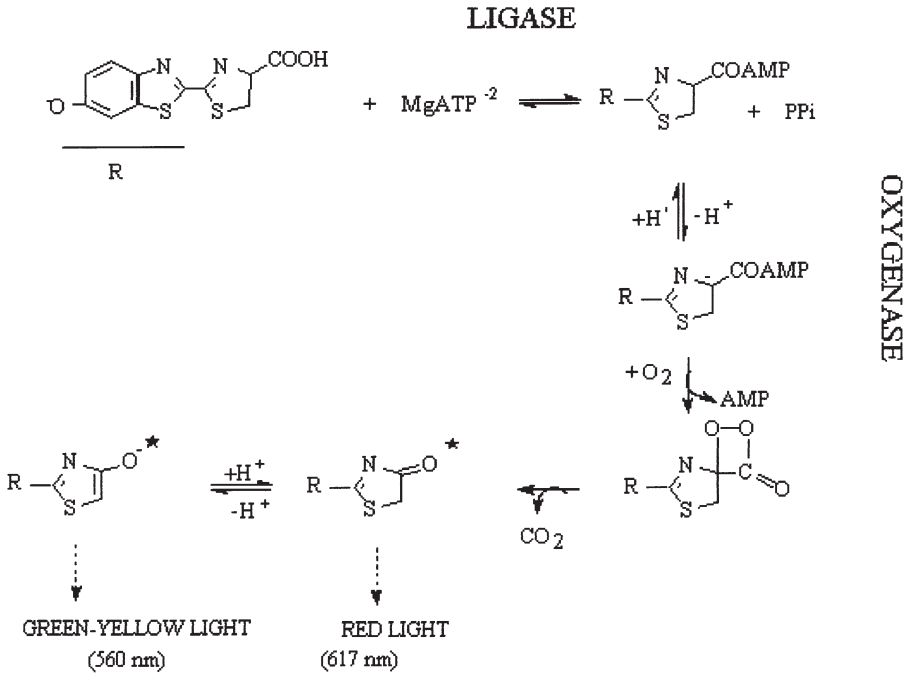


Figure 3. Mechanism of beetle luciferase-catalyzed reactions.

Substrates and products of the firefly luciferase reaction

The structure of firefly luciferin (fig. 2), a benzothiazole compound, has been determined, and it was synthesized in the laboratory along with dehydroluciferin, a side product of autooxidation (fig. 2) [63]. Dehydroluciferin is a potent competitive inhibitor of luciferin [64]. It remains tightly bound to the luciferase active site, possibly causing the observed fast decay of luminescence during the luciferase in vitro reaction. Oxyluciferin is a very unstable compound: its structure was deduced from the synthesis of analogs with chemiluminescence and fluorescence spectra matching the bioluminescence spectrum [65]. Recently, the cDNA for an enzyme which recycles luciferin from oxyluciferin in the light organ of fireflies was cloned and sequenced, but no similarity was found with other enzymes [66]. Luciferin, dehydroluciferin, and oxyluciferin display similar spectroscopic properties [65, 67]. In aqueous media, when the 6′phenolate proton in the excited state is allowed to dissociate, all display intense yellow-green fluorescence (λ_{max} = 530–540 nm); however, in non-polar solvents, where proton dissociation is not favored, the fluorescence is blue (λ_{max} = 430–450 nm) [66]. Both yellow-green and blue fluorescence bands also suffer hypsochromic shifts going from polar to more apolar solvents [65]. Since the firefly bioluminescence spectrum is in the yellow-green region (λ_{max} = 565 nm), the emitter likely has the phenolate in the ionized form [67–69]. Blue bioluminescence is never observed, probably because the phenolate group must be in the ionized form for efficient CIEEL chemiexcitation, a step necessary for bioluminescence [20, 60, 61].

Dehydroluciferin also serves as a substrate for adenylation; however, it cannot be further oxidized in the following bioluminescent steps [64]. Some luciferin analogs that produce different bioluminescence colors have also been synthesized. The 6′aminoluciferin emits red light with firefly luciferase [70]. The naphthyl and quinolyl analogs produce blue and red bioluminescence, respectively [71]. Most ATP analogs are inactive for bioluminescence. The 3-iso ATP analog was found to be active for bioluminescence, but to a lesser degree, and results in red bioluminescence [72].

CoA has an important stimulatory effect on the luminescent reaction of firefly luciferase. Luciferases can catalyze the transfer and esterification of the carboxyl group of deydroluciferin and luciferin from the adenylate forms to the thiol group of CoA in an analogous way to acyl CoA synthetases [73] (scheme 3). The stimulatory effect of CoA has been suggested to be due to the removal of dehydroluciferin from the inhibited active site increasing the turnover rate. The sulfhydryl moiety of CoA was found to be essential to its activity [74]. The participation of CoA as a substrate in the thioesterification reaction of dehydroluciferin and of other carboxylic acids in homologous ligases suggests that there might be a vestigial binding site for this substrate in beetle luciferases [20]. In addition, firefly luciferase can catalyze the production of diadenosine tetraphosphates as a side reaction [75, 76]. Physiologically, beetle luciferases have also been proposed to play an auxiliary role in oxygen detoxification [77].

