Firefly luminescence: A historical perspective and recent developments†

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Significant advances have occurred regarding our knowledge of firefly luciferase mechanisms. Although most of this progress was an outcome of molecular biology and structural studies, important achievements have also occurred on its fundamental chemistry. Those developments are here summarized and presented in a historical perspective.

1. Introduction

Few natural phenomena are as deeply fascinating as bioluminescence, the emission of light by living organisms. With its esoteric charm, it has attracted mankind since early times.[1–3]

While the vast majority of bioluminescent organisms live in the ocean, there are many terrestrial forms, notably beetles (Coleoptera) in the families Lampyridae (the fireflies), Phengodidae (rairoad worms) and Elateridae (click beetles).[4,5] Although there are about 1800 species of luminous beetles the fundamental knowledge of the biochemistry of beetle bioluminescence has been largely based on a single species *Photinus pyralis*, the common North American firefly.

Significant advances have occurred since the last review on firefly luciferase (Luc).[4] Our main purpose here is to summarize those developments, integrating them in an account of our present understanding of the firefly system summarized in a loosely chronological order.

2. First studies and luciferin adenylation

The French physiologist Raphael Dubois carried out the first studies on the biochemistry of the bioluminescence of Coleoptera.[1,6] In 1885, Dubois obtained a luminescent solution upon adding cold water to ground up abdomens of an Elateridae beetle. The light produced with cold water rapidly faded, but in contrast he could observe no light emission when using a hot water extract. However, when cooled, this solution increased the bioluminescence observed from the cold-water solution. As a result Dubois concluded that the solutions contained two different compounds: in the cold water solution both were intact, but in the hot water solution, the heat had destroyed one of the components. When the hot solution was cooled and added to the exhausted cold solution it became luminous again because the component that was used up in the cold solution was precisely the one that was not destroyed by the heat. Dubois called the molecule that was consumed in the bioluminescence reaction luciferin and the component that was destroyed by the heat luciferase. Those definitions were adopted to define the substrate responsible for the light emission (the molecule whose oxidation to oxyluciferin results in photon emission) and the enzyme, respectively.

The research of Dubois was followed by that of an American scientist, Newton Harvey.[3] Harvey studied several bioluminescence systems and showed that within each system there was specificity between the luciferins and the luciferases. One aspect that was, however, common to all systems was the dependence on oxygen, as first observed by Robert Boyle in the XVIII century. Using an evacuated bell jar, Boyle demonstrated that he could extinguish the luminescence of rotten wood (fungus) and meat (bacteria) by removing air[1]. Besides luciferin and luciferase, O_2 is required for all bioluminescence to occur.[7]

While at Princeton Harvey accepted William McElroy as a PhD student and this would represent the start of a life-long study of firefly bioluminescence. McElroy's research was seminal for a large number of future researchers starting the work at his lab.[8,9]

The light production in fireflies occurs in organs called lanterns that contain specialized photocytes, located between two rows of cells, one thin external and one interior filled with uric acid crystals that reflect the light produced by the photocytes. Large quantities of enzyme could be obtained from grinding firefly lanterns and, pragmatically, McElroy used massive numbers of fireflies to obtain the required enzyme.[9]

In 1947, confirming the results of Dubois and Harvey, McElroy observed that lantern extracts produced luminescence.[10] By that time the function of ATP as a high energy molecule had been proposed and McElroy experimented with the addition of ATP to a cold water extract whose bioluminescence had ceased, demonstrating for the first time that light emission was proportional

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to ATP.[8,10] The ATP requirement suggested that its hydrolysis could be important for the energetic of the light emission but this hypothesis was soon rejected. The energy associated to the emission of a mole of yellow-green photons, as in *Photinus pyralis*, was far superior to that corresponding to the hydrolysis of ATP.[11]

The conditions that influenced the bioluminescence reaction, namely temperature and pH, were studied but the interpretation of those results was limited, as McElroy acknowledged,[12] by the purity of the substrates and enzyme used. Nonetheless, the bioluminescence was found to be dependent on four components: oxygen, the enzyme, the substrate luciferin (LH_2) and ATP·Mg^{2+}. The reaction was shown to consist of two steps, a first step independent of O_2 in which ATP·Mg^{2+} and LH_2 reacted, and a second step corresponding to the oxidation and light emission (Fig. 1).[13]

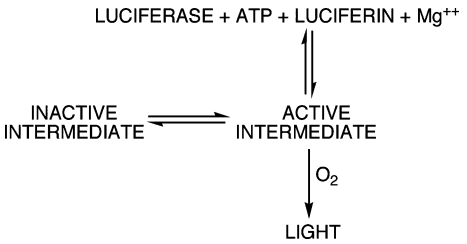

Fig. 1 Mechanism for firefly bioluminescence as proposed by McElroy group in 1953.[13] Despite its simplicity, this model already predicted the existence of two sequential steps; a first step involving luciferase, luciferin and ATP results in the formation of an intermediate (later identified as D-LH_2–AMP), which is then oxidized by O_2 in a second step with light emission.[13]

The crystallization of Luc allowed quantitative studies.[14] Noting the formation of PPi and AMP, Green and McElroy[14,15] concluded that LH_2 and ATP·Mg^{2+} form an adenylated intermediate, luciferyl–adenylate (LH_2–AMP). LH_2–AMP formation was supported by an LH_2-dependent ATP–PPi exchange reaction; the incorporation of radioactive PPi into ATP would result from the pyrophosphorolysis of the adenylated intermediate (Fig. 1, reaction (1)). According to this mechanism LH_2–AMP should accumulate under anaerobic conditions and indeed the readmission of O_2 to anoxic mixtures that contained all the components necessary to light production resulted in a brilliant flash of light.[13]

$$\text{Luc} + \text{ATP}\cdot Mg^{2+} + \text{D-}LH_2 \rightleftarrows \text{Luc}\cdot\text{D-}LH_2\text{–AMP} + \text{PPi}\cdot Mg^{2+} \quad (1)$$

$$\text{Luc}\cdot\text{D-}LH_2\text{–AMP} + O_2 \rightarrow \text{Luc} + CO_2 + \text{AMP} + \text{oxyluciferin} + \text{photon} \quad (2)$$

The LH_2–AMP mechanism was further supported by the first insights on LH_2 structure; 1500 fireflies were collected to obtain the 9 mg of LH_2 that allowed its partial characterization as a carboxylic acid with a phenol group (Fig. 2).[16] The carboxyl group was essential for ATP activation, which was prevented if LH_2 was converted into its methyl ester. The adenylation mechanism was established when Rhodes and McElroy obtained light production using chemically synthesized LH_2–AMP, thus bypassing the adenylation step.[17,18] The activation reaction was very specific for ATP, not occurring with UTP, CTP, GTP and ITP; only p_4A was able to promote a weak bioluminescence. Presently it is known that besides p_4A only dATP, ATPγS and Ap_5A can replace ATP although with weaker efficiencies.[14,19–23]

Like LH_2, a product formed during the bioluminescence reaction, at the time named oxyluciferin (now called dehydroluciferin, L), also produced an adenylated intermediate (dehydroluciferyl–adenylate, L–AMP) (Fig. 3, reaction (3)).[15,17,18]

$$\text{Luc} + \text{ATP}\cdot Mg^{2+} + \text{L} \rightleftarrows \text{Luc}\cdot\text{L–AMP} + \text{PPi}\cdot Mg^{2+} \quad (3)$$

In 1961 the chemical structure of LH_2 was determined.[24,25] Firefly luciferin, 2-(4-hydroxybenzothiazol-2-yl)-2-thiazoline acid, is a unique compound characterized by a highly reactive and easily oxidizable thiazoline ring (Fig. 2).[26] Its structure was confirmed by total synthesis. In the last step of synthesis of LH_2, 2-cyano-6-hydroxybenzothiazole is reacted with cysteine. When D-cysteine is used, D-LH_2 is obtained, whereas when L-cysteine is used, L-LH_2 is obtained. In the presence of Luc and ATP·Mg^{+2} both isomers

