Invited Review

Perspectives on Bioluminescence Mechanisms†

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Received 29 June 2016, accepted 24 August 2016, DOI: 10.1111/php.12650

ABSTRACT

The molecular mechanisms of the bioluminescence systems of the firefly, bacteria and those utilizing imidazopyrazinone luciferins such as coelenterazine are gradually being uncovered using modern biological methods such as dynamic (ns-ps) fluorescence spectroscopy, NMR, X-ray crystallography and computational chemistry. The chemical structures of all reactants are well defined, and the spatial structures of the luciferases are providing important insight into interactions within the active cavity. It is generally accepted that the firefly and coelenterazine systems, although proceeding by different chemistries, both generate a dioxetanone high-energy species that undergoes decarboxylation to form directly the product in its S0 state, the bioluminescence emitter. More work is still needed to establish the structure of the products completely. In spite of the bacterial system receiving the most research attention, the chemical pathway for excitation remains mysterious except that it is clearly not by a decarboxylation. Both the coelenterazine and bacterial systems have in common of being able to employ “antenna proteins,” lumazine protein and the green-fluorescent protein, for tuning the color of the bioluminescence. Spatial structure information has been most valuable in informing the mechanism of the Ca2+-regulated photoproteins and the antenna protein interactions.

BACKGROUND

In the beginning, there was just Mythology. A commonly held belief is that many stories from ancient times probably originated from encounters with natural phenomena. Reference to fiery sea monsters, for example, is found in many ancient myths almost certainly observations of bioluminescence of marine organisms, clearly in one case resulting from the disturbance of seawater by breaching whales. For example, in the Talmud:

“The body of the Leviathan, especially his eyes, possesses great illuminating power”. This was the opinion of Rabbi Eliezer, who, in the course of a voyage in company with Rabbi Joshua, explained to the latter, when frightened by the sudden appearance of a brilliant light, that it probably proceeded from the eyes of the Leviathan. He referred his companion to the words of Job xli. 18: ‘By his neesings a light doth shine, and his eyes are like the eyelids of the morning’. (B. B. l.c.). http://en.wikipedia.org/wiki/Leviathan

Bioluminescence is a very old science, the first written systematic observations and classification being from Aristotle (384-322 BCE) and Pliny the Elder (23-79 CE). A monumental account of the history can be found in the classic book by Harvey (1) (1887–1959), which should be required reading for all students for entry into this subject. My intent in this review is not to be comprehensive, but to highlight what I might call “punctuated points” in investigations into bioluminescence mechanisms, discoveries that have advanced understanding of this field into the modern era of the Science of Bioluminescence. For a first example, in the 17th century, Boyle and Hooke with the newly invented air pump showed that bioluminescence depended on the presence of air, now known to be the requirement for oxygen. In 1875 in Paris, Raphaël Dubois reported the first in vitro demonstration of bioluminescence. He made two extracts of the bioluminescent clam Pholas, one using hot water and another with cold water. After the light from the cold-water extract dimmed and after cooling the hot-water extract, mixing the two resulted in a bright light emission. The same was done with extracts from the light organ of the Jamaican click beetle. Dubois gave the name “luciferine” to the hot-water extract and the cold-water extract “luciferase” considering it to have enzyme properties. The definition of “luciferin” has been updated since. What is now called firefly or beetle luciferin is not stable in hot water so presumably, the beetle light organ extract in the hot water contained the cofactor ATP now known to be required for the bioluminescence of beetles, and the cold extract was both firefly luciferin and the firefly luciferase. Pholas luciferin, from the bioluminescent clam Pholas dactylus, is a relative of the well-known marine luciferin coelenterazine and is bound to a large protein pholasin, which is relatively stable in hot water. The structure of Pholas luciferin has only recently been identified (2,3).

During the 19th century, improvement in optics led to higher precision in spectroscopic measurements, and by 1900, it enabled Max Planck (1858–1947) to formulate an equation to fit more accurately the spectral emission from a blackbody, which then led to Albert Einstein’s (1879–1955) “invention” of the quantum, later renamed the “photon.” In 1909, the pioneering spectroscopist William Coblentz (1863–1972) investigated the properties of the light in bioluminescence. At that time, the belief persisted that any emission of light must always be accompanied by heat according to Planck’s equation for the blackbody. The question
was where does the bioluminescence energy go? Coblentz used a prism spectrometer with photographic detection and importantly calibrated the spectral sensitivity of detection to absolute photons by thermoelectric methods with reference to the blackbody. Coblentz rigorously excluded any production of heat, that is, emission in the infrared at wavelength longer that 0.7 microns (700 nm) (4). It was already known that the bioluminescence of different species of fireflies had different colors, some in the green and others more yellow-orange. He demonstrated that the spectral distribution of different firefly species in fact differed quantitatively, explaining the visual color variation. The spectral maxima Coblentz published are close to those determined fifty years later using modern instrumentation (5).

Both from bioluminescence and molecular fluorescence, the broadband spectra observed were soon accoutned for using the new quantum theory. Although Coblentz had shown a monomodal spectral envelope in firefly bioluminescence, in 1937 Eymers and van Schouwenburg (6) reported that the bioluminescence from a specimen of the ostracod or “sea-firefly” *Cypridina* was bimodal (Fig. 1) and analyzed it as two Gaussian distributions based on Einstein’s theory of oscillator strengths of radiative transitions in a diatomic. In 1950, Spruit-van der Burg (7) showed that the absolute spectra of bacterial bioluminescence were monomodal but also differed according to species, with a range of spectral maxima 472–496 nm.

![Figure 1.](image)

The invention <75 years ago of the phototube, and then the photomultiplier with even greater photon detectivity, enabled more quantitative measurements of bioluminescence. More recently and to be described in this review, the methods of structural biology have given great insight into bioluminescence mechanisms. Dynamic processes have also been revealed using laser spectroscopy. This review will cover the role of advances in technology which necessarily has been limited to study of those systems available in “chemical” as distinct from kinetics or enzymatic quantities, made possible of course by the technology of molecular biology, beginning around 1980 with the production of the recombinant proteins, aequorin, GFP (green-fluorescent protein) and luciferases, bacterial, firefly and Renilla.

We might say that the “modern” era of the science of bioluminescence began just before 1950 with the discovery of the requirement of ATP for the firefly reaction *in vitro* (8,9). Apparently it must have been ATP in the hot-water extract of firefly tails that was mistakenly named the firefly luciferin. Within a few years, the genuine firefly luciferin was isolated separately from the cold-water extract along with an active protein firefly luciferase. In the early 1950s, a different protein was extracted from a culture of bioluminescent bacteria that did not have the properties of firefly luciferase. Instead, the requirements for bioluminescence from this “bacterial luciferase” were found to be FMNH$_2$ and a long-chain aliphatic aldehyde, the aldehyde now designated as the bacterial luciferin. In the following 10 years, the chemical structures were determined of firefly (beetle) luciferin and cypridinid luciferin from the ostracod *Cypridina* (syn. *Vargula* hilgendorfi).

The chemical structures of about 10 presently known luciferins are mostly unrelated (9, 10). In fact, this was already becoming evident after the first three luciferins were characterized, the aliphatic aldehyde in bacterial bioluminescence followed later by firefly luciferin from *Photinus pyralis*, and cypridinid luciferin from the ostracod in 1966. The bacterial bioluminescence is equally efficient with any long-chain aliphatic aldehyde, but tetradecanal is the one most abundant in the cells. The three distinct luciferin structures explained why cross-reactions among bioluminescence systems were uncommon, a question raised in the 1920s.

