Review

The Secret of the Biochemical Reaction in the Abdomen of the Beetle: Bioluminescence of the Firefly

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Abstract:
Fireflies (*Lampyridae*) belong to a family of beetles that live widely in the humid tropical and subtropical regions of the world. They are best known for using a light organ in their abdomen to produce light, which they use to communicate with each other. Species can be distinguished by the pattern of light flickering that identifies members of a species. All larvae also glow to signal to predators that they are inedible.
The light emitted by fireflies is produced by converting chemical energy into light. In this phenomenon, called bioluminescence, the substance luciferin reacts with oxygen in the presence of the enzyme luciferase. In addition, bioluminescence is difficult to study because all animals stop glowing after they are captured. Bioluminescence is a very efficient process, converting up to 90% of energy into light, also called cold light. The complex process of bioluminescence is still not fully understood, so scientists are using interdisciplinary methods (from theoretical to experimental approaches) to study the problem.

Keywords: Firefly, bioluminescence, luciferin, luciferase, mechanism, enzyme

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1. Introduction

The term *luminescence* comes from a Latin root (*lumen*: light). It was first introduced in 1888 by the German physicist and historian of science Eilhard Wiedemann as luminescence for all light phenomena that are not solely due to an increase in temperature, i.e., incandescence (Valeur et al., 2011). Before discussing the historical development of the term luminescence, it should be noted that today's definition of luminescence is: "spontaneous emission of radiation by an electronically excited species (or a vibrationally excited species) that is not in thermal equilibrium with its environment" (Valeur et al., 2011).

The different types of luminescence are classified according to the type of excitation. Photoluminescence is the emission of light that results "from direct photoexcitation of the emitting species". Fluorescence, phosphorescence, and delayed fluorescence are known forms of photoluminescence. There are other types of luminescence that differ by the type of excitation: chemiluminescence, bioluminescence, electroluminescence, cathodoluminescence, radioluminescence, sonoluminescence, thermoluminescence, triboluminescence (Valeur et al., 2011; Jeran et al., 2020). This article is about bioluminescence and explains the general mechanisms of bioluminescence in the abdomen of beetles (fireflies).

Many nocturnal fireflies use discrete pulses of bioluminescence to find mates (Lewis et al., 2008; National Geographic, 2010). In some groups (e.g., the North American common eastern firefly, *Photinus pyralis*), it is notable that both sexes use precisely timed flash signals to encode information about species and sex (**Figure 1**). Primarily, males signal this by emitting flashing signals during flight. When the female responds with a flashing signal of her own, a dialogue ensues between the two as they court each other with flashing signals. Females of this species usually light up in response to the male's signals and are often immobile, although capable of flight. This courting continues until the male comes into contact with the female. The bioluminescence of fireflies is thought to date back to an early ancestor of cantharids and served as a warning sign of inedible larvae. The original significance of bioluminescence in fireflies is therefore thought to be as a warning to larvae of potential predators, but later the system was also used for courtship (Lewis et al., 2008).

Figure 1. A biochemical reaction in the beetle's abdomen; production of bioluminescence process (Credit: Art Farmer, permission under Creative Commons license) (Tancig, 2019).

2. Development of bioluminescence in nature

The beginnings of bioluminescence research in beetles can be traced to the French physiologist Raphael Dubois, who in 1885 produced a luminescent mixture by applying cold water to the abdomen of a beetle of the genus *Elateridae* (Fraga, 2008). The light emitted by the cold water faded quickly, while he could not achieve such a result with the hot water. Dubois then noticed that the resulting solutions contained different constituents. In the cold mixture, the components were intact, but in the hot solution, the heat destroyed one of the thermolabile components that are critical to light generation. Dubois called the component consumed in the light reaction luciferin and the component destroyed by the heat in the second mixture luciferase. These definitions still designate the substrate (luciferin) and the enzyme (luciferase) responsible for light emission (Fraga, 2008).
Dubois research was continued by the American scientist Newton Harvey, who studied the relationship between luciferins and luciferases. A common aspect of all systems was their dependence on oxygen, which was first discovered by Robert Boyle in the 18th century. Using the "evacuated bell jar", he succeeded in quenching the luminescence on the rotting wood and flesh of the bacteria by depriving them of air. So, in addition to luciferin and luciferase, oxygen is also needed to trigger bioluminescence. At Princeton University, William McElroy began a lifelong study of firefly bioluminescence. Light production in fireflies occurs in the light organ, which contains specialised photocytes located between two cell types. These are located between two cell types, one of which is thin and external, while the other is internal and filled with uric acid crystals that reflect the light emitted by the photocytes. McElroy confirmed the discoveries of Dubois and Harvey and investigated the conditions that affect the production of bioluminescence, such as temperature and pH, but interpretation of the results was limited. However, they discovered that bioluminescence depends on four factors. These are: oxygen, enzyme luciferase, substrate luciferin (LH_2), and ATP-Mg^{2+} (Fraga, 2008).