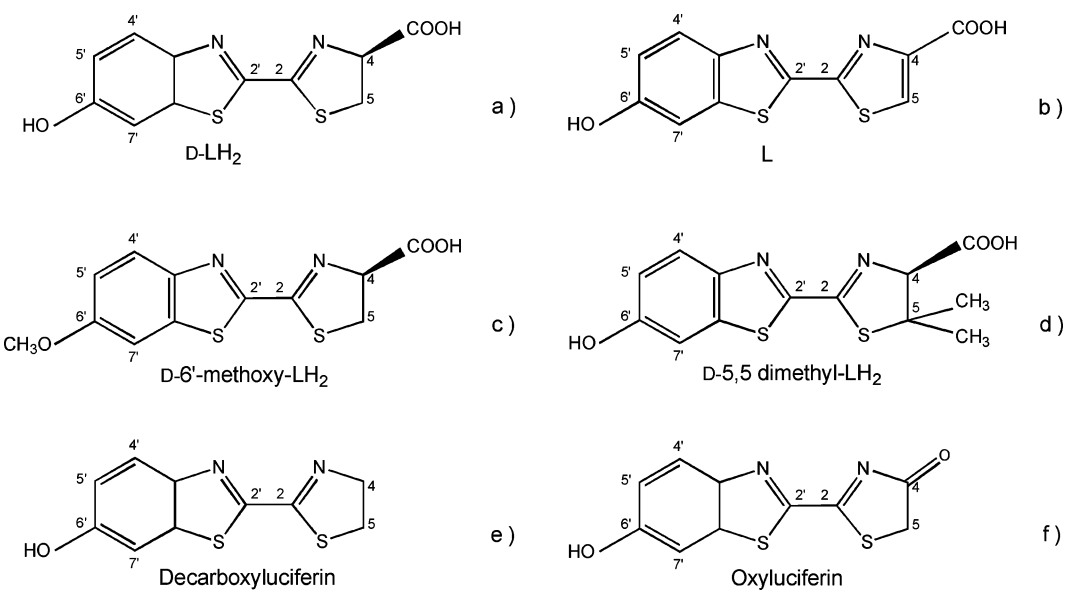

Fig. 2 Structures of D-luciferin (D-LH_2) (a), dehydroluciferin (L) (b), D-6′-methoxy-luciferin (D-6′-methoxy-LH_2) (c), D-5,5-dimethyl-luciferin (D-5,5-dimethyl-LH_2) (d), decarboxyluciferin (e) and oxyluciferin (f).

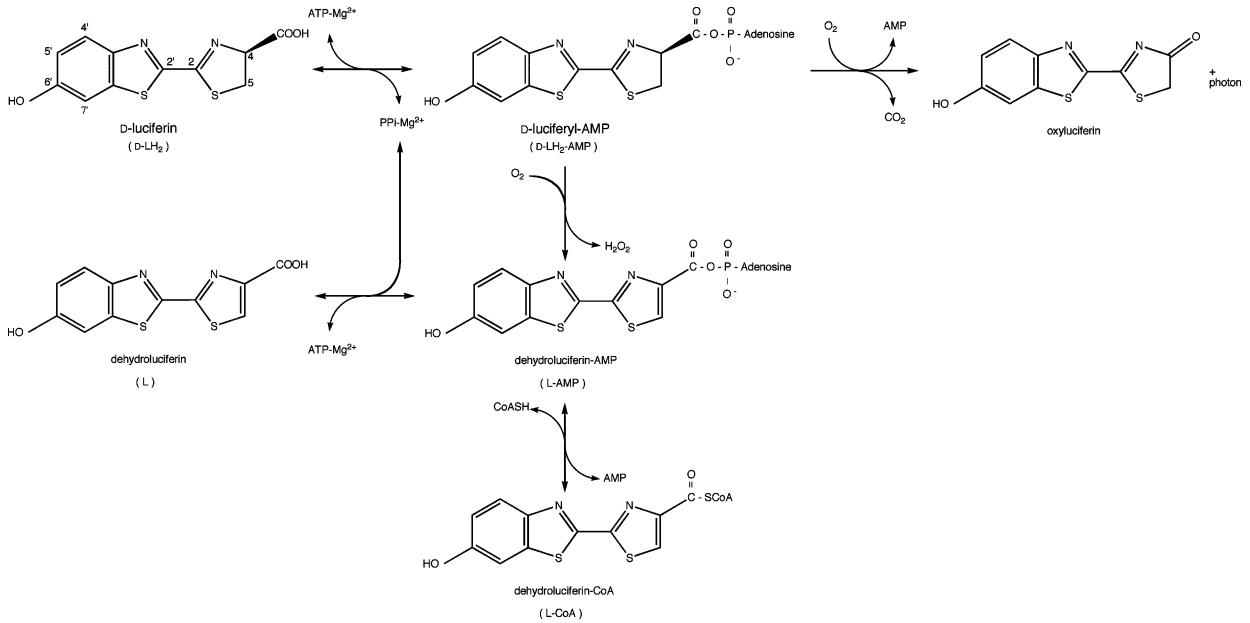

Fig. 3 The bioluminescent reaction involves the formation, from D-LH_2 and ATP, of an adenylyl intermediate (D-LH_2–AMP) and its subsequent oxidation with release of AMP, pyrophosphate (PPi), CO_2 and oxyluciferin, the light emitter. In parallel, D-LH_2–AMP is oxidized in a different way, giving rise to dehydroluciferyl–adenylate (L–AMP), which binds to luciferase inhibiting the light reaction. CoA can react with L–AMP giving rise to L–CoA.

were adenylated but only D-LH_2 resulted in O_2 consumption and respective light emission[27,28]. The non biolumiluminescent L-isomer behaved as an inhibitor of light emission.[29,30]

Recently Lembert presented evidence that light emission could be obtained from L-LH_2.[29] From the data it is not clear if L-LH_2–AMP is directly involved or if the chemical racemisation of L-LH_2–AMP into D-LH_2–AMP could account for the very dim light emission. The prompt racemization of D-LH_2–AMP into L-LH_2–AMP was first described by Seliger and coworkers and confirmed more recently by our group in Portugal for the enzyme-formed adenylate.[27,31] The existence of an equilibrium between free and enzyme bound adenylated could explain the light emission observed.[32]

3. The oxidation of LH_2–AMP and light emission

In contrast with the adenylation reaction, essentially clarified in the 1960s, except for the effect of oxygen concentration,[13] little was known with respect to the second step, the oxidation of LH_2–AMP.

In 1962, Seliger and McElroy observed a 1 : 1 stoichiometry between LH_2 and O_2 and proposed that O_2 would add to LH_2–AMP to form a linear hydroperoxide.[28,33] At that time the use of dipolar aprotic solvents was introduced for the study of luminol chemiluminescence and with this technique it was observed that LH_2–AMP was chemiluminescent in DMSO with base. Light production also occurred when instead of LH_2–AMP, methyl esters or phosphate anhydrides of LH_2 were used.[33] These results were in agreement with a non-energetic role of ATP. The function of ATP would be to increase the acidity of the C_4 proton of the thiazoline ring allowing sequential proton removal and carbanion formation; otherwise, the pH required to remove the C_4 proton from LH_2 would unavoidably destroy the molecule.[33]

Later in the decade, as a corollary of chemiluminescence models,[6,28,34,35] a mechanism for light emission was proposed, developed independently by the groups of Emil White and Frank McCapra. They postulated that the reaction of O_2 and the carbanion would result in the formation of a hydroperoxide on C_4 of the thiazoline ring (Fig. 4).[36,37] The subsequent removal of AMP, a good leaving group, would result in the formation of a cyclic peroxide with a carbonyl group (the dioxetanone ring), whose break-up generated CO_2 and excited state oxyluciferin. The collapse of the dioxetanone could fulfill the high energetic requirements of the bioluminescence reaction. The relative weakness of the peroxide O–O linkage, the strain energy stored in the ring and the formation of two carbonyl compounds all in one unimolecular reaction, would yield sufficient energy to populate the excited state of oxyluciferin.[6,7,34,38] According to the model, firefly oxyluciferin would correspond to 2-(6′-hydroxybenzothiazol-2′-yl)-4-hydroxybenzothiazole (Fig. 2) but the first experimental evidence for the dioxetanone mechanism was only obtained with 5,5-dimethyl-luciferin (5,5-dimethyl-LH_2, Fig. 2).[36,37] The use of LH_2 analogs allowed the identification of the corresponding oxyluciferin, whose fluorescence emission spectra matched the spectrum of the light emitted. Extending this line of evidence, the formation of labeled CO_2 was detected using either LH_2 labeled in the carboxylic group or $^{18}O_2$.[39–41]

While the dioxetanone mechanism anticipated the chemical structure of oxyluciferin, the first attempts to isolate this compound failed. According to White, the difficulties were the result of the tendency of thiazolines, the chemical group of oxyluciferin, to polymerize.[34,36,39,42] It was against those odds that Goto's group was able to obtain oxyluciferin in a state of purity that allowed the confirmation of the structure predicted by the dioxetanone mechanism.[43–46] Those results were validated by the group of DeLuca.[47,48] Using the increase in absorbance at 385 nm as