Coelenterazine was identified in the decade following, as the bioluminescence component in the photoprotein aequorin and as a substrate for the Renilla luciferase from the sea-pansy *Renilla reniformis*, and subsequently determined to be involved in the bioluminescence of a great number of marine organisms, many of which are coelenterates giving rise to the name “coelenterazine.” It is interesting that most marine luciferins are derivatives of imidazopyrazinone, although coelenterazine is most common. The first structure in this family to be determined was Cypridina luciferin, now cypridinid luciferin being the preferred name as suggested by Morin (11).

PHOTOCHEMISTRY AND PHOTOPHYSICS

The two fundamental laws of photochemistry apply equally well to bioluminescence and chemiluminescence mechanisms (9). The first law is obvious in that the light must be absorbed to initiate a photochemical reaction, and the second law is that for each photon absorbed, only one molecule is reacted. A restatement of this second law is that there is chemical equivalence for the photon, meaning that one photon yields up to one product molecule. This apparent photon stoichiometry is measured by the photochemical quantum yield, $\Phi_P = \text{product molecules/photons}$
absorbed, and must be \(<1.0\). For a bioluminescence (or chemiluminescence) reaction, the definition is the inverse, \(\Phi_b = \text{photons emitted/luciferin consumed or product molecule, and it also must be <1.0.}

The quantum yield or efficiency, for bioluminescence \(\Phi_b\) or chemiluminescence \(\Phi_C\), is a product of three efficiencies:

\[
\Phi_b \text{ or } \Phi_C = \Phi_Y \times \Phi_E \times \Phi_F
\]

where \(\Phi_Y\) is the chemical yield, \(\Phi_b\) the fraction of the light path products deposited into the excited state from which fluorescence emission with yield \(\Phi_F\) occurs as the bioluminescence light (12). The determination of \(\Phi_b\) is technically challenging, but a detailed calibration procedure directly traceable to the blackbody standard has been established (13). The chemiluminescence quantum yield of luminol was accurately determined and has become widely adopted as a secondary standard (13).

The first bioluminescence quantum yields determined were for firefly \(\Phi_b = 0.88\) (18) and bacteria \(\Phi_b = 0.05\) (19), remarkably much higher than known for any chemiluminescence reaction at that time. Recently the firefly has been reetermined with greater accuracy as being in the range \(\Phi_b = 0.4\)–0.6 (20, 21) depending on the type of beetle, and this range is consistent with the \(\Phi_b = 0.4\) for the product firefly oxy-luciferin anion (22). Importantly, these high values for the firefly \(\Phi_b\) give confidence that \(\Phi_Y\) must be also near unity and that the overall chemistry is the one of the light path. For the bacterial bioluminescence, the \(\Phi_b\) was later remeasured for the aldehyde as in the range \(0.1\)–0.13 (15, 23, 24), and 0.16 using a different calibration method (25), meaning that with this low efficiency some caution is needed in interpreting results of chemical investigation. The product of bacterial chemiluminescence is the aliphatic acid of the same carbon chain length as the aldehyde and with respect to this product \(\Phi_b = 0.1\)–0.13 (24, 26), meaning that there is a 1:1 stoichiometry between the aldehyde and carboxylic acid product. For the FMNH\(_2\), \(\Phi_b = 0.05\), which being only half that of the aldehyde, would mean that two flavins were involved in a stoichiometric relation, but a later NMR investigation showed that this second FMNH\(_2\) was not required in the light path (27). The structure of the emitter of bacterial chemiluminescence is not yet identified directly but it has a \(\Phi_b = 0.3\). Cypridinid luciferin has \(\Phi_b = 0.3\) (28), and the product cypridinid oxy-luciferin based on an analog has \(\Phi_b = 0.35\) (29).

**CHEMICAL EXCITATION**

The first solution chemiluminescence reported was that of an organic molecule lophine in 1877 by Radziszewski. By the mid-20\(^{th}\) century, the bright chemiluminescence reactions of luminol and the acridans were discovered and these three so-called classical chemiluminescences continue to be much investigated. A fundamental and still unsolved question in chemical excitation is how the system at its transition state chooses to deposit its exothermicity into the excited singlet state of the product molecule, the same state as achieved by absorption of a photon, in preference to the product ground state. Even the overall chemistry leading to lophine and luminol chemiluminescence remains unresolved (12).

In 1963, Ed Chandross published what has become a landmark discovery (30). The reaction of oxalyl chloride with concentrated \(\text{H}_2\text{O}_2\) generated a dim blue chemiluminescence, but the inclusion of diphenylanthracene increased the intensity many orders of magnitude and the chemiluminescence spectrum corresponded to the fluorescence of the diphenylanthracene. The diphenylanthracene was not consumed in the chemical reaction; it simply seemed to serve as an “antenna.” Other fluorescent dyes also work to be excited to their fluorescent state by a postulated high-energy intermediate. This discovery was commercialized as the well-known “light stick.” In the light stick, the fact of its utility is that the luminosity is quite bright and the color can be modulated depending on the fluorescence of the antenna dye. The Rauhut group at the American Cyanamid Company determined the typical \(\Phi_c > 0.2\) part as part of their development of the product including the shifting of color using different fluorophores (31). It was thought that the high-energy intermediate could be some form of cyclic peroxide having a highly strained ring system such as dioxetandione, which is essentially a \(\text{CO}_2\) dimer, the breakdown of which to \(\text{CO}_2\) would provide a calculated exothermicity well in excess of the \(\text{S}_0 \rightarrow \text{S}_1\) energy of the antenna dye. The dioxetandione structure was confirmed only very recently by low-temperature \(^{13}\text{C}\)-NMR experiments (32, 33). The dioxetandione should have high oxidation potential, so the excitation efficiency of the antenna dye would be expected to correlate with the reduction potential of the fluorophore within a collision complex in solution. Many investigations now support the idea that some form of electronic orbital overlap is involved in the excitation step (34).

Frank McCrampa proposed that a similar cyclic peroxide, a substituted dioxetane, to be the high-energy intermediate in the chemiluminescence of acridans and firefly bioluminescence (35, 36). The bioluminescence mechanism he proposed was through the decarboxylation of a firefly luciferin dioxetanone with the release of \(\text{CO}_2\) to form directly the oxy-luciferin in its \(\text{S}_1\) excited state. This dioxetane is too unstable to be observed directly, but the reaction carried out in the presence of \(^{13}\text{CO}_2\) produced one \(^{15}\text{O}\) in the liberated \(\text{CO}_2\) giving indirect support for the proposed luciferin dioxetanone structure (10). No \(\text{CO}_2\) is found as a product from bacterial bioluminescence, only the carboxylic acid of the same chain length as the aldehyde, and an \(^{13}\text{CO}_2\) experiment yielded this carboxylic acid with one \(^{15}\text{O}\) (37). The mechanism of excitation in bacterial bioluminescence does not apparently involve the intermediacy of a dioxetane (38).

In both the firefly and imidazopyrazinone marine bioluminescence systems therefore, the high-energy intermediate is assumed to be a dioxetane of fleeting existence (36). Another advance that became very important for mechanistic study was the synthesis of a stable dioxetane derivative by Kopcey and Mumford (39). Tetramethyl-dioxetane is stable at room temperature and generates chemiluminescence on thermolysis, “efficient enough to light up the room” was announced to this author on a visit, but it was pointed out that his sample of course was near molarity. In fact, a \(\Phi_c < 10^{-5}\) was measured some few years subsequently, but surprisingly, the triplet yield was far greater, \(\Phi_T \sim 0.01\).