3. Luciferin and luciferase

Luciferase enzymes are oxidative enzymes and belong to the class of oxidoreductase enzymes. The accepted name for the *P. pyralis* luciferase enzyme is *Photinus*-luciferin-4-monooxygenase (decarboxylating, ATP-hydrolyzing), but it is commonly referred to as firefly luciferase or simply as luciferase (Luc). The firefly luciferin substrate (LH_2) is (*S*)-2-(6′-hydroxy-2′-benzothiazolyl)-2-thiazoline-4-carboxylic acid (**Figure 2**). Luciferin occurs in the optical isomers *D*- and *L*-, but only the *D*-isomer reacts promptly via the bioluminescence pathway (Leitão et al., 2010). The general biochemical reaction catalysed by Luc is shown **Equation (1),**

$$D\text{-}LH_2 + ATP + O_2 \rightarrow AMP + PPi + CO_2 + oxyluciferin + h\nu \quad . \qquad (1)$$

The *D*-isomer of the luciferin substrate (*D*-LH_2) reacts with a molecule of adenosine 5′-triphosphate (ATP) in the presence of oxygen (O_2) to produce the light-emitting oxyluciferin, adenosine 5′-monophosphate (AMP), and inorganic pyrophosphate (PPi).

The overall reaction presented in **Equation (1)** proceeds in two successive steps. The first step (**Equation (2))** is an adenylation in which an enzyme-linked intermediate *D*-luciferyl adenylate (*D*-LH_2- AMP) is formed by reaction of *D*-LH_2 with ATP.

$$luciferase + D\text{-}LH_2 + ATP\text{-}Mg^{2+} \rightleftharpoons luciferase \times D\text{-}LH_2\text{-}AMP + PPi\text{-}Mg^{2+} \qquad (2)$$

The second step (**Equation (3**)) consists of oxidation and decarboxylation, in which the intermediate reacts with oxygen to form the product oxyluciferin, which emits light.

$$luciferase \times D\text{-}LH_2\text{-}AMP + O_2 \rightleftharpoons luciferase + AMP + CO_2 + oxyluciferin + h\nu \qquad (3)$$

As we could see, Luc could catalyse the reactions. In the presence of ATP, <u>*D*-</u>LH_2 is activated to *D*-LH_2-AMP, which is oxidized via a series of intermediates by O_2 to oxyluciferin,

CO_2, and AMP. In a side reaction, LH_2-AMP is oxidized by O_2 to *L*-AMP and hydrogen peroxide. *L*-AMP can be cleaved by PPi to give dehydroluciferin (L) and ATP.

Luciferase is a generic term for any enzyme that catalyzes a reaction that produces visible light. Light emission results from the formation of a product or intermediate in an electronically excited state; return to the ground state occurs by emission of a photon of light. Luciferases are so diverse that they catalyze many different reactions with very different substrates. What they all have in common is the involvement of oxygen. Luciferases are more distinct in comparison with proteases, all of which perform hydrolytic chemistry on peptide bonds. All luciferases emit light, but in very different ways. Therefore, luciferases from different organisms probably evolved independently and do not trace back to a common precursor enzyme. Bacterial luciferase, the first luciferase to be cloned and structurally characterized, is a flavin monooxygenase that uses flavin mononucleotide (FMN) to activate molecular oxygen, producing flavin C4a peroxide. Reaction of the peroxide with an aliphatic aldehyde substrate eventually yields the carboxylic acid and flavin-C4a hydroxide in the first excited singlet state. Light emission, loss of C4a hydroxide, and dissociation of FMN return the enzyme to its initial state. Firefly luciferase, on the other hand, catalyzes an oxidative reaction involving ATP, firefly luciferin, and molecular oxygen, producing the electronically excited species oxyluciferin. This excited species emits visible light, which the firefly uses for its reproductive behavior. Firefly luciferase was one of the first enzymes to be studied in detail (Baldwin, 1996).