Phylogeny and molecular evolution of beetle luciferases

Phylogeny

After the cloning of *P. pyralis* luciferase [78], many other firefly luciferases were cloned and sequenced [79–84]. All of them are single polypeptides that vary in length from 545 to 550 residues (table 2) and have an average molecular weight of ~60 kDa [20]. Except for the acetylation of the first methionine, no other post-translational modifications are observed in firefly luciferases [85]. With the exception of *Luciola* luciferases, all beetle luciferases have a C-terminal signal three-peptide SKL (fig. 4), which directs them to the peroxisomes. The firefly luciferases share 66–99% identity with each other. In general, the phylogeny based on sequence identity of these luciferases matches the phylogeny of Lampyridae based on bionomical data (fig. 5).

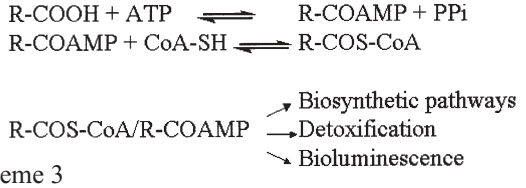
Click beetle luciferases emit bioluminescence ranging from the green to the orange region of the spectrum (λ_{max} = 532–593 nm) [18, 86]. They are the shortest polypeptides, with 542–543 residues (table 2) and are ~45% identical to firefly luciferases [87, 88]. The green, yellow-green, yellow, and orange light-emitting isozymes from the abdominal organ of the Jamaican click beetle *Pyrophorus plagiophthalamus* have been cloned. These isozymes share from 95 to 99% identity among themselves. More recently, the luciferase from the larval click beetle *P. termitilluminans*, which produces one of the greenest bioluminescences (λ_{max} = 534 nm) among elateroid beetles [88] was also cloned. It shares 82% identity with *P. plagiophthalamus* isozymes (fig. 4) [88]. Click beetle luciferases tend to aggregate, being active as monomers and dimers [54].

Phengodid luciferases emit bioluminescence spanning the widest range of colors among beetles (λ_{max} = 536–623 nm), including red light [19, 33]. A *Phengodes* luciferase has been cloned, although its sequence has not been published [89]. Recently, we cloned the red- and green-emitting luciferases from the head lantern and lateral lanterns of the Brazilian railroad worms *Phrixothrix hirtus* and *P. viviani* (fig. 1) respectively, which are 71% identical [90]. The luciferase from the Japanese relative *Ragophthalmus ohbai* is 56–66% identical to *Phrixohtrix* spp. railroad worm luciferases [91]. Although *Ragophthalmus* has been classified as a subfamily of either the Lampyridae [92] or Phengodidae, or as the independent family Ragophthalmidae [7, 93], the close identity of its luciferase with those of phengodid luciferases support their inclusion as a subfamily within the Phengodidae [fig. 5]. Phengodid luciferases have 543–546 residues (fig. 4, table 2), and are ~50% identical to firefly luciferases and 45% to click beetle luciferases.

Origin and molecular evolution

One of the most intriguing questions about bioluminescence is how such an exotic process originated during evolution. Bioluminescence has arisen at least 30 independent times [8]. Based on present biochemical data (table 1), bioluminescence in insects arose at least three times, but considering the unknown systems of Collembola, staphylinid beetles as well as those of other bioluminescent insects yet to be discovered mainly in the tropical forests, I anticipate the discovery of many other bioluminescent systems.

Early reports suggested that bioluminescence arose as an accidental by-product of oxygen detoxification when photosynthetic oxygen started to rise in the atmosphere [94]. According to such a hypothesis, luciferases arose from fortuitously luminescent oxygenases, involved in the removal of the increasingly toxic oxygen. A subsequent hypothesis suggested that luciferases arose from mixed-function oxygenases involved with the oxidation of increasing levels of unsaturated and aromatic compounds during early life history [95–97]. The new luminescent phenotype conferred a selective advantage on the species having it. Whereas the origin from an early oxygenase could be valid for bacterial luciferases [8], such a hypothesis is unlikely to apply to most other taxa since most of them originated after the oxygen pressure had almost reached the current level [98]. Indeed, Hastings [8] suggested that most luciferases originated much later, after the development of vision. Oxygenases are usually metalloproteins with prosthetic groups and no similarity with luciferases at the primary-structure level [98]. The current view is that most luciferases did not originate from oxygenases but, rather, the luminescent phenotype drove the evolution of the new oxygenase functions [20, 99]. Based on the homology with proteins such as calmodulin, the coelenterate photoproteins for example have been suggested to have originated from ancestral calcium-binding proteins [100]. In the case of beetle bioluminescence, luciferases clearly evolved from an acyl-CoA ligase that had another metabolic function, probably by gene duplication [20] (fig. 6). The similarity of the adenylation reaction of firefly luciferase and the activation of fatty acids by acyl-CoA synthetases (scheme 3) and of amino acids by aminoacyl-tRNA synthetases has long been noted [57]. Not surprisingly, with the advent of molecular biology, beetle luciferases were found to be homologous to many ligases that catalyze the adenylation of different carboxylic compounds and subsequent thioesterification (scheme 3) [101–106]. This family of enzymes