A stable and extremely efficient chemiluminescent dioxetane was synthesized by Paul Schaup (40, 41), one substituted by a spiroadamantyl group, a methoxy, and a phenyl group. Apparently, the spiroadamantyl substituent confers the stability and the phenyl moiety is the source of electron overlap, and the transfer of charge then triggers the reaction. The chemiluminescence on thermolysis in DMSO produced \(\Phi_c = 0.25\) and the product \(\Phi_b = 0.44\) so the calculated \(\Phi_F = 0.57\), making it a satisfactory
test of the triple \( \Phi \) product equation. A second derivative was designed for water solubility and to be able to be triggered by forming its anion via the enzyme alkaline phosphatase (Fig. 2). Although for this one, \( \Phi_C = 5 \times 10^{-3} \), the high sensitivity of photomultiplier-based instruments has proven this dioxetane to have great utility as a chemiluminescent label for diagnostic clinical application.

A general mechanism for triggering bioluminescence is considered to be similar by anion formation and intramolecular charge transfer, facilitated by the high degree of conjugation in the structures of firefly and coelenterazine luciferins. An early hypothesis was for a full electron redox transfer, chemically initiated electron-exchange luminescence (CIEL), but through a number of precedents of organic mechanisms, a charge-transfer initiated luminescence (CTIL) proposal has become generally acceptable. One concern was that only partial electron transfer or charge transfer would avoid the presence of radical intermediates with their consequent potential for deleterious side reactions.

In the last 15 years, these “pictorial” mechanisms have been supplanted by attempts using computational chemistry. It is computationally challenging to deal with luciferin located within the luciferase-binding site with the consequence of a multitude of interacting centers (42). This has not deterred some investigators addressing the system in totality with predictions, for example, that the structure of the excited product of the firefly chemiluminescence differs from the oxy-luciferin Franck-Condron state (43) or that there exists a biradical intermediate in the pathway of the oxidative decarboxylation of the Ca\(^{2+}\)-regulated photoprotein obelin (44). Other researchers less bold prefer to do away with the protein part altogether and deal with the smallest target, the dioxetane or dioxetanone in the gas or condensed states (45, 46).

The potential energy surface on thermolysis of the dioxetane is computed along the reaction coordinate. The O–O bond being weakest is assumed to first undergo a homolytic splitting to produce a biradical, and a torsional motion leads rupture of the C–C bond at the transition state where it is degenerate with the surface of the excited or ground state products. This point in the energy surface is called a “conical intersection.”

Thermolysis is not an option for bioluminescence; instead, the trigger appears to be intramolecular charge transfer into the O–O bond, thereby weakening it and leading to fracture at a lowered activation energy (47, 48). A high-level computation for firefly chemiluminescence in the gas phase concludes that CTIL controls the excitation step and excludes CIEL in aqueous solution (47). In the aprotic solvent dimethylsulfoxide made strongly basic, both firefly luciferin and coelenterazine generate chemiluminescence with \( \Phi_C \sim 0.01 \) (49,50). In the three bioluminescence systems under consideration here, the presence of an anionic center is key to formation of the high-energy species and its triggering.

Chemiluminescence and bioluminescence reactions are classified either as “direct” where the chemical exothermicity is directly released into the product as it is formed or “indirect” in other words the “light-stick” mechanism, a high-energy intermediate transferring its energy to a molecule not involved in the chemistry. The indirect mechanism is also called “sensitized” chemiluminescence by analogy to sensitized fluorescence, where the emitter species, the sensitizer may be at a far molecular distance from the excited “donor.” In some cases, Förster resonance energy transfer (FRET) is invoked to account for the process. It is generally conceded that bacterial bioluminescence is an indirect process although the details of the mechanism remain quite obscure. The bioluminescence of firefly luciferin, cypridinid luciferin, coelenterazine and its derivatives is a direct process.

### Bacterial Bioluminescence

In 1963, Hastings and Gibson made the first investigation of the light path in bacterial bioluminescence using the recently developed rapid mixing stopped-flow apparatus (51). In aqueous solution, FMNH\(_2\) was oxidized very fast to FMN by dissolved oxygen, but in the presence of a molar excess of bacterial luciferase, the FMN appeared more slowly. Bacterial bioluminescence requires the inclusion of a long-chain aliphatic aldehyde (RCHO), but Hastings and Gibson found that this could be delayed for some minutes with a diminished light yield in proportion to the delay time. They proposed that a metastable intermediate designated “intermediate II,” formed on O\(_2\) reaction of the FMNH\(_2\) bound to the luciferase (E), which they called intermediate I, and that II would probably be an O\(_2\)-FMNH\(_2\)-luciferase complex. They formulated a linear mechanism for the bioluminescence with direct production of an excited state IV* (9):

\[
E \rightarrow FMNH_2(\text{I}) + O_2 \rightarrow E \rightarrow FMN + H_2O_2(\text{II})
\]

\[
\text{II} + RCHO \rightarrow FMNH - OOC(H)R
\]

\[
(\text{III} \rightarrow E \rightarrow FMNH - peroxyhemiacetal)
\]

\[
\text{III} \rightarrow FMNH + 4a - OH + (\text{IV*}) \rightarrow h + FMN + H_2O
\]

Except for II, the following intermediates are too short-lived to apply direct structural identification, and proposed structures are based on spectral properties among other considerations (52,53). Intermediate II was proven by \(^{13}\)C-NMR only 25 years later to be 4a-hydroperoxy-FMNH: luciferase (27). In correspondence with the generally believed pathway of flavoprotein oxidation, it is reasonable to propose that III is luciferase-bound 4a-peroxyhemiacetal-FMNH (54). The structure of IV as 4a-hydroxy-FMNH: luciferase (luciferase–hydroxylflavin) would be consistent with the observation that the final products FMN and H\(_2\)O would result from a simple dehydration (52,53). In spite of the passing of 50 years and the investigations by many in the community, our knowledge here has not advanced very much. There are a number of recent reviews (54–63) but the story is far from finished. I will summarize what I feel is reliable information and also allow some comments.

As written, the light reaction was assumed to be a direct chemiluminescence, III decomposing to the excited or fluorescent state of the product IV bound within the luciferase. However, the present state of knowledge indicates that a sensitized chemiluminescence is more likely, and this is certain when the antenna proteins are included. In this review, I intend to assemble evidence that this may also be the case without the presence of these antenna proteins.
Recently, there has been a reclassification of the bioluminescent bacteria based on phylogenetics (64), but here to lessen confusion, I will use the earlier names and abbreviations, for example, VH-luciferase from Vibrio (or Benekea) harveyi, VF-luciferase (PF- or AF-) from Vibrio (now Alivibrio) fischeri, PL-luciferase from Photobacterium leiognathi and PP-luciferase from Photobacterium phosphoreum. Their bioluminescence spectra are all broadband (FWHW ~ 100 nm) with a type-dependent spectral maxima in the blue–green range 472–505 nm, with the notable exception of A. sifaeae (previously P. fischeri strain S1), which has a yellow bioluminescence with maximum at 545 nm.