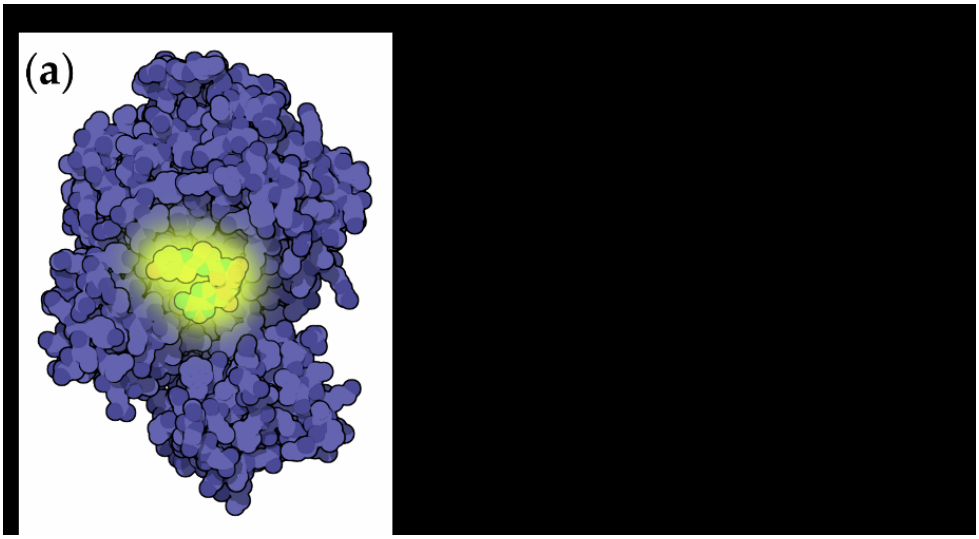

Figure 2. (**a**) Firefly luciferase with the chromophore in yellow (permission under Creative Commons license) (Goodsell, 2006). (**b**) Skeletal structure of firefly luciferin: (4*S*)-2-(6-hydroxy-1,3-benzothiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid. The visible emission occurs when oxyluciferin transitions from the excited state to the ground state. The colour of the discharge is different even if the luciferin used is the same; this may be due to changes in pH value or differences in the structure of the luciferase involved (Hewitt et al., 2019).

The fold in which polypeptide enzymes form is unique in that it consists of two domains, a large *N*-terminal domain (containing residues 4-436) and a *C*-terminal domain (containing residues 440-544). The larger, lower domain consists of a β-barrel and two β-sheets interleaved with α-helices forming a five-layered αβαβαβ structure. The upper *C*-terminal domain, on the other hand, consists of five β-strands and three α-helices and is folded into a compact structure that is connected to the lower *N*-terminal domain by a disordered loop connecting residues 435 and 441. Between the two domains, there are also three disordered loops connecting residues 539-529 (in the *C*-terminal domain), 198-204, and 355-359 (in the *N*-terminal domain), but these are not visible in electron density. The regions with the greatest sequence conservation are most likely involved in the catalytic mechanism of the

enzyme (Baldwin, 1996). Based on previous analyzes, the active site is thought to consist of residues on the surfaces of both domains, and the two domains combine to form an active site after substrate binding. For the firefly bioluminescence reaction to proceed with high yield, water must be excluded from the active site (Baldwin, 1996).
One fascinating aspect of firefly bioluminescence is the colour of the emitted light. *Luciola mingrelica* (found in southern parts of Russia) and *Luciola cruciata* (native to the Japanese region) firefly luciferases resemble *Photinus pyralis* luciferase (from North America) with a maximum light intensity (I_{max}) of 562–570 nm, but *Luciola lateralis* (native to the Japanese region) firefly luciferase catalyzes such a reaction and emits green light with an I_{max} of 552 nm (Baldwin, 1996). Interestingly, the luciferase from the American firefly *Photinus pyralis* crystallizes in active form at low ionic strength, whereas the *Luciola firefly* luciferase is inactivated at the same conditions. They investigated both the presence and absence of proton acceptors using fluorescence emission spectra and concluded that the colored bioluminescence depends on the arrangement of the luciferase molecules. The hypothesis is that the red colour comes from the keto anion form of the product molecule and the yellow-green light comes from the enol anion form of the product molecule. This hypothesis is consistent with the emission of red light in the bioluminescence reaction at lower pH values, which led the researchers to believe that the different light colours in fireflies were due to spectral mixing of these two products. However, they later isolated luciferases from different organs of the beetle *Pyrophorus plagiophthalamus*, where they found that each emitted a different colour of bioluminescence and each spectrum showed a single peak, rather than a superposition of two or more spectra. Then they selected four luciferases from the aforementioned beetle with different colours of bioluminescence, cloned them, and sequenced their *c*DNA. The amino acid sequences of the selected luciferases were 95-99% identical, and less than two or three amino acid changes were required to produce spectral shifts of up to 50 nm at I_{max}. These isolated luciferases emit light with sharp emission spectra with spectral maxima at 546 nm (green colour), 560 nm (yellow-green colour), 578 nm (yellow colour), and 593 nm (orange colour) (Baldwin, 1996). This finding suggests that the light emission from each enzyme originates from a single molecular species in the surrounding enzymes and that the resulting colour differences are due to the different microenvironments of the enzyme complexes and oxyluciferin. A change in the colours of bioluminescence may also be caused by the tertiary structure of the luciferin molecule. In a similar study, the *c*DNA of the firefly *Luciola cruciata* was mutagenized, and five different mutants with different colours of bioluminescence were obtained. The isolated colours ranged from green to red, and the mutations consisted of single amino acid changes. The sequence changes of the mutant luciferase from the firefly *Luciola cruciata* were compared with enzymes isolated from the beetle *Pyrophorus plagiophthalamus* (Jamican click beetle (Stolz et al., 2003), and no common amino acid sequence affecting light colour was found. This suggests that bioluminescence may be affected by the overall tertiary structure of the enzyme (Baldwin, 1996).