Scheme 3

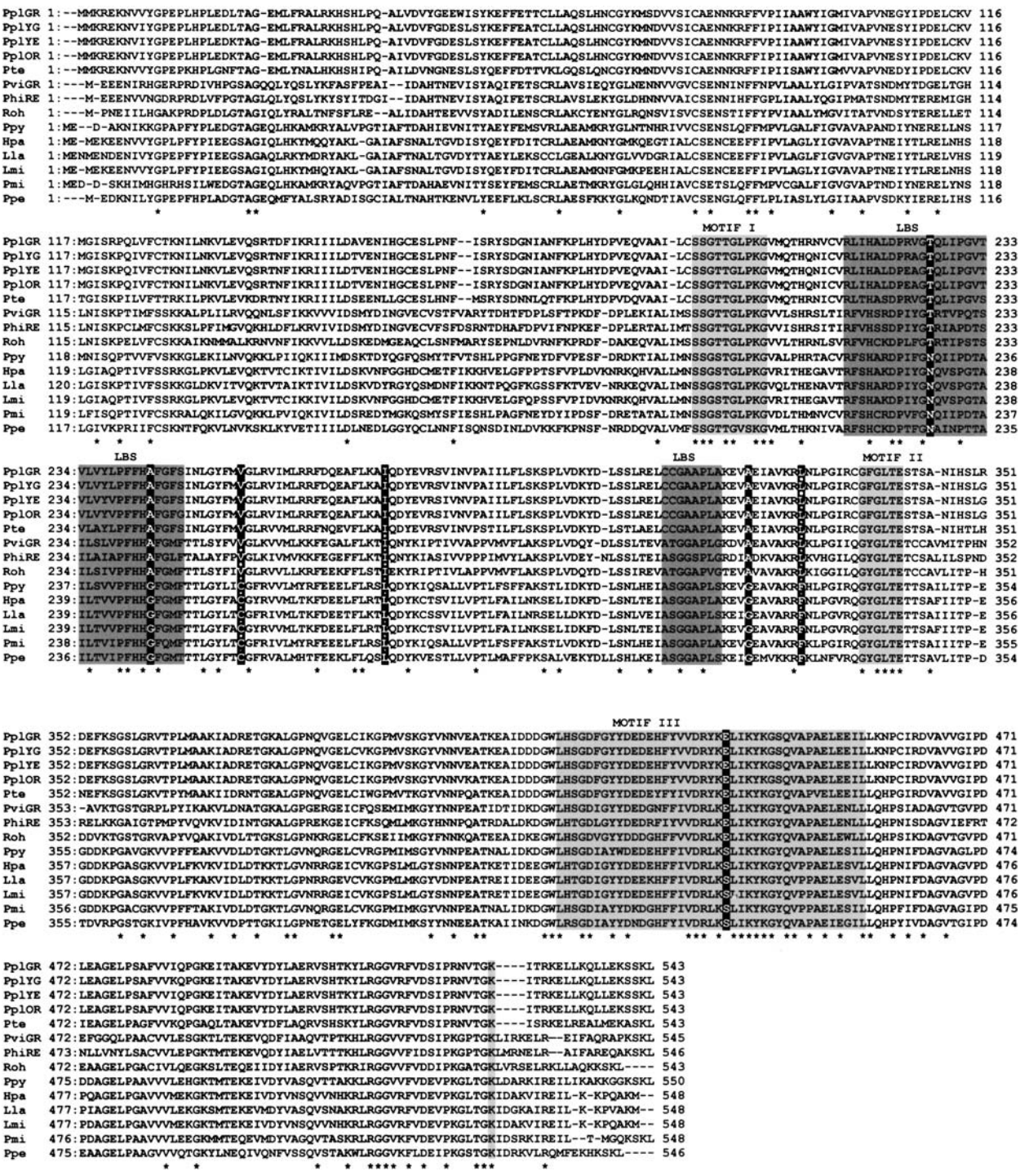


Figure 4. Multialignment of beetle luciferases primary structures: Ppl, *Pyrophorus plagiophthalamus* (light-emitting isoenzymes: GR, green; YG, yellow-green; YE, yellow; OR, orange). Pte, *Pyrearinus termitilluminans* larval click-beetle luciferase; PviGR, *Phrixothrix vivianí* green light-emitting luciferase; PhiRE, *Phrixothrix hirtus* red light-emitting luciferase; Roh, *Ragophthalmus ohbai* larval luciferase; Ppy, *Photinus pyralis* luciferase; Hpa, *Hotaria parvula* luciferase; Lla, *Luciola lateralis* luciferase; Lmi, *Luciola mingrelica* luciferase; Pmi, *Pyrocoelia miyako* luciferase; Ppe, *Photuris pennsilvanica* luciferase; * invariant residues; light shading, conserved motifs among adenylate-forming ligases; dark shading or LBS, putative luciferin-binding site; black shadow, conserved residues differing between pH-sensitive and pH-insensitive luciferases. The multialignment was generated using the Genetyx Mac program.