The in vitro bioluminescence of all types is also a broadband with type-dependent maxima but over a smaller range 485–505 nm, including the luciferase from the yellow type A. sifaeae. There would be expected to be a product present in the reaction having a high fluorescence yield and spectral distribution corresponding to the bioluminescence. A qualifying candidate with matching fluorescence with maximum around 490 nm was recovered from the total mixed fluorescence of a complete reaction (24,65). A detailed kinetics study compared the appearance of this fluorescence, called the “fluorescent transient,” with the bioluminescence signal and showed that the light path could not be linear (66). Although the rates of appearance and decay qualified the fluorescent transient as a product, its correlation with the bioluminescence kinetics indicated three distinct pathways to bioluminescence. A protein–protein interaction was invoked to model a quadratic relationship between the decay rates of the fluorescent transient and bioluminescence intensities. McCapra (54) has incorporated a luciferase–luciferase interaction in a novel excitation mechanism and provided a critical appraisal of the many extant speculations, including his own!

Fluorescence anisotropy decay experiments confirmed the presence of luciferase fluorescent transient dimer association. The decay of the fluorescence anisotropy, r of a protein, is a single exponential function, \( r(t) = r_0 \exp(-t/\varphi) \), and the parameter \( \varphi \) is called the rotational correlation time (67). Conveniently, for a fluorophore rigidly bound to a macromolecule, \( \varphi \) (2°C) in ns units is numerically and coincidentally, nearly the same as the macromolecule mass in kDa units. Bacterial luciferases alone have no visible fluorescence, only from tryptophan residues, which have a spectral maximum around 330 nm. Using either the tryptophan fluorescence or the extrinsic fluorophore ANS as markers, VH-luciferase and VF-luciferase both with mass about 77 kDa show the expected \( \varphi \approx 80 \text{ ns} \) (2°C). However, the fluorescent transients observed from both VH- and VF-luciferase reactions show anomalously high values, \( \varphi > 100 \text{ ns} \), even as high as 150 ns. This would be simply explained as the presence of luciferase dimers (150 kDa) and was confirmed by filtration of the reaction mixtures through a 100-kDa cut-off membrane, where the fluorescent species did not pass through (68). On the other hand, the fluorescent transient in the PL-luciferase reaction having \( \varphi = 79 \text{ ns} \) (2°C) was not retained by the 100-kDa cut-off (69). A bioluminescence-fluorescent transient kinetics investigation into the PL-luciferase bioluminescence would seem called for, as it should produce a less complicated kinetics model than that for VH-luciferase (66).

There is no doubt that the fluorescent transient is the bioluminescence emitter in the reaction initiated with FMNH₂. With the three types of luciferase, the fluorescence envelope is almost the same as the in vitro bioluminescence, and they all have the same efficient \( \Phi_\text{F} = 0.30 \) and corresponding long fluorescence decay lifetime, \( \tau = 10 \text{ ns} \) (4°C), and an excitation spectral maxima at 366 nm consistent with its having a formal electronic structure of a dihydroflavin (55,70). It is surely no coincidence that the same properties are observed for what I call the luciferase photoflavin, \( \Phi_\text{F} = 0.3 \) and \( \tau = 10.2 \text{ ns} \) (71). Balny and Hastings (72) reported that the VH-luciferase peroxyflavin intermediate was only weakly fluorescent but continued irradiation at 370 nm would enhance the fluorescence to about the same efficiency as FMN, \( \Phi_\text{F} = 0.25 \), with a spectrum the same as the bioluminescence. Following studies showed similar behavior, \( \Phi_\text{F} = 0.17 \) (73), \( \Phi_\text{F} = 0.28 \) for VF-luciferase photoflavin and \( \Phi_\text{F} = 0.33 \) for PL-luciferase photoflavin (68,70,71). Tu (73) developed methods for stabilization of these luciferase intermediates that were later employed for many studies, including the C³NMR identification of the 4a-peroxy-FMN: luciferase intermediate (27). The same NMR identification could be feasible on the PL-luciferase photoflavin and could provide important information.

In all current presentations of the mechanism of bacterial bioluminescence from FMNH₂, the luciferase-bound 4a-OH-FMN (IV) structure is written as the final excited product and origin of the bioluminescence spectrum. This conclusion is popular but is supported more by assertion than direct evidence. There have been many efforts to produce model substituted flavins that associate with luciferase but generally fail to qualify based on \( \Phi_\text{F} \) or spectral distribution. In fact, 4a-substituted flavins are notoriously nonfluorescent. An example though distant is the blue-light receptor FMN-binding proteins containing the so-called LOV domain. These were first discovered in plants but are now found in many types of organisms. They control response to light via a conformation change due to the ligand FMN photochemically induced to form a 4a-adduct with a proximate cysteine residue side chain in the binding cavity. The flavin fluorescence is quenched and the responsible adduct structure is directly proven from C³-NMR and crystal structure (74,75). The adduct bond is weak, and the blue-light receptor slowly recovers.

Such promising analogs for the bacterial bioluminescence emitter are ones with substituents at the 4a,5-positions, which are demonstrably fluorescent in the case of the flavoenzyme lactate oxidase (76,77), and have some qualifying spectral properties (62).

The fluorescence of the final product FMN is quenched on VH-luciferase, but it is agreed that the bioluminescence emitter must at least be related to the FMN structure because the analogs iso-FMNH₂ and 2-thioFMNH₂ produce bioluminescence at shorter or longer wavelengths, respectively (78,79). FMNH₂ is certainly identified as the emitter in the case where the reaction is carried out in the presence the “antenna protein” variant that has FMN as a ligand (80,81). The first in this class of antenna proteins was called “lumazine protein” (LumP) discovered in some bacteria of the genus Photobacterium that had bioluminescence maxima around 475 nm instead of around 490 nm like most types (56). Lumazine protein was named because the bound fluorophore was identified as 6,7-dimethyl-8-ribityl-lumazine. An homologous yellow fluorescence protein (YFP) was isolated from the yellow bioluminescence bacteria A. sifaeae, having FMN or riboflavin as a ligand (80–82). The in vitro bioluminescence resulting on inclusion of the antenna protein matches the fluorescence of the bound fluorophore, flavin or the lumazine (56,69,71,82).

The in vitro bioluminescence shift on inclusion of LumP shows a titration behavior, and less than 10 µm of LumP is sufficient for a full shift implying a protein–protein association to be
involved. The fluorescent transient is a possible source of bioluminescence excitation of LumP whether or not it is initially excited in a direct chemiluminescence or functions as a sensitizer having proximity to a supposed high-energy intermediate. A fluorescence anisotropy experiment was employed to detect this energy transfer complex. The PL-luciferase fluorescent transient had the expected $\phi = 79$ ns (2°C) according to its mass of 77 kDa, but on inclusion of LumP, not an increase but a spectacular reduction to a single fast $\phi \sim 4$ ns is displayed (56, 71). Theory shows that energy transfer provides a channel of anisotropy loss in addition to rotational diffusion (83). The contribution of the fast channel here correlates with the effect of shifting the bioluminescence spectrum. On the assumption that the approximations made for FRET in free solution are valid for resonance coupling within the luciferase:LumP complex, a Förster separation of the lumazine ligand and the flavin species in the active site of the luciferase was estimated as $< 20$ Å.