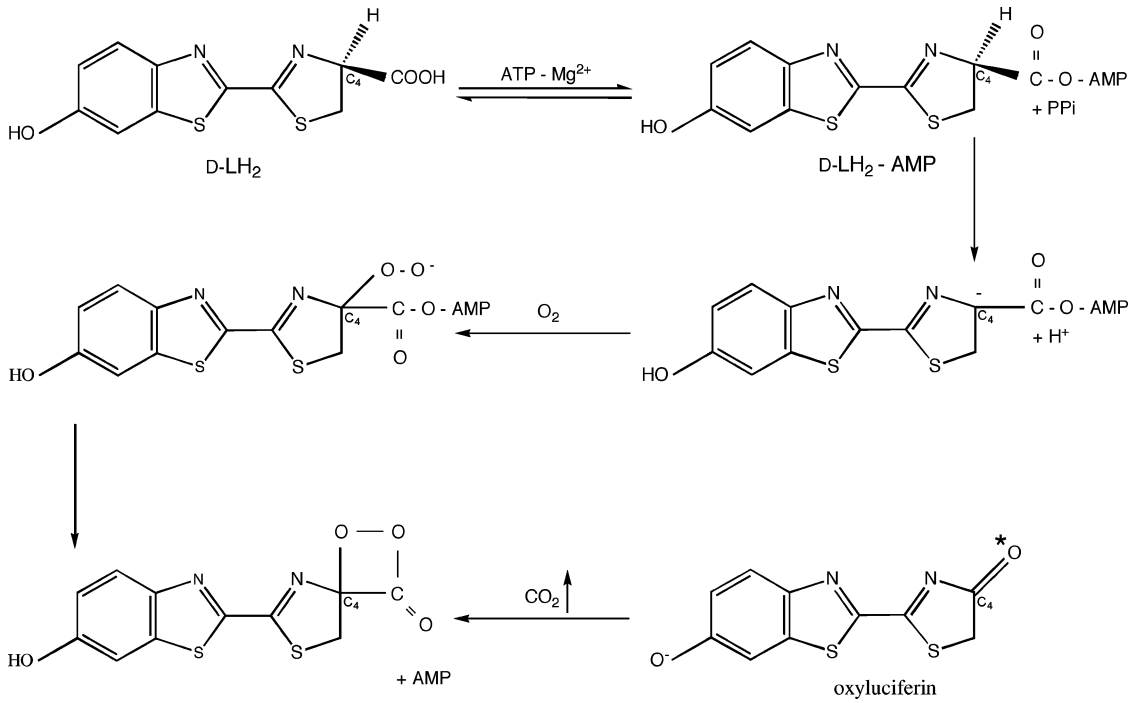

Fig. 4 Mechanism of the bioluminescence reaction as proposed by Emil White and Frank McCapra. Following D-LH_2 adenylation, proton removal and peroxide formation, a dioxetanone ring is created whose break-up results in the production of excited state oxyluciferin (represented by *). The decay of excited oxyluciferin to the ground state is the process responsible for light emission.

a measure of oxyluciferin formation, they proved that it was parallel to photon production.[47,48] In spite of this, the unequivocal identification of oxyluciferin as a reaction product came only in 1980 when oxyluciferin was first isolated as the product of LH_2 chemiluminescence.[41]

In 1998, using RP-HPLC and analyzing spent mixtures, Fontes and coworkers were able to identify four main enzymatic products, the previously described L and L–AMP, and two unknown compounds attributed to degradation products of oxyluciferin.[49,50] This work was the prelude to the isolation of enzymatically formed oxyluciferin[51]; similar approaches were used to identify oxyluciferin from enzyme reaction mixtures of D-5,5-dimethyl-LH_2 adenylate and in the analysis of chimeric luciferase enzymes.[52,53]

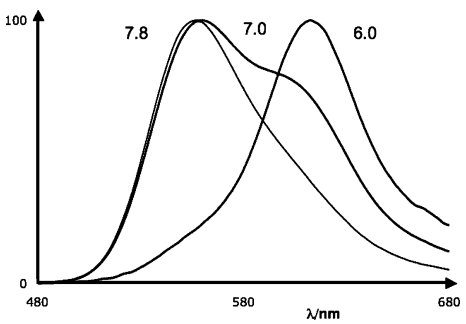

Fig. 5 *Photinus pyralis* bioluminescence spectra at pH 7.8, 7.0 and 6.0. The spectra are normalized; the intensity of emission is significantly lower at acid pH.

4. Emission spectrum and quantum yield

Among reaction products, the photon is undoubtedly the most important; few can argue that if not for the light, Luc would not have been rescued from the obscure beetle biochemistry.

Following the discovery of the ATP requirement, the first reference to the *in vitro* emission spectra of Luc dates from the late 1940s; the light emission produced at pH 7–8 had a maximum at 562 nm extending from 500 to 650 nm.[28,54,55] This spectrum is easily red shifted by diverse factors including pH, metal cations, increase in the temperature and the substitution of LH_2, ATP or by replacing LH_2–AMP by several analogues.[23,27,55,56]

Among these factors the most attention has been given to the effect of pH. In *Photinus pyralis* the emission spectrum is red-shifted as the pH is acidified, having a maximum at 620 nm at pH 5–6 (Fig. 5).[56] Different models were advanced with the common belief that the shifts in the emission spectrum resulted from modifications in Luc structure. Indeed that conclusion is strongly supported by the fact that despite the fact that all beetles use D-LH_2 as substrate the emission can vary greatly according to the species.[4,55] In addition, changes in the enzyme amino acids can result in dramatic shifts of the emission spectrum.[4,57–60]

One model was proposed by White and coworkers on the basis of fluorescence and chemiluminescence studies of LH_2 analogs. According to White the different emission spectra corresponded to different tautomers of the emitter oxyluciferin; hence red emission would result from oxyluciferin in the keto form while green emission would result from oxyluciferin in the enol form.[41,61] Basic residues in the enzyme active site would promote this tautomerization; in fact, Luc active site methylation resulted in a red emitting enzyme.[62] However, Branchini and coworkers later demonstrated that green emission could be obtained from

5,5-dimethyloxyluciferin, a compound where tautomerization cannot occur and which is constrained to the keto form.[52]

McCapra and coworkers proposed that, instead, the different colors result from different conformations of oxyluciferin depending on the angle between the benzothiazole and thiazolines rings along the C_2–$C_{2'}$ axis.[63] An angle of 90° between the rings would correspond to the lowest energy state and to red emission, while an angle of 0° would correspond to the highest energy state and green light, with the structure of Luc active site determining the angle. However X-ray studies as well as higher order molecular quantum mechanical calculations do not support this hypothesis.[60,64,65]

The consensus of all recent discussions is, evidently, that the enzymatic microenvironment of the keto form of oxyluciferin in its excited state determines the precise resonance form from which emission occurs.[60,64,65] From the results of the most recent computations, Nakatani and coworkers propose that the emission from the keto form of oxyluciferin is spectrally tuned by its protonation state and resonance structure imposed by Luc.[65] A further analysis is outside the scope of this review.

Another aspect of bioluminescence requiring discussion is its efficiency, commonly referred to as its quantum yield (Q).[34] Q is defined as the ratio of the number of photons emitted by the reaction to the number of molecules that reacted, *i.e.* the number of luciferin molecules oxidized. This value can be factored into three components: the fraction of the reaction that produces the potential light emitter (the oxyluciferin) Φ_p, the fraction of oxyluciferin that is formed in a excited state Φ_{ex} and finally the fraction of those excited states that produce light Φ_{fl} (*i.e.* oxyluciferin fluorescence yield); the overall efficiency Q resulting from: $Q = \Phi_p\Phi_{ex}\Phi_{fl}$.[34,41]

The first Q quantification for Luc bioluminescence dates from 1960; using high Luc and ATP concentrations in order to achieve complete LH_2 consumption, firefly Q was determined as 88 ± 12%.[56] This value was dramatically influenced by the pH and dropped at acid pH, a likely consequence of a decrease in the oxyluciferin fluorescence yield.[66] As expected, the bioluminescence Q was far superior to the one observed by White for the chemiluminescence of the ethoxyvinyl ester of LH_2 ($Q = 0.09$ with non-limiting O_2).[41]

It is clear that with a quantum yield as high as 0.88, each component involved in the overall emission (Φ_p, Φ_{ex}, Φ_{fl}) must be highly efficient.[56,67] This requires that a reaction without side products be coupled to a very efficient excited state formation followed by an efficient emission. Taking this into consideration, it is somehow surprising that this value was not re-examined until recently. This is even more evident considering that this Q determination predated the elucidation of LH_2 and oxyluciferin structures and, more important, that the D-LH_2 used in the assays was still obtained from fireflies and therefore a mixtures of enantiomers. This motivated a Japanese group to proceed with a new Q determination; they obtained a maximum value of 41%, based on the luminol standard.[68]

5. Firefly luminescence and CIEEL mechanism

The dioxetanes and dioxetanones proposed as intermediates in bioluminescence and chemiluminescence reactions were anticipated to be highly unstable molecules. With the synthesis of 3,3,4-trimethyl-1,2 dioxetane, Kopecky and Mumford[69] were the first to prove that such compounds could actually be prepared. In agreement with the involvement of these compounds in light emitting reactions, the thermal decomposition of the trimethyl-1,2-dioxetane resulted in weak blue light emission, a result amply confirmed in the following years for the numerous dioxetanes produced in different laboratories.[70]