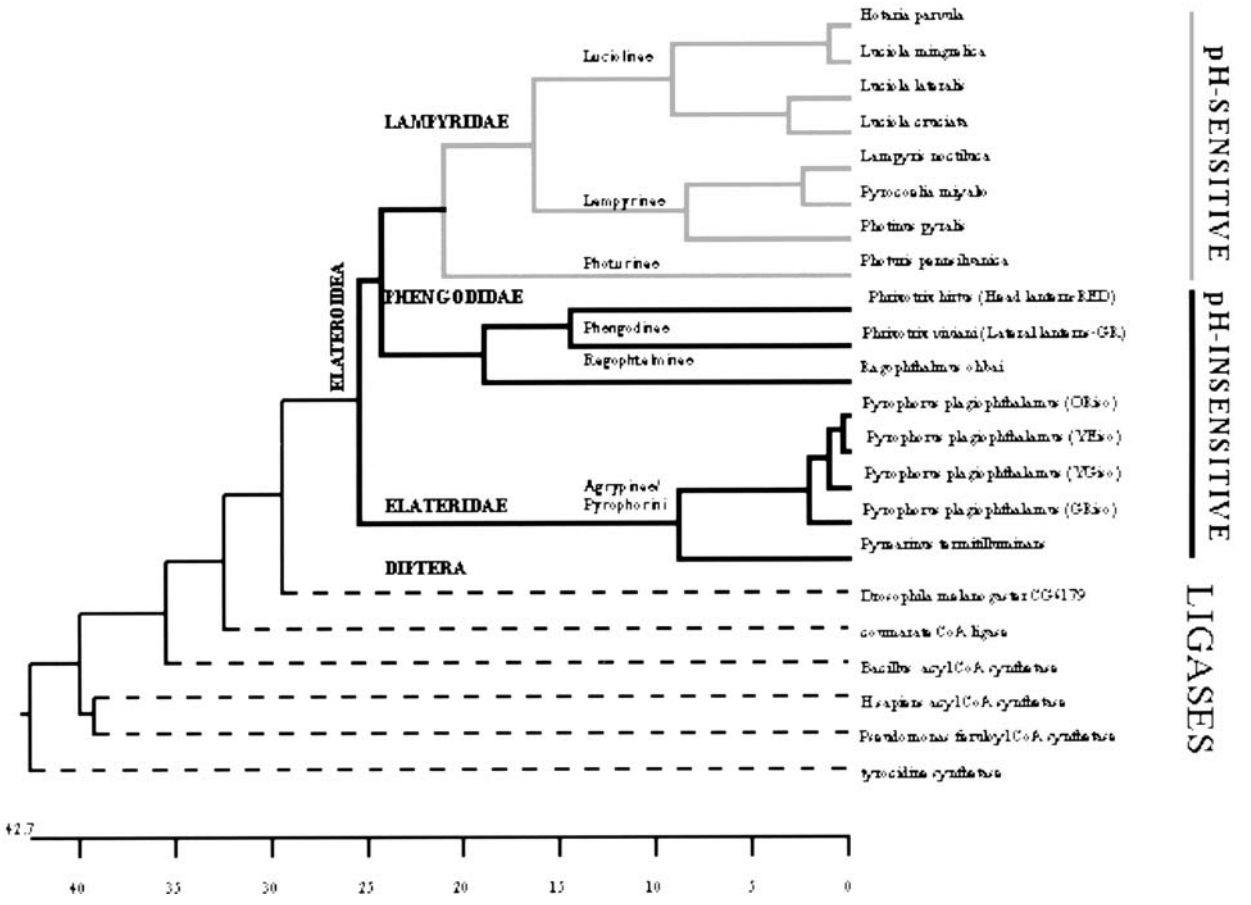


Figure 5. Phylogenetic tree of beetle luciferases and homologous ligases. Filled lines are used for luciferases assuming a constant rate of substitutions (a molecular clock), whereas dashed lines are used when a molecular clock cannot be assumed. The phylogenetic tree was generated by the neighbor-joining method using a multialignment generated by the ClustalW algorithm.

includes peptidyl synthetases found in bacteria and fungi, which catalyze the non-ribosomal adenylation of amino acids and the subsequent thioesterification to an enzyme-bound 4′-phosphopantetheine during the synthesis of cyclic peptides and antibiotics, and the acyl-CoA synthetases or ligases, which catalyze the adenylation of fatty acids and aromatic acids followed by the thioesterification to CoA during the metabolism of lipids and other biosynthetic pathways [20]. Beetle luciferases are more closely related to the latter group, which includes long-chain acyl-CoA synthetases, acetate CoA synthetases, coumarate: CoA ligases, and aromatic acid synthetases (fig. 5). The closest enzymes are coumarate: CoA synthetases, which are involved in phenylpropanoid metabolism during the synthesis of lignin and other biologically important compounds in higher plants [107]. Although aminoacyl-tRNA synthetases have no similarity with CoA ligases or peptide synthetases, they were recently found to catalyze the transfer of their aminoacyl group to CoA and other thiols, suggesting that they originated from ancestral forms of thioester-dependent synthetases functionally similar to the present-day non-ribosomal peptide synthetases [108].