A few years after these studies, the recombinant proteins became available allowing a more systematic examination of these fluorescence effects (69, 82, 84). The rec-LumP from P. leiognathi has the same properties in shifting the bioluminescence of rec-PL-luciferase as the natural LumP. Both riboflavin and FMN also bind to apo-lumazine protein but more weakly than the natural ligand. The spectral properties are the same as the YFP, but they have no bioluminescence shift property. Neither rec-LumP nor the rec-flavoproteins have association with the rec-PL-luciferase prior to reaction reflected by no change in the 20-ns rotational correlation time of the LumP. This is different from the LumP association with PL-luciferase published previously and may indicate that some factor, perhaps the product long-chain carboxylic acid, accompanies the otherwise purified natural luciferase (69). However, both the rec-PL-luciferase per-oxylavin (intermediate II) and its fluorescent transient reveal again the fast anisotropy decay component, $\phi = 2.5$ ns, on addition of the rec-LumP. An analysis of fluorescence titration shows a stoichiometry of 1:1, luciferase:LumP. The addition of the flavoproteins in contrast has no effect on the anisotropy decay indicating again, no association and consequently no energy transfer, the reason for no bioluminescence shift. The protein–protein association behavior or the lack of any was confirmed by chromatography and SDS-PAGE (68, 69).

Both forms of the natural YFP with either FMN or riboflavin as the bound fluorophore are active for shifting the blue (maximum 495 nm) in vitro bioluminescence of A. siiue luciferase, toward the yellow to match the 545-nm maximum of the bound flavin fluorescence and the in vivo bioluminescence (69, 80, 81, 85, 86, 87). The primary sequences of YFP and LumP are very similar (88, 89) and their spatial structures are homologous (90, 91). Yet as already mentioned, the ligand exchange of LumP with FMN or riboflavin eliminates the association with the PL-luciferase intermediates and consequently the property of shifting the bioluminescence to longer wavelength (69, 92).

There is a significant advantage in using the yellow bioluminescence system for more precise recovery of the energy transfer parameters (92). The donor fluorescence spectrum from the fluorescent transient is well separated from the absorption spectrum of the acceptor YFP. This allows time-resolved fluorescence of the donor to be used to determine the energy transfer rate rather than the more indirect extraction from fluorescence anisotropy decay measurement. At a 1:1 stoichiometry that achieves the complete bioluminescence shift, the fluorescence lifetime of the donor changes from 10 to 0.25 ns. This energy transfer rate 4 ns$^{-1}$, is 10 times faster than the number for the PL-luciferase plus LumP system (0.4 ns$^{-1}$) observed as the rapid 2.5-ns component of anisotropy decay. On the assumption stated above about the proper application of FRET theory, the Förster separation of donor and acceptor is estimated as 15 Å. The 10 times ratio of energy transfer rates between the two bioluminescence experiments is the same as the ratio of donor–acceptor spectral overlaps and thus validates the acceptance of the FRET approximation for the complex.

The inclusion of an antenna protein in the bioluminescence reaction also has marked effect on the bioluminescence kinetics (56, 93). To initiate the reaction, an aliphatic aldehyde like tetradecanal is added, and to stabilize the intermediates before reaction for more precise study, an aliphatic substance like dodecanol has to be included (73). Evidently, these additives must interfere in the binding process as the model requiring a close approach of the antenna protein to the active site or the binding of the stabilizing agent presumably to the same binding site as for aldehyde might be expected to result in kinetics changes. It is found that without the presence of the dodecanol stabilizer, no evidence of protein–protein association is observed (93). It is suggested that the aldehyde or the stabilizer through a hydrophobic effect induces a structural modification, favoring the interaction.

The three-dimensional structures of lumazine protein and its riboflavin and FMN variants have been determined by X-ray crystallography (90, 91) and can be viewed at the Protein Data Bank (94). A computationally docked spatial model has been produced of a lumazine protein complex with VH-luciferase having bound FMN (91, 95). This model shows a separation of only 10 Å between the FMN in the luciferase-binding site and the lumazine ligand, providing a very favorable circumstance for the FRET mechanism of bioluminescence color shift. It is, however, only plausible that this model is representative of the true structure of the purported donor–acceptor complex because experimentally, lumazine protein associates hardly at all with VH-luciferase, and only with PL-luciferase reaction intermediates, and the complexation does not take place without inclusion of a long-chain aliphatic.

The VH-luciferase structure with FMN bound was determined by soaking the FMN into the luciferase crystals (95). The FMN is located internally within the luciferase $\alpha$-subunit, as predicted by a host of earlier studies, and in a large cavity that has an opening to solvent. The FMN is in a planar conformation, noncovalently bound but with H-bonding to residues lining the cavity. The nature of the FMN interactions is in remarkably complete agreement with interpretations of the H-bonding from the earlier $^{13}$C-NMR study of FMN/VH-luciferase in solution (96). An attempt to reduce the FMN within the crystal produced cracking, commonly interpreted as due to a protein conformational change for bound FMNH$_2$. The $^{13}$C-NMR study of FMNH$_2$-luciferase also indicated a different set of H-bond interactions than from the FMN ligand (96).

Although the spatial structures of the lumazine protein variants are quite homologous overall, there are some small variations around the binding site. They have identical spectroscopic properties to the native proteins and the variations among the ligands can be rationalized by examination of interactions in the binding site (97). The lumazine derivative is buried in a hydrophobic pocket held in place by an extensive hydrogen bond...
network (98). The lumazine derivative in water has a fluorescence maximum around 490 nm, but in solvents of lower polarity, it is blueshifted toward that of lumazine protein itself. The opposite is found for flavins, less polar solvents cause a shift from about a 535 nm maximum to longer wavelength, although by only a few nanometers. The yellow fluorescence protein fluorescence maximum at 542 nm must be a result, therefore, of additional interactions such as from the hydrogen bond network, although this is not as extensive as for the lumazine in lumazine protein, explaining in part the weaker affinity of flavins for the lumazine apoprotein. Another distinctive property is the high fluorescence yield of the bound flavin compared to almost all other flavoproteins, which are mostly hardly fluorescent. It might be informative to computationally model luciferase complexed with FMN bound to the lumazine apoprotein because these holoproteins fail to associate and produce any bioluminescence shift.

I have not included here the many speculations on chemical mechanisms of excitation in bacterial bioluminescence because these have been critically reviewed recently (54,60). If the conclusion of the “light-stick” indirect chemiluminescence is considered most likely, then the possibility is that the 4a-peroxy substituent, which closes to the luciferyl-AMP ester then, following a luciferase conformational change, addition of molecular oxygen proceeds with Pyralis luciferase showed that a red bioluminescence color is also found naturally in a specific photophore, a lumazine protein complex. However, absent the luciferase which could provide the fluorescent sensitizer, a chemiluminescence model reaction gives little light alone (100). Energy transfer processes in bioluminescence is certainly known, in the bacterial reaction with the antenna proteins, and in the case of the coelenterazine bioluminescence systems to be described in the following section.

FIREFLY AND COELENTERAZINE

These two bioluminescence systems are discussed together as they have a common direct excitation mechanism, decarboxylation of a luciferin dioxetanone to form the S1 state of the product, the bioluminescence emitter. Their chemistries of course differ as the two luciferins have different chemical structures. What is called “firefly” luciferin is common to all the beetles; the firefly itself is a beetle (order: Coleoptera) and not a true fly (order: Diptera). Firefly luciferin is found only in these terrestrial organisms. There are many bioluminescent species within Diptera, but their luciferin structures are unknown. The chemical structure of firefly luciferin is 6′-hydroxybenzothiazol-1-dihydrothiazole-carboxylic acid (Fig. 3a). Firefly luciferase is a bifunctional enzyme (Fig. 3a); the first step is addition of ATP and formation of the luciferyl-AMP ester then, following a luciferase conformational change, addition of molecular oxygen proposed to form a 4-peroxy substituent, which closes to the firefly luciferin dioxetanone (101–103). These last two intermediates are short-lived and have not been directly identified.