Curiously, as the number of dioxetanes studied increased, it also became evident that although their decomposition generated excited states, those excited states were predominantly triplet states. In solution, triplet states are quickly quenched and the energy is dissipated through non-radiative pathways, that is, without photon emission. It was clear that dioxetane and dioxetanone decomposition failed to explain the efficient formation of singlet states observed in bioluminescence.[70,71]

In the 1970s Schuster observed that the decomposition of diphenoyl peroxide and dimethyl dioxetanone could be catalyzed by compounds with low oxidation potential and high fluorescence yields, so-called activators.[72] In their presence, peroxides whose decomposition produced mainly triplet states, could lead, according to Schuster, to a highly efficient production of singlet excited fluorescers with yields comparable to those observed in bioluminescence reactions. The emission spectrum obtained under those conditions corresponded to the fluorescence spectrum of the activator used and the catalytic effect of the activator was inversely proportional to its ionization potential. To account for these results, the "Chemical Initiated Electron Exchange Luminescence" (CIEEL) mechanism was proposed. According to the CIEEL theory, an electron was transferred from the fluorescer, as an electron donor to diphenoyl peroxide as an acceptor, provoking its decomposition and forming a fluorescer pair, radical cation/radical anion pair with the extrusion of CO_2. Back electron transfer would leave the fluorescer in the singlet-excited state.

Surveying the literature, it is clear that results similar to the ones described by Schuster were previously observed in other chemiluminescence systems.[71,73] In fact Rauhut[38] and McCapra[71] described a similar dependence between the luminescence yield and electron donor capacity of the fluorescer in the highly efficient peroxy/oxalate system. The CIEEL mechanism had, however, the virtue of grouping under a common mechanism distinct systems, and could potentially explain the discrepancy between the low quantum yields observed in chemiluminescence and the high quantum yields of bioluminescence.[74]

The application of the CIEEL mechanism to firefly bioluminescence was an interesting step. Koo *et al.*[75] proposed that in the firefly electron transfer should occur intramolecularly between the deprotonated phenolic group of LH_2 and the dioxetanone ring. Indeed, the phenolic group of LH_2 was known to be deprotonated in the excited state and analogues of LH_2, like D-6′-methoxyluciferin (D-6′-methoxy-LH_2), lacking an electron donor group, were unable to produce significant light production.[75,76]

The potential of the CIEEL mechanism was explored in the 1980s, namely with the development of several molecules with the chemical characteristics of LH_2 but its relevance as a unifying model for bioluminescence has been questioned.[71,77] Indeed, in a re-examination of Schuster's model system diphenoyl peroxide, Catalani and Wilson[78] found that its quantum yield was over estimated by many orders of magnitude, a result not contested by Schuster.[78] The quantum yield for this reaction is in fact 2×10^{-5}; the originally reported value was 0.1,[79] evidently a

very low value for a mechanism proposed for the highly efficient bioluminescence.[71,78] For the firefly few experimental studies have attempted to check the CIEEL mechanism.[80] Usually this mechanism is presented, in the absence of an alternative, as responsible for light emission, but in fact experimental evidence for it are scarce. In 1980, White and coworkers, referring to the Schuster proposal and the non bioluminescence of the 6′-*O*-methyl ether of luciferin commented: "The two groups cite negligible or nonchemi- and bioluminescence of the 6′-*O*-methyl ether of luciferin in support of the electron transfer mechanism. The fluorescence efficiency of *O*-methyloxyluciferin has not been reported; however, in the event that the efficiency is low, the observations can be explained simply by the low fluorescence of the emitter. The claim that the methylated ketone itself fluoresces efficiently is not supported by references cited".[41] With those simple terms White refuted the CIEEL mechanism in Luc. In fact taking as a model the fluorescence of 6′-methoxy-luciferin, we can predict that the fluorescence of the respective oxyluciferin will be rather low,[81] contributing to the lack of luminescence with these compounds. Taking this into consideration the application of the CIEEL mechanism with complete electron transfer remains to be proven.[82]

6. Firefly luciferase is an acyl-CoA ligase

The similarities between firefly luciferase biochemistry and that of several ligases, including aminoacyl-tRNA synthetases and acyl and acetyl CoA synthetases, were already evident in the 1960s.[47,83] These enzymes have common properties, the most evident the formation of a highly reactive adenylate. Moreover, Luc could catalyze, in a mechanism identical to the one of acetyl and acyl CoA synthetases, the synthesis of L–CoA from L–AMP (reaction (4)).[83,84]

$$\text{Luc·L–AMP} + \text{CoA} \rightleftarrows \text{Luc} + \text{L–CoA} + \text{AMP} \quad (4)$$

Indeed those similarities were confirmed by the first molecular biology studies; beetle luciferases are homologous to many ligases that catalyze the adenylation of different carboxylic acids and subsequent thioesterification (see[4,5] for review). These enzymes were grouped under the name of "acyl-adenylate/thioester-forming" enzyme family.[85]

While the relationship between Luc and this large class of ligases has been regarded as an example of homology between different biochemical pathways, this perspective was recently changed. Indeed, Oba and colleagues demonstrated that Luc is a functional fatty acid CoA ligase.[86,87] These authors were also able to clone and characterize orthologous genes of Luc gene in *Drosophila* (CG6178) and in the mealworm *Tenebrio molitor*, whose protein products also possess acyl-CoA ligase activity.[88,89] The case of the mealworm experiments, a distant non-bioluminescent relative of fireflies, is particularly interesting, since protein extracts of this animal were shown to produce bioluminescence in the presence of ATP·Mg^{2+} and D-LH_2.[90,91] However and contrary to the prospect that the luciferase-like mealworm protein could catalyze a bioluminescence reaction, no light was observed with D-LH_2.[89] According to Oba, Luc and the *Drosophila* gene constitute a new family of acyl-CoA ligases belonging to the group of 4-coumarate: CoA ligases, with lauric acid as the preferred substrate. On the other hand, it is still unclear what the relevance of Luc is for beetle fatty acid metabolism since Luc is preferentially expressed in the lantern.[92]

Assuming that Luc evolved from a CoA ligase, several questions remain to be clarified, notably how can a monooxygenase evolve from a ligase? Some hypotheses have been advanced mainly on the basis of the chemiluminescence of LH_2–AMP.[91] As mentioned, and in spite of a lower quantum yield, LH_2–AMP is able to emit light in an enzyme free environment.[33] These results demonstrate that LH_2–AMP is intrinsically prone to oxidation and that Luc may function simply as an adenylation catalyst. Indeed, luminescence was observed with LH_2–AMP and albumin, and it is well know that Luc, in contrast to other oxygenases, does not contain any oxidative cofactor based on heme or Fe ^{III}. In this respect the reaction resembles an "autoxidation" (non-enzyme catalyzed reaction).[91,93]

In our view, although interesting, this evaluation is perhaps reductionist. Several ligases, including a chimeric protein constructed using N and C domains of Luc and the orthologous *Drosophila* gene, fail to elicit bioluminescence, and it is known that even in aprotic solvents, LH_2–AMP chemiluminescence only occurs with addition of a strong base.[33,53] Moreover, as mentioned, L-LH_2 is very efficiently adenylated to L-LH_2–AMP without significant bioluminescence (the emission observed is probably a result of racemization);[28,29] if light emission was a consequence of a non-catalyzed oxidation of enzyme formed LH_2–AMP the process should not be stereospecific. Probably Luc also plays a key role in the removal of the active proton from activated LH_2, with subsequent oxidation by O_2. Nevertheless, until now, all the efforts to identify the residue(s) involved in that presumed proton removal failed and it is unclear how Luc could efficiently activate O_2 without the aid of a cofactor.[4,94]

As recently stated by Day and coworkers, another aspect deserving consideration "is whether beetle luciferin was ever a productive substrate for the formation of luciferin–CoA *via* a beetle luciferin–CoA ligase activity where the oxidation reaction did not significantly compete with the ligase activity? If so, what metabolic pathway utilized the luciferin–CoA thioester?"[91]

In fact the enzymatic synthesis of luciferyl–CoA (LH_2–CoA) was first described in 2004 by our group in Portugal.[31] The mechanism for LH_2–CoA formation was analogous to the one observed for L–CoA, with the exception that LH_2–CoA synthesis from D-LH_2 occurred only under low O_2 concentrations, whereas L-CoA formation (from D-LH_2) occurs in the presence of O_2. Moreover, whereas L does not contain a chiral alpha carbon, LH_2 conserved the asymmetric center and could form either D-LH_2–CoA or L-LH_2–CoA.