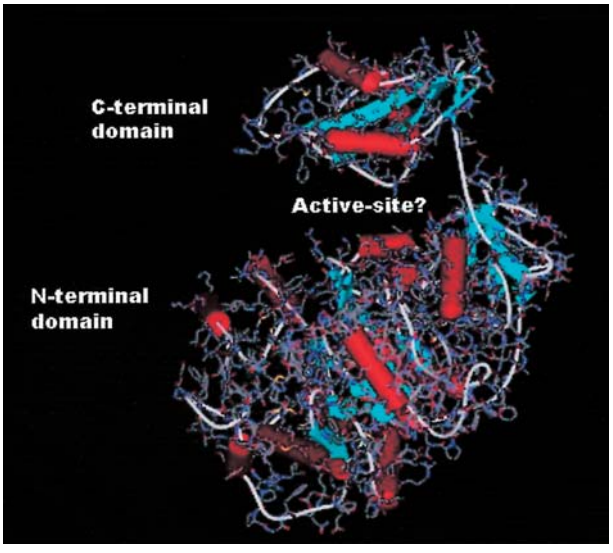


Figure 6. Three-dimensional model of *Photinus pyralis* firefly luciferase. The model was generated from the LC1.pdb template using the Lite-viewer program.

In principle, the ancestral ligase could catalyze the adenylation of luciferin, which was a natural or adventitious substrate, producing adenyl-luciferin [20]. Many ligases catalyze the adenylation of substrates other than their original one [109, 110]. The synthesis of adenyl-luciferin must have been the key step in the development of bioluminescence, because this compound is weakly chemiluminescent in basic aqueous medium [111]. Thus, a ligase able to adenylate luciferin would, in principle, produce weak luminescence. Indeed, we found ligases with luciferase-like activity in the fat body of non-luminescent larvae of *Tenebrio molitor* and other beetles [112]. These luciferase-like enzymes produce a low-level red chemiluminescence in the presence of beetle luciferin and ATP. Although the chemiluminescence quantum yield of such ligases is apparently too low to confer a selectable bioluminescent phenotype, such an enzyme could be paralogous, exemplifying what looks like a putative beetle protoluciferase. Unsurprisingly, an unidentified gene product with high similarity to beetle luciferases was found in the *Drosophila* gene bank (fig. 5). The next step toward the development of a new and efficient luciferase active site was the alignment of a basic residue in proximity to the luciferin C4 proton to increase the chemical yields of the oxygenase reaction and chemiexcitation and to enhance the hydrophobicity to increase the emission yield. Luciferases then coevolved with the anatomical structure of the photogenic organs to produce different luminescence patterns (glow or flashes) and bioluminescence colors (green to red) suitable for different photic environments and biological functions.

Structure-function in beetle luciferases

Studies to understand the relationship between structure and luciferase enzymatic functions have been undertaken for a long time. Two sulfhydryl groups in firefly luciferase were originally suggested to be important for catalysis in bioluminescence [113, 114], however, recent investigations show that no sulfhydryl group is essential for activity [115, 116]. The deletion of the last C-terminal 12 residues in firefly luciferase results in complete loss of activity [117], showing that the C-terminal domain is essential for activity. Recent spectroscopic studies using oxyluciferin suggest that the active site is more hydrophilic than originally thought [118, 119].

Three-dimensional structure and the active site

Recently, a great deal of information about the structure of firefly luciferase was gained through the crystallization of *P. pyralis* firefly luciferase without bound substrates (fig. 6) [120]. Its three-dimensional structure shows a unique topology with a main N-terminal (aa 1–436) domain connected by a flexible loop (aa 436–440) to a smaller C-terminal domain (aa 440–550; fig. 6). The main N-terminal domain consists of a compact domain containing a distorted antiparallel β barrel and two β sheets flanked by α helices forming an $\alpha\beta\alpha\beta\alpha$ five-layered structure. The C-terminal consists of an α + β structure. The surfaces of the N- and C-terminal domain facing each other form a cleft where many conserved residues are found and which is thought to be the active site. During the bioluminescence reaction, firefly luciferases undergo considerable conformational change and the N- and C-terminal domains are likely to come close enough to sandwich the substrates [120].