Coelenterazine is widespread in marine bioluminescent organisms. It is a derivative of imidazopyrazinone as are a number of other marine lucifers (3). It was with having similar spectroscopic properties to the first discovered cypridinid luciferin that led to the identification of coelenterazine in aequorin bioluminescence.

Coelenterazine bioluminescence comes in two “flavors.” The first is the Ca2+-regulated photoproteins as in aequorin, in which a coelenterazine hydroperoxide is stabilized within a protein cavity, primed for triggering for bioluminescence on the addition of Ca2+. The second is for various luciferases for which coelenterazine is a substrate, adding oxygen on binding to generate bioluminescence without the involvement of Ca2+. These luciferases are named for the organism from which they originate, and among the types, they mostly have low sequence homology. Renilla luciferase from the sea-pansy *Renilla reniformis* is the most well-known one of these (104). The chemical mechanism of these imidazopyrazinone luciferins, cypridinid luciferin (Fig. 3b), coelenterazine (Fig. 3c), etc., is the same. The photo-proteins can just be regarded as a luciferase having stabilized the reaction intermediate (104–106).

The chemical structure of the directly excited product and emitter of firefly bioluminescence has been in contention for 60 years. An early proposal was that the reaction could be a sensitized bioluminescence as the firefly luciferin anion itself is highly fluorescent, ΦY = 0.62 (107,108). However, its fluorescence maximum at 537 nm was considered inconsistent with the bioluminescence maximum at 562 nm using the Pyralis luciferase, the only type of firefly luciferase available at that time. In later years, other firefly species have been discovered with bioluminescence at 546 nm, so the argument against sensitization becomes not so strong. For a direct process, there would be expected to be a product having a qualifying fluorescence. One was found in the reaction mixture and designated “oxy-luciferin,” but this was a “red herring” because it turned out to be a decomposition product “dehydroluciferin.”

The genuine firefly oxy-luciferin was identified much later as 6′-hydroxybenzothiazol-2′-4-hydroxythiazole (Fig. 3a) (109). The oxy-luciferin structure can be viewed as that of the firefly luciferin minus the terminal “CO,” an important recognition that the reaction was probably a decarboxylation. The production of CO2 would yield a calculated enthalpy exceeding the energy of the S0→S1 transition of the oxy-luciferin. A landmark observation was the result of using oxygen-18 for the oxygenation step. Product analysis finding one atom of 18O in the released CO2 provided support for the hypothetical luciferin dioxetanone intermediate (10).

As already mentioned, different species of fireflies and other beetles have bioluminescence colors ranging from yellow-green through orange. The absolute spectral distribution of 35 species of fireflies was measured to have spectral maxima ranging from 546 to 594 nm (110). Also first reported almost 200 years ago, warming a firefly would produce a red bioluminescence color (107). A red bioluminescence color is also found naturally in a specific photophore of the “railroad worm” (111). The first studies with Pyralis luciferase showed that a red in *vitro* bioluminescence was induced by increase in temperature, a more acidic pH from the optimum of around 8–6.8, or by addition of Hg2+ or Cd2+. It was concluded at that time that the 546–594 nm range could reflect different perturbation of a single molecular S1 level due to different environmental polarities of the binding cavity in the different species of luciferase (107–109,112). A luciferase conformational alteration could also be produced by temperature, acidity or by the Hg2+, which is known to attach to Cys residue side groups, and these changes could allow a different molecular species to be the origin of the red bioluminescence. Such a rationalization is consistent with the recently determined spatial structures of a firefly luciferase, one having a bound luciferin analog and the other the oxy-luciferin and AMP products (113).
Oxy-luciferin is very unstable; it was not until recently that its chemical, and spectroscopic properties could be confidently assessed (22,114–117). In aqueous solution over the physiological pH range, oxy-luciferin can exist in two neutral forms due to keto-enol tautomerism and four anionic forms by acid–base equilibrium. Much attention has been given to identifying the oxy-luciferin structure responsible for the green to yellow or for the red bioluminescence, by determining again, which form has...
fluorescence spectral properties matching the bioluminescence. It is certainly an anion because the neutral forms have a blue fluorescence and therefore can be eliminated from consideration. There are several possibilities, one of the three monoanions, or the dianion. Attempts at spectral measurements were initially frustrated by the chemical instability of oxy-luciferin.

Until recently, it was generally accepted that the red emitter was the 6'-phenolate keto-oxy-luciferin. As the phenol moiety has a pKₐ ~ 8 and probably a more acidic pK⁺, and allowing for tautomerism the enol would not be expected to be acidic. This proposal was supported using an analog, 5,5-dimethyl-luciferin-AMP as a substrate with Pyralis luciferase because it produced a red bioluminescence. This substitution would block any tautomerism (117). Surprisingly, this derivative with another type of luciferase gave the normal yellow bioluminescence leading to the proposal that the keto-oxy-luciferin species was the emitter over the complete range, shifted by a variety of perturbing environments in the luciferases from different species. Hirano (118) proposed an ion-pair interaction of the 6'-O⁻ with a protonated amino acid residue located in the binding cavity, and in the available spatial structure (113) Ser317 and Gln340 could fulfill this role. The strength of this interaction could be clearly modified by cavity site polarity, and the spectral properties of an oxy-luciferin analog in a variety of solvents were offered as supporting evidence. However, the proposal of a single electronic state responsible for the complete spectral range of bioluminescence is at variance with the different Gaussian parameters required for fitting the red bioluminescence versus the shorter wavelength spectral envelopes.

The question has now been raised again by the finding that oxy-luciferin-enol is a strong photoacid, that is the pK⁺ < 0 (119). Evidence from other spectroscopic studies was assembled in support of the oxy-luciferin enolate as the yellow emitter. Also, any formation of a dianion was discounted and it was proposed by others that the red bioluminescence to be from the enolate but the yellow from the neutral keto-phenol-oxy-luciferin (110,116,120) (to be continued...).

The in vivo bioluminescence absolute spectral distributions from 35 different firefly species having maxima 546–594 nm were shown to be Gaussian with the same parameters (110). This was basis for the hypothesis that differences in permissivity within the cavity environment of the luciferases from the different species perturbed the S₁ level of the oxy-luciferin to different extents. No contribution from a second red Gaussian component that could induce a longer wavelength shift was evident. An apparently contradictory result has recently appeared (121–123).

Absolute bioluminescence spectra from the in vitro reaction using Pyralis luciferase, the luciferase from the Japanese firefly _Luciola cruciata_, and several of its mutants that produced emission colors orange through the red were found to exhibit asymmetry in their spectral envelopes, and required three Gaussian functions for a precise fit. These components had wavelength maxima at 560, 610 and 640 nm. No such asymmetry was reported in the collection mentioned above of the in vivo bioluminescence from the 35 firefly species (110). An explanation can be offered here that luciferase being a very hydrophobic protein and the firefly photophore having a very dense concentration of material would constrain the luciferase conformation and its cavity structure so that the emission envelope fulfilled its known biological “courtship” function in efficiently matching the visual spectral sensitivity of the required species of female firefly (124).

In free solution, the protein conformation would be less restricted leading to a population of different cavity states, thereby favoring the emission of several Gaussian spectral envelopes.