Indeed, this ambiguity was recently clarified and, according to Nakamura and coworkers, Luc functions as an unusual enzyme, recognizing D-LH_2 for light emission and L-LH_2 for the formation of LH_2–CoA, according to the reactions (5) and (6):[30]

$$\text{Luc} + \text{ATP·}Mg^{2+} + \text{L–}LH_2 \rightleftarrows \text{Luc·L–}LH_2\text{–AMP} + \text{PPi·}Mg^{2+} \quad (5)$$

$$\text{Luc·L–}LH_2\text{–AMP} + \text{CoA} \rightleftarrows \text{Luc} + \text{L-}LH_2\text{–CoA} + \text{AMP} \quad (6)$$

Recently the chemical synthesis of LH_2–CoA was described by Fraga and coworkers.[95] This compound could, with the addition of AMP and in the presence of oxygen, result in the emission of light. However, the kinetics of LH_2–CoA bioluminescence with AMP

was markedly different from the flash profile of the "canonical" ATP reaction. Curiously, the rate of light production increased with incubation time, reaching a plateau after 10–20 minutes. Since the synthesis did not exclude the thioester racemization and chiral chromatography was not used, in what configuration of the LH_2–CoA was obtained is unclear. Meanwhile, an other path to obtain light from LH_2–CoA was described; using enzyme formed L-LH_2–CoA, Nakamura and coworkers were able to obtain light production taking advantage of thioester isomerization followed by unspecific hydrolysis.[96]

The Luc synthesis of L-LH_2–CoA appears to be relevant for the biosynthesis of D-LH_2, an old topic of discussion.[27] The spontaneous formation of LH_2 from 2-cyano-6-hydroxybenzothiazole and cysteine is generally accepted as the main route for its biosynthesis. But bearing in mind that the origin of the thiazoline ring is cysteine, it has always been difficult to explain the LH_2 configuration.[91,97] Natural aminoacids have L configuration and this implies that any process for LH_2 biosynthesis from cysteine must comprise a chiral inversion of its alpha carbon. Whether this would result from the activity of a specific racemase was an open question.[91]

Ohmiya's group proposed that this inversion could result from the stereospecific formation of L-LH_2–CoA from L-LH_2 followed by racemization and hydrolysis.[97] Supporting this mechanism, it was observed that soluble fractions of the light organs of fireflies were able to catalyze the CoA dependent inversion of L-LH_2 into D-LH_2 according to the mechanism present in Fig. 6.[97]

The similarities between this mechanism and the well-known *in vivo* epimerization of 2-arylpropionic acids (APA), known as profens, are evident.[98] These drugs, active in the *S* configuration, are converted from the *R* to the *S* configuration when administered to mammals. The mechanism for this chiral inversion involves three steps: a stereoselective activation of *R*-APA by formation of the acyl–CoA thioester in the presence of CoA and ATP followed by the enzymatic epimerization of *R* thioester into the *S* thioester and finally the release of free active *S*-APA by hydrolysis of the thioester. Interestingly, a Japanese group, motivated by this recent discovery, demonstrated that Luc could also catalyze the enantioselective thioester formation of 2-arylpropanoic acid.[99]

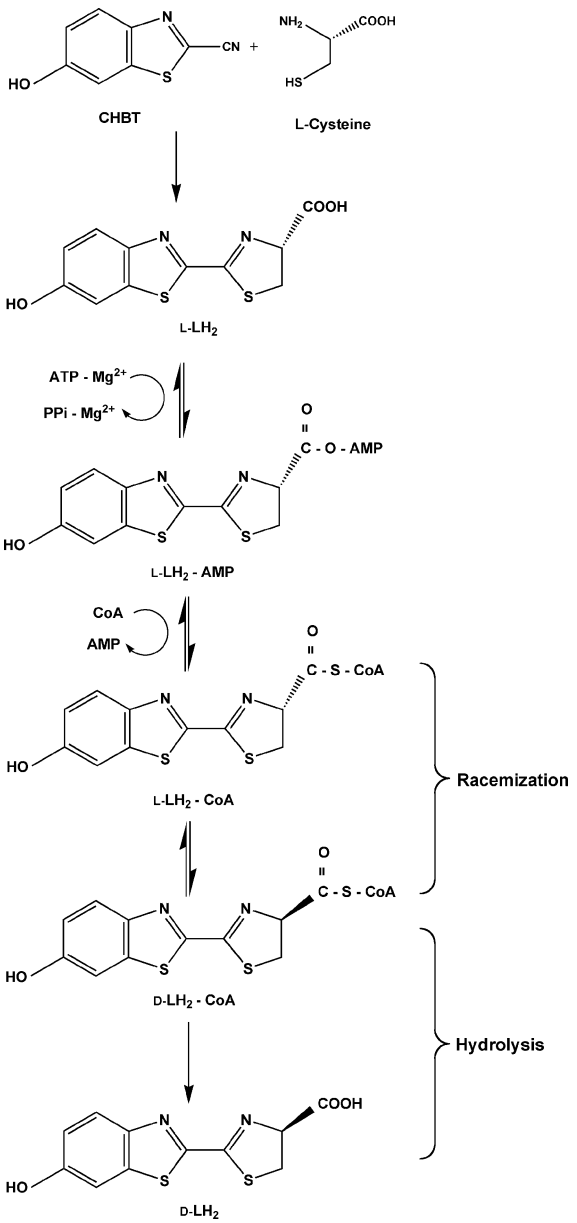

Fig. 6 Synthesis of L-LH_2–CoA and the proposed mechanism for the formation of D-LH_2.[97] Figure adapted with the permission of the authors.

7. Synthesis of mono and dinucleoside polyphosphates by firefly luciferase

Dinucleoside polyphosphates are a group of compounds with an internal phosphate chain inaccessible to the hydrolytic activity of unspecific phosphatases. As an example, diadenosine tetraphosphate (Ap_4A) has two adenosines linked by a chain of four phosphates attached to the 5′OH of the pentoses. These metabolites, first described in the 1960s, are ubiquitous in prokaryote and eukaryote cells and their synthesis primarily results from a side reaction of several aminoacyl-tRNA synthetases.[100]

Testing the hypothesis that enzymes capable of forming adenylated intermediates with concomitant PPi release should catalyze the synthesis of such compounds, Sillero and coworkers showed that Luc could also catalyze the synthesis of Ap_4A and derivatives according to the reaction:[22,101]

$$\text{Luc}\cdot LH_2\text{–AMP} + \text{ATP} \rightleftarrows \text{Luc} + LH_2 + Ap_4A \quad (7)$$

This mechanism was, however, revised and replaced by another in which L–AMP, an oxidation product of LH_2–AMP, functions as the real intermediate.[22,50]

$$\text{Luc}\cdot\text{L–AMP} + \text{ATP} \rightleftarrows \text{Luc} + \text{L} + Ap_4A \quad (8)$$

While starting from the same substrates as bioluminescence, Ap_4A synthesis has different characteristics from the light production reaction, including an acid pH optimum and a lower reaction rate.[22]

The general role of dinucleoside polyphosphates for firefly bioluminescence, and more generally to cell biology, remains largely undefined.[100] Regardless of the large number of biological effects described, a broad physiological function is still to be discerned.[22] In fact, although more than thirty years has passed since their identification, there is no report of an enzyme that

specifically catalyses the synthesis of these compounds. Their synthesis results from a secondary reaction of the enzyme under conditions usually non-optimal for its canonical activity, for example at different pH (as occurs with Luc),[102] in the presence of Ni^{2+} or upon cellular stress.[100] Contrasting with the apparent absence of anabolic pathways, there are a significant number of enzymes that specifically degrade this class of compounds, suggesting that the enzymes function to prevent their accumulation of the compounds. Dinucleoside polyphosphates are resistant to conventional phosphatases and their accumulation would be otherwise inevitable.