The structure of the ATP-binding site in beetle luciferases was deduced by comparison of the primary structures of luciferases and acyl-CoA ligases. In peptide synthetases, three putative contact sites of the adenine moiety of ATP were identified through photoaffinity labeling using 2-azidoadenosine triphosphate: these involve the peptides corresponding to G399–A409, W425–R437, and L505–L517 in firefly luciferase [121]. Acyl-CoA ligases show three main highly conserved regions which could be involved with the adenylation function: motif I [197SSG(S/T)TGLPKGV209], motif II [340YGLTE 344], and motif III [419L–457L] [122, 123] (fig. 4). Motif I consists of a flexible loop, originally associated with ATP binding. In the motif I of several ligases, the residues corresponding to G200, G203, and K206 of firefly luciferase were shown to affect dramatically the rate of ATP/[P^{32}]PPi exchange, suggesting their possible involvement in coordinating pyrophosphate release during the adenylation reaction [122, 124]. In firefly luciferase, the substitution of several of these residues could be tolerated without dramatic losses of activity [125]. However, the substitution S198T had an altered optimum pH and increased affinity for ATP, suggesting the involvement of this residue in ATP binding [125]. Of note, in the three-dimensional structure of firefly luciferase, S198 is hydrogen bonded with E344 of motif II. Other conserved residues among adenylate-forming enzymes, including the residues corresponding to G315 and R337 of firefly luciferases, were also shown to affect the rate of ATP/[P^{32}]PPi exchange and the K_m for ATP, suggesting their possible involvement in the adenylation reaction [123–126]. In firefly luciferase the invariant residue K529 was shown to be very important for the adenylation [127]. According to a proposed active-site model, this residue is located near the luciferin carboxylate and the ATP phosphates and could be involved in the stabilization of the pentavalent transition state of the adenylation step.

Much less is known about the luciferin-binding site and the structural determinants of the oxygenase function. The motif 244HHGF247 was shown to be in close proximity to luciferin through photoinactivation studies using a lu-

ciferin analog [128]. Some insights were also gained through the crystallization of firefly luciferase in the presence of bromoform, a competitive inhibitor of luciferin [129], and on the relative orientation of AMP and phenylalanine in the three-dimensional structure of the phenylalanine-activating domain of gramidicin-S-synthetase [130]. One of the two binding sites for bromoform was suggested to constitute the luciferin-binding site. Based on these studies, two active-site models were proposed [130–132]. Although there is general good agreement between these models, important differences are evident. According to these active-site models, the residues R218, H245–F247, A313–G320, G339–I351, and K529 make the binding site for luciferin [131, 132]. The residues A313, A348, I351, and F247 form the hydrophobic surrounding of luciferin, the latter residue probably interacting by π-stacking with the luciferin aromatic rings [131, 132]. However, whereas in one of the models the guanidinium ion of the residue R218 was suggested to interact with luciferin phenolate [131], in the other model, R337 was closer to the phenolate to make such interaction [132]. According to the first model [131], the hydroxyl group of T343 is close to the ATP phosphates and the luciferin carboxylate, and may assist in their stabilization and partial reactions [124]. However, since the three-dimensional structure in the presence of the substrates is still unknown, these models must be considered with caution. In particular, predicting the exact orientation of luciferin based on the coordinator of different molecules such as bromoform and phenylalanine and an enzyme that shares only 16% identity with firefly luciferase is difficult.

The structural origin of bioluminescence colors

Among all luciferases, beetle luciferases are the most remarkable because they are the only luciferases that can produce a wide range of colors, from green to red [20]. Furthermore, only *Phrixothrix* and a few other railroad worm luciferases can naturally produce red light. Most other bioluminescent organisms produce different colors through the use of accessory fluorescent proteins or inner filters [2]. In beetle luciferases, color differences are essentially determined by the primary structure, which in turn affects the active-site environment around the emitter. How such differences affect the emission spectra has been the subject of much debate.

Three mechanisms have been proposed to govern bioluminescence colors [20] (fig. 7): (i) the active-site polarity [118]; (ii) the presence of basic residues assisting excited oxyluciferin tautomerization [133, 134], and (iii) the geometry of the active site governing the angle of rotation of oxyluciferin thiazoline rings along the C2-C2′ bond [135].

The polarity hypothesis was based mostly on the observation that the fluorescence spectrum of luciferin and its analogs, like that of other fluorescent compounds, is affected by the nature of the solvent [64, 65, 119]. The fluorescence spectrum in solvents of distinct dielectric constants can be shifted as much as 40 nm, suggesting that similar differences in the luciferase active-site microenvironment can equally affect the emission spectrum of bioluminescence. In non-polar solvents, the spectrum is shifted toward blue, whereas in more polar solvents it is shifted toward red. However, polarity fluctuations around

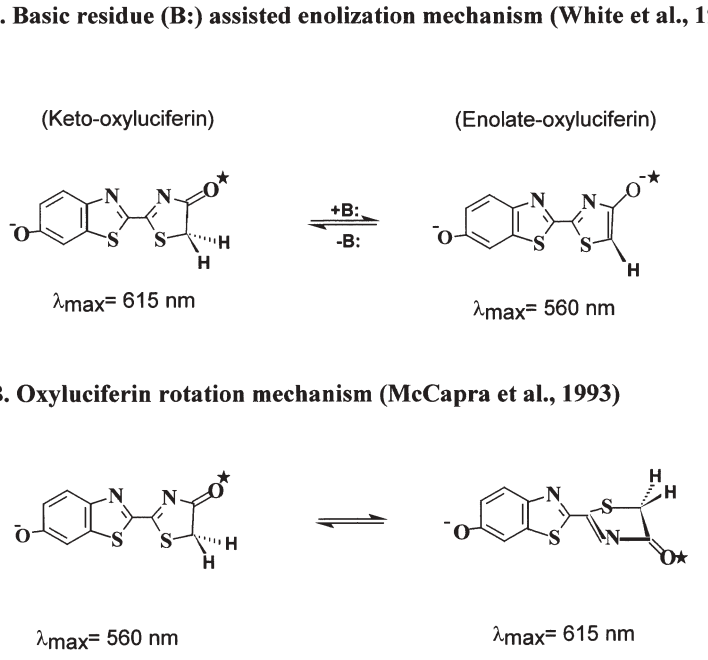


Figure 7. Mechanisms of bioluminescence color determination.

a single emitter within the active site are unlikely to account for larger-scale differences such as the green to red bioluminescence spectra observed in railroad worm luciferases.