The application of X-ray crystallography to determine spatial structures of Ca²⁺-regulated photoproteins has provided significant advances in understanding the mechanism of this bioluminescence (106,125). The crystallographic method is only feasible if good single crystals of the proteins can be grown, and in this regard, the Ca²⁺-regulated photoproteins have been very cooperative. Aequorin from the jellyfish _Aequorea_ was the first of these to be classified as a photoprotein and given this status because Ca²⁺ addition alone was sufficient to trigger the bioluminescence without any requirement for molecular oxygen (10,125). It was proposed in 1962 that oxygen was already bound within the protein cavity along with the substrate, not known to be coelenterazine at that time (126). Many others in this class now with the preferred descriptive name “Ca²⁺-regulated photoproteins” have been identified since, from about 26 different organisms altogether (127,128). The ones with determined spatial structure and in other respects most studied in detail are aequorin, obelin from two species of the hydroid _Obelia_ and clytin from the hydrozoan jellyfish _Clytia_ (63,106,129).

The spatial structures of aequorin and the obelins for the first time directly visualized a noncovalently bound coelenterazine structure in the active site with a peryoxo attachment to the C-2 position (130–133). A¹³C-NMR investigation earlier had announced a corresponding finding that the sp² bond at the C-2 of coelenterazine within aequorin changed to sp³ on addition of oxygen (134). Interestingly, the same¹³C-NMR experiment was carried out contemporaneously with bacterial luciferase, revealing the C-4a chemical shift on oxygenation of the bound FMNH₂ (27).

In protein structures determined by X-ray crystallography, a separation of ~3.5 A between a potential donor and acceptor is interpreted as the presence of an H-bond. The H atom itself cannot be distinguished in X-ray structures except in some cases with exceptional resolution, much <1.0 Å. In the obelin structure (Fig. 4, upper left), H-bonding is inferred at the distal C2-peroxo oxygen to a proximal side group of Tyr190 (Y190), which is bifurcated to the imidazole N of His-175 (106). A similar picture is evident in the structures of aequorin and other Ca²⁺-regulated photoproteins (63,129,130,135–137).

It was suggested that this H-bonding is the stabilizing factor in the Ca²⁺-regulated photoproteins, the basis for a proton-relay hypothesis for how Ca²⁺ binding triggers the bioluminescence (106). Proton relay is a favored suggestion for many enzyme mechanisms (138). The Ca²⁺-regulated photoproteins belong to the large family of calcium-binding proteins that have a secondary structure with several EF-hand calcium-binding loops. In an X-ray structure determination, bound Ca²⁺ can be recognized in the electron density maps as occupying a specific position within the consensus loop, and for this to happen, the residues forming the loops prior to binding need to move to accommodate the incoming Ca²⁺. Comparing the loop structures in obelin before and after Ca²⁺ addition, the movement of residues is largest in the loop sequence adjacent to His-175 supporting this idea that the binding of Ca²⁺ there destabilizes the H-bond network (Fig. 4, upper right). It is proposed that the shift of His-175 makes the H-bond to Y190 more ionic, which in turn leaves a negative charge on the peryoxo group, and this according to the model chemiluminescence mechanism of McCapra (50) will...
cause the peroxy to undergo irreversible nucleophilic addition onto the C-3 of coelenterazine to form the dioxetanone (and the rest is photochemistry).

The cypridinid luciferin model of McCapra (50) was the chemiluminescence of a substituted imidazopyrazinone in the aprotic solvent DMSO made strongly basic with potassium t-butoxide. The emission was a single band with maximum at 455 nm, which corresponded to the fluorescence of the major product, assigned as its amide anion. Goto (139), however, in addition to using DMSO, reported that the chemiluminescence of cypridinid luciferin in the protic solvent diglyme made weakly basic with acetate had a maximum at 430 nm and that this spectrum was close to the fluorescence of the neutral amide that had a 421 nm maximum. With the strong base t-butoxide in this protic solvent, the product fluorescence had a maximum at 530 nm. It was concluded that the two bands observed in the \textit{in vivo} bioluminescence (Fig. 1) were from both the neutral (466 nm) and anionic (548 nm) products, respectively. Hirano (140) extended the chemiluminescence model studies and revealed the influence of R2 substituents (Fig. 5) on the acidity of the amide N. This substitution position influences the acidity of the N-1, and therefore, the extent of protonation of the dioxetanone, and whether or not in the bioluminescence of imidazopyrazinone luciferins, the neutral or anionic excited product is dominant.

Usami and Isobe (141) investigated a model chemiluminescence using low-temperature solvent conditions, which allowed the intermediates to be trapped and identified using $^{13}$C-NMR (Fig. 5). A $^{13}$C-enriched imidazopyrazinone derivative ($R^1 = \text{benzyl, } R^2 = \text{p-methoxyphenyl, } R^3 = \text{t-butyl-methyl}$) was reacted with singlet oxygen ($^1O_2$) at $-95^\circ\text{C}$ in methylene chloride and the product identified as having a 2-hydroperoxy substituent. In a mixed solvent at $-78^\circ\text{C}$ (Fig. 5, lower path), the dioxetanone derivative itself was structurally identified. On warming to $-50^\circ\text{C}$, a broad chemiluminescence emission was observed having a maximum at 400 nm, but it shifted to a 475 nm maximum as the temperature rose to $-10^\circ\text{C}$. It was proposed that the neutral dioxetanone derivative was less stable than the anionic dioxetanone and that there were these two pathways to the

Figure 4. Two-dimensional representation of the ligand interactions within the binding cavity. Upper left: WT obelin; Lower left: Y138F obelin. Upper right: Ca$^{2+}$-discharged obelin; lower right: Ca$^{2+}$-discharged Y138F obelin. Implied hydrogen bonds are the dotted lines, and the numbers in bold are the separations in A units. W$_1$ and W$_2$ are protein-bound water molecules. (Adapted from reference 137, and reproduced with permission of the International Union of Crystallography).
excited product, with the neutral species occurring in the lower pathway being less stable (29,140,141). Singlet oxygen was employed for the oxygen addition because it has no spin prohibition and reacts readily with the imidazopyrazinone at -95°C and, depending on conditions, it will go via the upper pathway to the 2-peroxyl derivative or via the lower to the neutral dioxetanone. Under basic conditions, the neutral dioxetanone may lose a proton to form the anion, but no reprotonation is feasible from the excited anion product (142).

The bioluminescence of aequorin and the fluorescence of the protein-bound product are the same with a maximum at 465 nm, and assigned as from the coelenteramide anion. All Ca²⁺-regulated photoproteins have a unimodal bioluminescence band with maximum in the range 470-495 nm depending on the type. However, obelin shows a small shoulder around 400 nm and W92F obelin produces a violet bioluminescence color, and the spectral distribution is bimodal with maxima at 400 and 475 nm (132, 135). The shorter wavelength band corresponds to the fluorescence of coelenteramide in the neutral state, and its bioluminescence intensity is greater than the band at 475 nm, but is the lesser if the reaction is carried out in D₂O. This last result is consistent with a rate-determining protonation of the neutral and anionic dioxetanones in the chemiluminescence model (Fig. 5).

In the coelenterazine binding cavity of obelin (Fig. 4, upper left), in addition to the H-bond to Tyr190 (Y190) already mentioned that stabilizes the hydroperoxide, there is an H-bond between N-1 and Tyr138 and onward to a protein-bound water, W₂. The overall nature of the obelin binding cavity does not

![Figure 5](image)

**Figure 5.** Chemiluminescence of an imidazopyrazinone derivative at low temperature reveals two pathways accounting for the two bioluminescence bands from a Cypridina specimen (Fig. 1) and the two chemiluminescence bands of cypridinid luciferin. (Adapted from reference 141). The asterisks symbolize the excited electronic state of the products.