Despite this, several experimental results observed with Luc and other enzymes can only be accurately explained by considering the synthesis of these compounds. As an example, the continuous consumption of ATP after the end of the luminescence is probably the result of Ap_4A and not the consequence of an ATPase activity. The report of Ap_4A in firefly lanterns is relevant[103] and the synthesis of this compound by Luc when used as a reporter gene was recently described.[104]

8. Molecular and structural studies

Prior the advent of molecular biology, enzyme structural studies utilized the chemical modification of the residues involved in catalysis. This technique, when applied to the Luc mechanism, resulted in the conclusion that the cysteines, initially regarded as essential for catalysis, were in fact not relevant.[105,106] It was in this context that Luc gene was cloned and recombinant Luc expressed in rabbit reticulocytes.[107] The Luc gene is made of seven exons separated by 6 introns with extensions between 43 and 58 base pairs, coding for a protein with 550 amino acids and a molecular weight of about 60 kDa.[108] When expressed in eukaryote cells Luc is targeted to the peroxisomes, a consequence of a C terminal peptide (SLK), the first peroxisomal signaling sequence discovered.[109]

The strategy used to clone *Photinus pyralis* luciferase has since been applied to the cloning of other genes in species of Coleoptera, and many sequences are now available (albeit representing only a small fraction of the total number of species)[4] While the vast majority of the cloned genes are from Lampyridae some luciferases of the Phengodidae and Elateroidae were also cloned.[4]

The crystal structure of Luc was the first of its superfamily of adenylate forming enzymes to be determined.[110] In the absence of substrates, this enzyme adopts a two domain structure; a large N terminal domain and a short C terminal domain separated by a wide cleft (Fig. 7). Taking in consideration the homologies between Luc and non-bioluminescent members, and assuming that the regions involved in the catalytic process are conserved within the superfamily, it was proposed that Luc active site has amino acids common to the other enzymes of the family and present on the surface of the two domains. In the open structure those residues were too far apart and it was postulated that following substrate binding the two domains would approach and those residues would form, together with others resides deep inside the domains, the active site.[110] The possibility of a large conformational change during the bioluminescence reaction was also supported by earlier experiments.[47]

Indeed, the crystallographic studies obtained for the non-bioluminescence enzymes of the firefly superfamily confirmed this hypothesis. In the presence of substrates the two domains were always in close contact involving the substrates. The first of those structures to be determined was that of the phenylalanine activating subunit of gramicidin S from *Bacillus brevis* (PheA).[111] Despite its low sequence similarity to Luc, 16%, the tertiary structures of these two enzymes are very similar. In the case of PheA with the substrates in place, the data show the small C terminal domain rotated to enclose the substrate. Similar results were obtained for the activating subunit of 2,3 dihydroxybenzoate of *Bacillus subtilis*,[112] the yeast acetyl-CoA synthetase (ACS) with AMP[113] and the 4-chlorobenzoyl-CoA ligase (CBAL) from *Alcaligenes sp. AL3007* with 4-chlorobenzoate.[114]

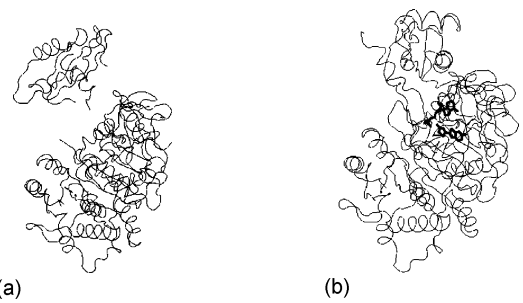

Fig. 7 (a) *Photinus pyralis* luciferase structure without substrates. In this structure it is evident the cleft separating a small C-terminal (top right) from the large N terminal domain. (b) *Luciola cruciata* luciferase structure in the presence of L–AMP analogue, DLSA. As referred in the text the C terminal is much closer to the N terminal domain completely involving the intermediate.

The structure of the Japanese firefly *Luciola cruciata* was recently obtained in the presence of the L–AMP analog 5′-*O*-[*N*-(dehydroLuciferyl)-sulfamoyl] adenosine (DLSA), as well as with both $ATP{\cdot}Mg^{2+}$ and oxyluciferin with AMP. This work confirmed that the spatial arrangement of the two domains in those three complexes was similar to the one described for PheA in the presence of phenylalanine and $ATP{\cdot}Mg^{2+}$ and different from the one described for Luc in the absence of substrates.[60] The same study also demonstrated that, with regard to the three-dimensional structure of the enzyme, the complexes $Luc{\cdot}ATP{\cdot}Mg^{2+}$ and Luc·oxyluciferin·AMP were similar and different from the complex Luc·DLSA.[60]

The determination of the crystal structures of Luc greatly helped the ongoing structure–function and mutagenesis studies.[58,115,116] Using the coordinates obtained from the crystallographic studies, two models for the active site were advanced by the Branchini and Ugarova groups.[94,117] According to those models LH_2 binding site should include R218, H245–F247, A313-G320, G339-I351 and K529, with a hydrophobic region composed of the residues A313, A348, I351 and F247. While similar overall, the two models differed in the role of arginine 218 (R218). According to Branchini this arginine would interact with the phenol group of LH_2, while in Ugarova model that interaction appears to be mediated by another arginine at position 337.[118]

Taking into consideration the similarities between *Photinus pyralis* and *Luciola cruciata*, it is reasonable to conclude that the crystallographic studies of Luc in the presence of substrates by Nakatsu and coworkers can help define with more precision the nature of the active site in *Photinus pyralis*.[60] In fact those studies confirmed the proposed models and demonstrated that the residues in proximity to LH_2 in *Photinus pyralis* were: F249, T253, L286, E311-S314, R337-Y340 and A348.

Associated with those models, site-directed mutagenesis studies have identified a group of residues that are essential for the catalysis and the emission spectrum.[4,94,119–123] The substitution of residues in the active site or interacting with those in the active site results in inactive mutants or a different emission spectrum. However those studies did not allow a clear definition of a mechanism, even a speculative one.

From the bulk of structure–function studies we would single out those involving the lysine residues in the positions K529 and K443 (*P. pyralis* numeration). Both are highly conserved and essential for activity in all the enzymes of the Luc superfamily. According to Branchini and coworkers, K529 participates in the adenylation reaction, but is not important for the oxidation reaction.[123] Supporting this, its replacement by another amino acid results in low activities when LH_2 and ATP·Mg^{2+} are used as substrates, but higher activity when LH_2–AMP is used. Lysine 443, however, appears to have a function complementary to the one of Lysine 529, being relevant for LH_2–AMP oxidation but not for the adenylation. The most intriguing part of this dichotomy is these two lysines are located at opposite ends of the C terminal domain of Luc. This result led the authors to propose that, after LH_2–AMP formation, its oxidation requires a C terminal rotation in order to replace K529 for K443 at the active site. What the relevance of this conformational change is for light emission is still to be discovered.

Clearly the clarification of the enzyme mechanism that results in light emission will be one of the more exciting fields of study for the future.

9. Kinetics of the bioluminescence reaction

Enzyme catalysis of a light emitting reaction offers a unique tool for investigating the mechanism of enzyme action.[124] Since every photon results from a catalytic event it is possible to continuously monitor the rate of light emission or luminescence without aliquot removal or other experimental constraints that usually limit other enzyme studies.

Despite the broad range of observable patterns, Luc kinetics can be clearly subdivided in two: those obtained with low and with high substrate concentrations.[125,126] Whereas with low substrate concentration (LH_2 and ATP·Mg^{2+} in the nM range) the kinetics are characterized by a relatively steady light emission, with high substrate concentrations (LH_2 and ATP·Mg^{2+} in the μM range) there is a rapid rise of intensity to a maximum, in the first few seconds, and a prompt decay to about 5–10% of the peak, followed by a slow decay that may last for hours or even days (Fig. 8).[125,126] This flash pattern should not be confused with firefly *in vivo* flashes, whose kinetics and mechanisms are different.

The initial rapid decrease in the *in vitro* rate has been interpreted as a consequence of product inhibition.[13] Indeed, the addition of fresh Luc to inhibited mixtures is capable of restoring light emission.[127] Despite this, the identity of the product responsible for the inhibition remains to be clarified.

Oxyluciferin, the light emitter and main product of LH_2 oxidation, is usually considered "the product" responsible for the fast decay in light production.[126,128–130] This conclusion, is however, not properly supported. Indeed, when oxyluciferin inhibition was studied, it was described as competitive with respect to D-LH_2 with a K_i as high as 0.23–0.25 μM, a value similar to the one obtained

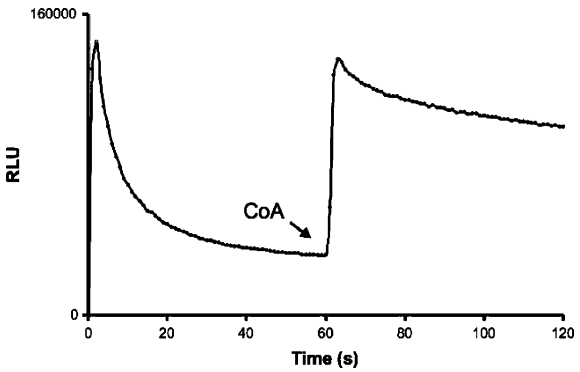

Fig. 8 The kinetic profile of Luc with high substrate concentrations and the effect of injecting CoA.[137] Figure adapted with the permission of the authors and the publisher.

for L or decarboxyluciferin.[130–132] To have a proper perspective of the degree of this inhibition, it should be mentioned that anesthetics in a wide range of concentrations are able to inhibit Luc by competing with LH_2 with an IC50 also in the μM range.[133,134]

Besides the magnitude of oxyluciferin inhibition, the competitive character of oxyluciferin inhibition is also open to discussion. As stated by DeLuca "If oxyluciferin is a true competitive inhibitor, the above results make it difficult to understand why the enzyme is so rapidly inhibited by small amounts of product in the presence of a large excess of luciferin".[48] Lemasters and Hachenbrock, discussing the same, explained the apparent contradiction with: "DeLuca and Marsh have demonstrated by optical rotary dispersion that a large conformational change in luciferase occurs after addition of substrates and initiation of the reaction. Such conformation may underline the difference in the inhibitory mechanism observed by Goto and ourselves since Goto measured reaction velocity upon initiation of luminescence while we measure luminescence after one to several minutes. Alternatively, our results may indicate that oxyluciferin is not the active species in product inhibition of luminescence".[129]