The tautomerization hypothesis (fig. 7) was originally proposed to explain the green-red shift observed in firefly luciferases upon decreasing pH. This theory was supported by spectroscopic data obtained with oxyluciferin and modified analogs [133, 136–139]. According to this hypothesis, the natural emitter of yellow-green light would be the phenolate in either the enol or enolate forms, whereas the red-light emitter would be the phenolate in the ketonic form [139]. The main lines of evidence supporting such an assumption were based on chemiluminescence studies of oxyluciferin analogs in aprotic solvents: (i) the 5,5-dimethyloxyluciferin analog, in which enolization is blocked by the two methyl groups, produce only red light [139] and (ii) the chemiluminescence of adenyl-luciferin and other esters is red. The presence of basic residues in the active site of firefly luciferase would assist oxyluciferin tautomerization [134]. Although chemical-modification studies originally suggested that histidine or cysteine residues could be the basic residues assisting oxyluciferin tauromerization [114, 134], no such residues were ever found to be essential for such a function. Recently, a study with 5,5-dimethyloxyluciferin-adenylate as a substrate for luciferases showed that the ketonic form can emit both green and red light depending on the enzyme, making the tautomerization hypothesis unlikely as a basis to explain green-red color shifts [140].

Finally, the third hypothesis (fig. 7) was proposed to explain the continuous range of colors observed in many beetle luciferases. According to this hypothesis, the excited oxyluciferin is in the lowest energy level in the 90° twisted conformer, emitting red light, whereas the planar conformer has the highest energy emitting green light. Intermediate colors would be obtained between these two extreme conformations. Although demonstrating that excited-state rotation of the thiazoline rings affects bioluminescence spectra has not yet been possible, the recent finding that the keto form of oxyluciferin can emit both green and red light favor this hypothesis [140]. However, a pitfall of this hypothesis is that it cannot explain the observed yellow-green fluorescence of oxyluciferin in aqueous solution, where free rotation of the thiazoline rings to the lowest energy state is expected to occur.

Many insights into the structural nature of bioluminescence color determination have been gained through the construction of chimeric proteins using fragments of luciferases that produce different colors of light, and, more directly, through mutagenesis studies (table 3). In click beetle *P. plagiophthalamus* isoenzymes, which are 95–99% identical, bioluminescence colors ranging from green to orange are determined by the region between residues 220–247, in particular the combined effect of the substitutions V224A, R223E, and S247G [141]. A good correlation between the substitution affecting the color and polarizability has been suggested for these isoenzymes [142]. In firefly luciferases, the region between residues 209–318 constitutes the main determinant of bioluminescence color between green and yellow [143]; however, many single substitutions along the region 200–452 dramatically affect the spectrum, resulting in red mutants (table 3) [144–147]. Many of these residues are concentrated in the β sheet B subdomain which is part of the active site (fig. 8). Other residues such as His433 and Pro452 are located far from the active site but may promote color shifts by long-range interactions with active-site residues [144]. The residues His245 and Thr343 were suggested to be critically important for bioluminescence color determination in firefly luciferases [148], though the latter was not found to be critical for railroad worm luciferases [V. R. Viviani, unpublished data]. Through the construction of chimeric luciferases using the green- and red-emitting luciferases of *Phrixothrix* railroad worms, we found that the region between residues 215 and 344 have a major effect on the bioluminescence color [149, 150] (fig. 8). Guanidine was found to induce considerable blue shifts from the red-emitting luciferase, but not from the green-emitting ones, suggesting that the red-emitting luciferase may lack some important arginine residue or that the active site might be less basic than in the green-emitting luciferases [149]. The invariant residue R215 (R218 in firefly luciferase) was shown to have an important role for green-light emission in *Phrixotrix* railroad worm [149] and firefly [151] luciferases but not in click beetle luciferases [150] (table 3). R215 was suggested to help to align oxyluciferin in a productive conformation for green-light emission [149, 151].

Beetle luciferases fall into two main groups, according to the sensitivity to pH of the bioluminescence spectra [152]: (i) the pH sensitive, which includes firefly luciferases, undergo a red shift at lower pHs, as well as higher temperatures and in the presence of heavy-metal cations [153] and (ii) the pH insensitive, which includes click beetle and railroad worm luciferases [152]. In pH-sensitive luciferases, many single substitutions result in red mutants that are pH insensitive. However, no single substitution in pH-insensitive luciferases resulted in real red mutants or made them pH sensitive [154]. The comparison of pH-sensitive with pH-insensitive luciferases showed a set of conserved residues that differs between these two groups of luciferases (fig. 4) and could be involved in the pH sensitivity [90]. Among them, the residues T226 in pH-insensitive luciferases and the corresponding N229 in pH-sensitive luciferases were found to be key residues involved in keeping a favorable active-site core for green-light emission [154]. Mutagenesis

Table 3. Mutants affecting the bioluminescence spectrum of beetle luciferases.