4. Conclusion

Fireflies, which provide light in the ecosystem, have also impressed scientists. The mysterious "cold" light emission will always stimulate curious minds, and the colour issues of bioluminescence and the details of the enzyme-catalyzed reaction could be clarified through interdisciplinary approaches. Exploration of the general mechanisms will have important implications for the development of applications, especially in medicine.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Baldwin TO. Firefly luciferase: The structure is known, but the mystery remains. Structure. 1996; 4: 223-228. DOI: https://doi.org/10.1016/S0969-2126(96)00026-3
2. Fraga H. Firefly luminescence: A historical perspective and recent developments. Photochem Photobiol Sci. 2008; 7: 146-158. DOI: https://doi.org/10.1039/B719181B
3. Goodsell DS. Molecule of the month: Luciferase. 2006. Accessed 5.8.2023. Available from https://pdb101.rcsb.org/motm/78. DOI: http://doi.org/10.2210/rcsb_pdb/mom_2006_6
4. Hewitt L, Lacey E, Layton C, White E. Luciferin – The glowing group of molecules responsible for bioluminescence. Molecule of the month. – November 2019. 2019; 1-4. DOI: https://doi.org/10.6084/m9.figshare.9988682.v1
5. Jeran M, Nemec V, Drab M. Physical approach to the characteristics of luminol chemiluminiscence reaction in water. In: Kralj-Iglič V, editor. Socratic lectures: 3rd International Minisymposium (Peer reviewed proceedings). Ljubljana, Slovenia, University of Ljubljana, Faculty of health sciences. 2020; pp. 68-77. Available from https://www.zf.uni-lj.si/images/stories/datoteke/Zalozba/Sokratska_2020.pdf
6. Leitão JMM, Esteves da Silva JCG. Firefly luciferase inhibition. J. Photochem Photobiol B: Biology. 2010; 101: 1-18. DOI: https://doi.org/10.1016/j.jphotobiol.2010.06.015
7. Lewis SM, Cratsley CK. Flash signal evolution, mate choice, and predation in fireflies. Annu Rev Entomol. 2008; 53:293-321. DOI: https://doi.org/10.1146/annurev.ento.53.103106.093346
8. National Geographic (2010), Fireflies. Accessed 5.8.2023. Available from https://www.nationalgeographic.com/animals/invertebrates/facts/fireflies
9. Stolz U, Velez S, Wood KV, Wood M, Feder JL. Darwinian natural selection for orange bioluminescent color in a Jamaican click beetle. Proc Natl Acad Sci USA. 2003; 100: 14955-14959. DOI: https://doi.org/10.1073/pnas.2432563100
10. Tancig M. Fireflies – Nature's Fireworks. University of Florida, IFAS Extension. 2019. Accessed 7.8.2023. Available from https://nwdistrict.ifas.ufl.edu/hort/2019/07/11/fireflies-natures-fireworks/
11. Valeur B, Berberan-Santos MN. A brief history of fluorescence and phosphorescence before the emergence of quantum theory. 2011; 88:731-738. DOI: https://doi.org/10.1021/ed100182h