![Figure 6](image)

**Figure 6.** Stereoview representation of the Clytia GFP-clytin complex derived from crystal structures of clytin and Clytia GFP, NMR mapping of the interaction surfaces and computational docking. This research was originally published by Titushin et al. (129).
change much after Ca\(^{2+}\)-discharge (Fig. 4, upper right), except for H175 which shifts and reorients to the vertical from the plane, and Tyr138 moves out of the cavity being replaced by W2 for H-bonding to the now amide nitrogen. The Tyr138 is not essential for the bioluminescence because Y138F obelin has only a 30% lower light yield, a 10 nm spectral shift to 493 nm, and several times slower bioluminescence kinetics rate constants. In the cavity structure of Y138F obelin (Fig. 4, lower left), there is no H-bond to N-1 but following the bioluminescence with Ca\(^{2+}\), the same W2 has moved into the amide H-bonding position (Fig. 4, lower right) (137). It is proposed that the function of the water molecule in this location is to catalyze the 2-hydroperoxycoelesterazine decarboxylation reaction by protonation of a dioxetane anion and speed its decomposition to the neutral excited-state product and violet emitter, according again to the chemiluminescence model of Fig. 5.

The organisms using coelesterazine for bioluminescence have in common with bacterial bioluminescence of being able to tune the emission using an antenna protein. The story of GFP is well known, and the mechanism of its action was first investigated systematically in the case of the luciferase-coelesterazine bioluminescence system of the sea-pancy, Renilla reniformis (143–146). Renilla luciferase is not a Ca\(^{2+}\)-regulated photoprotein, but like the firefly system, the addition to Renilla luciferase of its luciferin, coelesterazine, in the presence of oxygen generates a broad blue bioluminescence with maximum 485 nm and \(\Phi_b = 0.07\) (146). The emission corresponds to the fluorescence of the luciferase-bound product coelesteramine anion. However, the inclusion of Renilla GFP at only \(\mu M\) concentrations shifts the bioluminescence maximum to 509 nm with a spectral distribution the same as the fluorescence of Renilla GFP. It was proposed that for these \(\mu M\) concentrations to be effective, there would have to be a protein–protein complex formed for an efficient FRET between the Renilla luciferase and the GFP.

In the more than 20 years following these experiments with Renilla bioluminescence, there have been many advances that allow modern biophysical techniques to be recruited. Recently, we employed the recombinant proteins, clytin and Clytia GFP from the jellyfish Clytia gregaria, for a study of their bioluminescence properties (147). The Ca\(^{2+}\)-triggered clytin bioluminescence in vitro is a broad spectrum with maximum at 475 nm. The inclusion of \(\mu M\) concentrations of Clytia GFP is enough to shift the bioluminescence maximum to 500 nm where it is a match for the fluorescence of the Clytia GFP, the same observation as found with the Renilla system and bacterial bioluminescence with the inclusion of lumazine protein. The effect is less with obelin and Clytia GFP, suggesting specificity in the interaction, as also observed with the above mentioned other cases (144). The same argument used previously was that the spectral effects at these concentrations of Clytia GFP implied the presence of a protein–protein complex with \(K_D\) in the \(\mu M\) range but a weak complex was detected by observing the fluorescence intensity (150). This intriguing phenomenon of weak complex to be able to engage in this bioluminescence FRET with a micromolar interaction must be one formed transiently by the Clytia GFP dimer and Clytia GFP with \(K_D \approx 2\) nm (84). Within the dimer, the two fluorophores have a coupled electronic state transition. It needs to be noted that GFPs present a special case of “homeoenergy transfer” in which the donor–acceptor electronic transitions are the same, distinct from the cases of “heteroenergy transfer” in bacterial bioluminescence described above. A FRET analysis of fluorescence anisotropy decay experiments resulted in a Förster separation of 32 Å between the Clytia GFP fluorophores in satisfactory agreement with 25 Å in the spatial structure of the dimer (84,129). Many attempts failed at obtaining crystals of a clytin-Clytia GFP complex, and from fluorescence anisotropy measurements, there is also no evidence of any interaction with Ca\(^{2+}\)-discharged clytin (84).

A weak interaction of clytin with Clytia GFP with \(K_D \approx 2\) nm was detected by NMR chemical shift perturbation analysis, and the structure of the complex was estimated by computational docking of the spatial structures constrained by the NMR-identified residues on the protein surfaces involved in the association (129). Complete NMR assignments were determined using \(^{13}C\) and \(^{15}N\)-enriched proteins with reference to the crystal structures. The weak complex was detected by observing the \(^{15}N\)-HSQC chemical shifts that were altered on mixing the two proteins, first with \(^{15}N\)-enriched clytin and the Clytia GFP unlabelled, then the reverse. These chemical shifts are remarkably sensitive to subtle conformational changes, so in this way just those residues that were engaged in the interaction were mapped and entered as constraints for the computational docking.

A low-energy computational structure shows a separation of 45 Å between the putative donor and the GFP fluorophore as acceptor, which combined with a large spectral overlap would provide very efficient FRET (Fig. 6). In addition, as the GFP is dimeric, the active complex can accommodate two clytins and might naturally occur as a heterotetramer (63,84,129).

It was proposed that such a weak complex to be able to engage in this bioluminescence FRET with a micromolar interaction must be one formed transiently by the Clytia GFP dimer with a short-lived (milliseconds) intermediate in the clytin reaction pathway (84). The same appears to be the case with the Aequorea GFP effect on aequorin bioluminescence. Aequorea bioluminescence is the same as Aequorea GFP fluorescence (148) but in vitro, added GFP has no effect nor is there any detectable aequorin–GFP complex (144). However, a recombinant aequorin–GFP fusion protein does generate the same green GFP emission as in vivo (149). Stopped-flow experiments, one using this fusion protein as well more recently, clytin and Clytia GFP in free solution, revealed that in the course of the <10 ms rise time of the bioluminescence after Ca\(^{2+}\) addition, the efficiency of bioluminescence FRET measured by the 510/470 nm intensity ratio increased at the same rate as the rise of bioluminescence intensity (150). This intriguing phenomenon of “GFP-FRET” is a rich subject for a productive physical–chemical study (… the “Endless Frontier”).

CONCLUSION

The three bioluminescence systems that have received most study from the point of view of mechanism are ones that could provide material in “chemical” quantities, viz. the bacteria, firefly and coelesterazine reactions. The first important step was finding the different chemical structures of the different luciferins, then defining the chemical steps in the light pathway and determining
quantum yields, always critical for elucidating any photochemical process. Chemiluminescence models provided a basis for mechanistic proposals. The discovery of the “light-stick” reaction as involving a high-energy intermediate, a highly strained cyclic peroxide, suggested the idea of a dioxetane intermediate in the firefly and cypridinid luciferins reactions, and later for coelenterazine. Oxygen-18 experiments supported the dioxetane proposal and that the product excitation originated from a decarboxylation. No evidence has been found for such a pathway for bacterial bioluminescence.

The short lifetime of most bioluminescence intermediates hinders the determination of their chemical structure. However, conditions for stabilization of a bacterial luciferase intermediate allowed its identification by $^{13}$C-NMR as 4a-hydroperoxyflavin. Intermediates following in the reaction have not been directly identified including a highly fluorescent species that is the origin of the bioluminescence. A great advantage of study of the Ca$^{2+}$-regulated photoproteins is that they hold a stable coelenterazine hydroperoxide that has been directly visualized in their spatial structures derived from X-ray crystallography. The application of modern biophysical techniques has become more feasible by the availability of many recombinant proteins involved in bioluminescence.

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