This conformational hypothesis proposed by Lemasters is certainly interesting but was not supported by a recent structure determination that clearly shows that the complex Luc–oxyluciferin has a structure similar to the one with ATP·Mg^{2+}.[60] Furthermore, the hypothesis that other active species can be relevant for the inhibition profile has meanwhile gained significance. In the late 1990s, Fontes and coworkers reported that L–AMP was a significant secondary, (16%), product of Luc bioluminescence.[22,49,50] The idea that L is a product of "autoxidation" of LH_2 was rejected; no L–AMP or L was formed in the absence of Luc or ATP, a clear indication that adenylation was a prerequisite for oxidation.[50] Moreover, if there were doubts concerning the biosynthesis of L–AMP, those were removed by the study that demonstrated that its synthesis is stereospecific, resulting only from D-LH_2–AMP oxidation with H_2O_2 as co-product (Fig. 3).[135]

As mentioned, L–AMP is a powerful inhibitor, IC50 = 6 nM, and while it may not account for the lion's share of the inhibition, it clearly accounts for a significant fraction of it. In addition, L–AMP appears to behave as a truly non-competitive inhibitor to LH_2 and ATP.[17] This non-competitive character was recently supported by a structural work using the L–AMP analogue, DLSA.[60] Apparently Luc adopts a "closed" conformation in the

complex with the analogue and in fact Branchini and coworkers demonstrated that DLSA behaved as truly non-competitive inhibitor in respect to D-LH_2 ($K_i = 34 \pm 5$ nM) and ATP ($K_i = 41 \pm 3$ nM) and has a competitive in respect to LH_2–AMP ($K_i = 340 \pm 50$ nM).[136]

L–AMP synthesis can also explain the activating effect of some compounds in Luc luminescence, namely CoA. When added initially to the reaction mixture CoA is able to prevent the fast reaction decay and can, when added later to the reaction mixture, promote secondary flashes.[84,137–139] Those effects, first observed in the 1960s, were at the time explained by its reaction with L–AMP (then named oxyluciferyl–adenylate) forming L–CoA (then named oxyluciferyl–CoA) and allowing Luc turnover.[84] However this hypothesis was replaced by another in which the CoA effect was associated with an allosteric site.[139,140]

Surveying the literature, it is intriguing that compounds with similar characteristics such as acetyl-Coenzyme A, dephospho-Coenzyme A and dethio-Coenzyme A could provoke such different effects and that the removal of the thiol group was essential for CoA-promoted activation.[140] In fact, in a reevaluation of those two models it was found that, as originally proposed, the effect of CoA is a result of its reaction with L–AMP.[22,84] Accordingly, L-CoA is a less powerful inhibitor than L–AMP. Its rate of synthesis is consistent with the rapid effects observed (secondary flashes, see Fig. 8 and[137,138]) and only analogs able to react with the L–AMP were able to promote activations.[137,141] Moreover, CoA is unable to antagonize oxyluciferin inhibition, clearly refuting the proposed existence of an allosteric action mechanism.[142]

From the above it is clear that the importance of L–AMP synthesis for Luc kinetics cannot be ignored but the magnitude of its contribution remains to be determined. Is L–AMP the only and main inhibitor or does oxyluciferin as a major product play also a significant role? What is the function of several other activators, like PPi[143] and cytidine nucleotides?[138] Are their effects really due to allosteric effects as proposed? The clarification of those questions will certainly be interesting.

10. Perspectives

It has been a long way since the pioneer experiments of Dubois to the present knowledge on Luc bioluminescence. However, and despite impressive advances, several points remain to be clarified, especially in what concerns the enzyme mechanism underlining light emission. In addition, thioester and dinucleoside polyphosphate synthesis, dark reactions whose significance cannot be denied, also require clarification at the enzyme level. Are the residues involved in light emission also involved in the synthesis of acyl-CoA, L-LH_2–CoA and L–CoA, or did evolution recruit different residues to catalyse different reactions? The structures recently determined and the increasing number of sequences available may answer these questions and finally reveal the mystery behind one of nature's most spectacular displays, firefly bioluminescence.

11. Abbreviations

ACS, acetil-CoA synthetase; APA, 2-arylpropionic acids; Ap_4A, diadenosine tetraphosphate; CIEEL, chemical initiated electron exchange luminescence; CBAL, 4-chlorobenzoyl-CoA ligase; DLSA, 5′-*O*-[*N*-(dehydroLuciferyl)-sulfamoyl] adenosine; L, dehydroluciferin; L–AMP, dehydroluciferyl–adenylate; L–CoA, dehydroluciferyl–CoA, Luc, firefly luciferase; LH_2, firefly luciferin; LH_2–AMP, luciferyl–adenylate; LH_2–CoA, luciferyl–CoA; PheA, phenylalanine activating subunit of gramicidin S from *Bacillus brevis*; PPi, inorganic pyrophosphate.

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References

1 E. N. Harvey, in *A history of luminescence: from the earliest times until 1900*, Dover Phoenix, 1957.
2 O. Shimomura, in *Bioluminescence: Chemical Principles and Methods*, World Scientific Publishing Company, 2006.
3 E. N. Harvey, in *Bioluminescence*, Academic Press, 1952.
4 V. R. Viviani, The origin, diversity, and structure function relationships of insect luciferases, *Cell. Mol. Life Sci.*, 2002, **59**, 1833–1850.
5 K. V. Wood, The chemical mechanism and evolutionary development of beetle bioluminescence, *Photochem. Photobiol.*, 1995, **62**, 662–673.
6 F. McCapra, The chemistry of bioluminescence, *Proc. R. Soc. London, Ser. B*, 1982, **215**, 247–272.
7 T. Wilson and J. W. Hastings, Bioluminescence, *Annu. Rev. Cell Dev. Biol.*, 1998, **14**, 197–230.
8 W. D. McElroy, From the precise to the ambiguous: light, bonding, and administration, *Annu. Rev. Microbiol.*, 1976, **30**, 1–21.
9 J. W. Hastings, Firefly Flashes and Royal Flushes: Life in a full house, *J. Biolumin. Chemilumin.*, 1989, **4**, 29.
10 W. D. McElroy, The energy source for bioluminescence in an isolated system, *Proc. Natl. Acad. Sci. USA*, 1947, **33**, 342–345.
11 W. D. McElroy and H. E. Seliger, The chemistry of light emission, *Adv. Enzymol.*, 1963, **25**, 119–166.
12 W. D. McElroy and B. L. Strehler, Factors influencing the response of the bioluminescent reaction, *Arch. Biochem.*, 1949, **22**, 420–433.
13 J. W. Hastings, W. D. McElroy and J. Coulombre, The effect of oxygen upon the immobilization reaction in firefly luminescence, *J. Cell. Comp. Physiol.*, 1953, **42**, 137–150.
14 A. A. Green and W. D. McElroy, Crystalline firefly luciferase, *Biochim. Biophys. Acta*, 1956, **20**, 170–176.
15 W. D. McElroy and A. Green, Function of adenosine triphosphate in the activation of luciferin, *Arch. Biochem. Biophys.*, 1956, **46**, 399–416.
16 B. Bitler and W. D. McElroy, The preparation and properties of crystalline firefly luciferin, *Arch. Biochem. Biophys.*, 1957, **72**, 358–368.
17 W. C. Rhodes and W. D. McElroy, The synthesis and function of luciferyl–adenylate and oxyluciferyl–adenylate, *J. Biol. Chem.*, 1958, **233**, 1528–1537.
18 W. C. Rhodes and W. D. McElroy, Enzymatic synthesis of adenyl-oxyluciferin, *Science*, 1958, **128**, 253–254.
19 R. T. Lee, J. L. Denburg and W. D. McElroy, Substrate binding properties of firefly luciferase. II. ATP, binding site, *Arch. Biochem. Biophys.*, 1970, **141**, 38–52.
20 J. D. Moyer and J. F. Henderson, Nucleoside triphosphate specificity of firefly luciferase, *Anal. Biochem.*, 1983, **131**, 187–189.
21 G. Momsen, Firefly luciferase reacts with P-1,P-5-Di (adenosine-5′-) pentaphosphate and adenosine-5′-tetraphosphate, *Biochem. Biophys. Res. Commun.*, 1978, **84**, 816–822.
22 A. Sillero and M. A. G. Sillero, Synthesis of dinucleoside polyphosphates catalyzed by firefly luciferase and several ligases, *Pharmacol. Ther.*, 2000, **87**, 91–102.