Luciferase/substitution*	λ_{max} (nm)		Reference
	pH 8	pH 6	
pH-sensitive			
Lampyridae (fireflies)			
Photinus pyralis	565	617	20
R218K/Q/A	608		151
H245F/A/Q/N/D	595–617	612–620	131
T343A	617	560–617	148
Luciola cruciata	562	609	144
S286N (S284)	607	614	144
G326S (G324)	609	611	144
H433Y (H431)	612	612	144
Luciola mingrelica	570		
S286F/K/Y/Q/L (S284)	608–621	611–623	146
Pyrocoelia miyako	550	605	143
N230S/T (N229)	605	605	154
G247A (G246)	550	559	155
Hotaria parvula	568		
H433Y (H431)	612		145
pH-insensitive			
Phengodidae (railroadworms)			
Phrixothrix viviani	548	548	90
R215S (R218)	585		149
T226N (N229)	574	578	154
A243G (G246)	553	553	155
Phrixothrix hirtus	623	623	90
R215S (R218)	617		149
T226N (N229)	611	611	154
A243G (G246)	621	614	155
Ragophthalmus ohbai	550	550	91
T226S/N/H/E/V/F (N229)	565–590	587	154
A243G (G246)	559	612	155
Elateridae			
Pyrophorus plagiophthalamus			
Green isoenzyme	546		87
V224A (F227)	560		87, 141
V224A/R223E/L238V	578		87, 141
V224A/R223E/L238V/S247G593			87, 141
Pyrearinus termitilluminans	536	536	88
R215S (R218)	536		149
T226N (N229)	546	541	154
A243G (G246)	547	548	155

* In parenthesis: corresponding residue in *Photinus pyralis* firefly luciferase.

studies and three-dimensional models of pH-insensitive luciferases suggest that T226 and R215 (N229 and R218 in firefly luciferase) may interact helping to keep an active-site conformation suitable for green-light emission [155]. The residue G247 in firefly luciferases (G246 in *P. pyralis* firefly luciferase), which is substituted by the bulkier A243 in pH-insensitive luciferases, affects the pH sensitivity probably by influencing the flexibility of active-site segments [155]. Originally, the pH sensitivity was explained on the basis of the protonation state of a putative basic residue assisting oxyluciferin tautomerization [133, 152]. However, recent evidence suggests that pH sensitivity results from a flexible active site stabilized by protonable basic residues [82] generating two emitting species depending on its conformation, whereas pH insensitivity is the result of a more rigid active site probably stabilized by hydrophobic interactions [154].

Therefore, although the current evidence suggests that the conformation and dynamic changes of the active site play an important role in bioluminescence color determination in different luciferases, how the conformation of the active site affects the bioluminescence spectra in different luciferases remains a fertile area of investigation.

Application of insect luciferases in biomedical research and biotechnology

During the past 50 years, the firefly luciferase has been extensively used for sensitive applications involving measurement of ATP, such as to monitor biomass, to analyze biological fluid microbial contamination, to assess cell viability, and to assay enzymes involving ATP generation or degradation [12]. Many of these techniques are currently used in industry [156]. After the cloning of firefly luciferase cDNA, a new set of applications using the firefly luciferase gene as one of the most sensitive reporters of gene expression in living cells and tissues appeared. These applications include the analysis of transcriptional activity of promoters in different cell lines, analysis of the efficiency of transformation or transfection, and study of pathogen dissemination in vegetal and animal tissues [157, 158]. The use of luciferase genes associated with the development of very sensitive CCD-cameras allows one to image living cells, animals, and plants during normal and pathological conditions, and during embryonic development, and to study patterns of circadian expression, among other applications [159]. Examples of biomedical applications include the use of luciferase genes to study the progression and regression of viral and bacterial diseases such as HIV and mycoplasmas [160, 161], and the non-invasive assessment of therapeutic gene expression and of tumor proliferation and regression in animal models [162, 163]. These methodologies are helping to develop faster and more efficient methods for drug screening in the pharmaceutical industry. Luciferases are also being successfully used as biosensors for environmental pollutants such as arsenite, mercury, lead, phenols, agrochemicals, and xenoestrogens that may act as endocrine disruptors to humans and wildlife [157, 164]. To date, most applications have used mainly firefly luciferases, which emit in the yellow-green region of the spectrum. However, demand is increasing for luciferases which emit different colors, in particular red-emitting luciferases for uses in mammalian cells and other tissues rich in pigments such as hemoglobin that absorb shorter wavelengths [165]. Certainly, new insect luciferases that naturally emit in the red and blue ends of the spectrum and luciferases with different biochemistries offer great potential to extend the range of applications in the field of biotechnology and biomedicine.

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