23 M. Deluca, N. J. Leonard, B. J. Gates and W. D. McElroy, The role of 1N-ethenoadenosine triphosphate and 1N-ethenoadenosine monophosphate in firefly luminescence, *Proc. Natl. Acad. Sci. USA*, 1973, **70**, 1664–1666.
24 E. H. White, G. F. Field, W. D. McElroy and F. McCapra, Structure and synthesis of firefly luciferin, *J. Am. Chem. Soc.*, 1961, **83**, 2402–2403.
25 E. H. White, G. F. Field and F. McCapra, Structure and synthesis of firefly luciferin, *J. Am. Chem. Soc.*, 1963, **85**, 337–343.
26 L. J. Bowie, Synthesis of firefly luciferin and structural analogs, *Methods Enzymol.*, 1978, **57**, 15–28.
27 H. E. Seliger, W. D. McElroy, E. H. White and G. F. Field, Stereospecificity and firefly bioluminescence, a comparison of natural and synthetic luciferin, *Proc. Natl. Acad. Sci. USA*, 1961, **47**, 1129–1134.
28 W. D. McElroy and H. H. Seliger, Mechanism of action of firefly luciferase, *Fed. Proc.*, 1962, **21**, 1006–1012.
29 N. Lembert, Firefly luciferase can use L-Luciferin to produce light, *Biochem. J.*, 1996, **317**, 273–277.
30 M. Nakamura, S. Maki, Y. Amano, Y. Ohkita, K. Niwa, T. Hirano, Y. Ohmiya and H. Niwa, Firefly luciferase exhibits bimodal action depending on the luciferin chirality, *Biochem. Biophys. Res. Commun.*, 2005, **331**, 471–475.
31 H. Fraga, J. C. Esteves da Silva and R. Fontes, Identification of luciferyl adenylate and luciferyl coenzyme a synthesized by firefly luciferase, *ChemBioChem*, 2004, **5**, 110–115.
32 K. Ayabe, T. Zako and H. Ueda, The role of firefly luciferase C-terminal domain in efficient coupling of adenylation and oxidative steps, *FEBS Lett.*, 2005, **579**, 4389–4394.
33 H. H. Seliger and W. D. McElroy, Chemiluminescence of firefly luciferin without enzyme, *Science*, 1962, **132**, 683–685.
34 E. H. White, E. Rapaport, H. S. Howard and T. A. Hopkins, The chemi and bioluminescence of firefly luciferin: an efficient chemical production of electronically excited states, *Bioorg. Chem.*, 1971, **1**, 92–122.
35 F. McCapra, Chemical mechanisms in bioluminescence, *Acc. Chem. Res.*, 1976, **9**, 201–208.
36 T. A. Hopkins, H. H. Seliger, E. H. White and M. W. Cass, Chemiluminescence of firefly luciferin. A model for bioluminescent reaction and identification of product excited state, *J. Am. Chem. Soc.*, 1967, **89**, 7148–7150.
37 F. McCapra, Y. C. Chang and V. P. François, Chemiluminescence of a firefly luciferin analogue, *Chem. Commun.*, 1968, 22–23.
38 M. M. Rauhut, Chemiluminescence from concerted peroxide decomposition reactions, *Acc. Chem. Res.*, 1968, **2**, 80–87.
39 P. J. Plant, E. H. White and W. D. McElroy, The decarboxylation of luciferin in firefly bioluminescence, *Biochem. Biophys. Res. Commun.*, 1968, **31**, 98–103.
40 O. Shimomura, T. Goto and F. H. Johnson, Source of oxygen in CO_2 produced in bioluminescent oxidation of firefly luciferin, *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 2799–2802.
41 E. H. White, M. G. Steinmetz, J. D. Miano, P. D. Wildes and R. Morland, Chemi- and bioluminescence of firefly luciferin, *J. Am. Chem. Soc.*, 1980, **102**, 3199–3208.
42 W. D. McElroy, H. H. Seliger and E. H. White, Mechanism of bioluminescence, chemiluminescence and enzyme function in the oxidation of firefly luciferin, *Photochem. Photobiol.*, 1969, **10**, 153–170.
43 N. Suzuki, M. Sato, K. Nishikawa and T. Goto, Synthesis and spectral properties of 2-(6′-hydroxythiazol-2′-yl)-4-hydroxythiazole, a possible emitting species in the firefly bioluminescence, *Tetrahedron Lett.*, 1969, **53**, 4683–4684.
44 N. Suzuki and T. Goto, Firefly bioluminescence. II. Identification, of 2-(6′-hydroxybenzothiazol-2′-Yl)-4-hydroxthiazole as a product in bioluminescence of firefly lanterns and as a product in chemiluminescence of firefly luciferin in DMSO, *Tetrahedron Lett.*, 1971, 2021–2024.
45 N. Suzuki, M. Sato, K. Okada, T. Goto, N. Suzuki, M. Sato, K. Okada and T. Goto, Studies on firefly bioluminescence - I. Synthesis, and spectral properties of firefly oxyluciferin. A possible emitting species in firefly bioluminescence, *Tetrahedron*, 1972, **28**, 4065–4074.
46 N. Suzuki and T. Goto, Studies on Firefly Bioluminescence - II. Identification, of oxyluciferin as a product in bioluminescence of firefly lanterns and in chemiluminescence of firefly luciferin, *Tetrahedron*, 1972, **28**, 4075–4082.
47 M. Deluca, Firefly luciferase, *Adv. Enzymol.*, 1976, **44**, 37–68.
48 B. J. Gates and M. DeLuca, The production of oxyluciferin during the firefly luciferase light reaction, *Arch. Biochem. Biophys.*, 1975, **169**, 616–621.
49 R. Fontes, A. Dukhovich, A. Sillero and M. A. Sillero, Synthesis of dehydroluciferin by firefly luciferase: effect of dehydroluciferin, coenzyme A and nucleoside triphosphates on the luminescent reaction, *Biochem. Biophys. Res. Commun.*, 1997, **237**, 445–450.
50 R. Fontes, B. Ortiz, A. de Diego, A. Sillero and M. A. Gunther Sillero, Dehydroluciferyl–AMP is the main intermediate in the luciferin dependent synthesis of Ap_4A catalyzed by firefly luciferase, *FEBS Lett.*, 1998, **438**, 190–194.
51 J. C. G. E. da Silva, J. M. C. S. Magalhaes and R. Fontes, Identification of enzyme produced firefly oxyluciferin by reverse phase HPLC, *Tetrahedron Lett.*, 2001, **42**, 8173–8176.
52 B. R. Branchini, M. H. Murtiashaw, R. A. Magyar, N. C. Portier, M. C. Ruggiero and J. G. Stroh, Yellow-green and red firefly bioluminescence from 5,5-dimethyloxyluciferin, *J. Am. Chem. Soc.*, 2002, **124**, 2112–2113.
53 Y. Oba, T. Tanaka and S. Inouye, Catalytic properties of domain-exchanged chimeric proteins between firefly luciferase and Drosophila fatty Acyl-CoA Synthetase CG6178, *Biosci., Biotechnol., Biochem.*, 2006, **70**, 60364–60370.
54 W. D. McElroy and C. S. Rainwater, Spectral energy distribution of the light emitted by firefly extracts, *J. Cell. Comp. Physiol.*, 1948, **32**, 421–425.
55 H. H. Seliger and W. D. McElroy, The colors of firefly bioluminescence: enzyme configuration and species specificity, *Proc. Natl. Acad. Sci. USA*, 1964, **52**, 75–81.
56 H. H. Seliger and W. D. McElroy, Spectral emission and quantum yield of firefly bioluminescence, *Arch. Biochem. Biophys.*, 1960, **88**, 136–141.
57 N. K. Tafreshi, S. Hosseinkhani, M. Sadeghizadeh, M. Sadeghi, B. Ranjbar and H. Naderi-Manesh, The influence of insertion of a critical residue (Arg356) in structure and bioluminescence spectra of firefly luciferase, *J. Biol. Chem.*, 2007, **282**, 8641–8647.
58 N. Kajiyama and E. Nakano, Isolation and characterization of mutants of firefly luciferase which produce different colors of light, *Protein Eng.*, 1991, **4**, 691–693.
59 V. Viviani, F. G. C. Arnoldi, F. T. Ogawa and M. Brochetto-Braga, Few substitutions affect the bioluminescence spectra of Phixotrix (Coleoptera: Phengodidae) luciferases: a site-directed mutagenesis survey, *Luminescence*, 2007, **22**, 362–369.
60 T. Nakatsu, S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata and H. Kato, tructural basis for the spectral difference in luciferase bioluminescence, *Nature*, 2006, **440**, 372–376.
61 E. H. White, E. Rapaport, T. A. Hopkins and H. H. Seliger, Chemi- and bioluminescence of firefly luciferin, *J. Am. Chem. Soc.*, 1969, **91**, 2178–2180.
62 E. H. White and B. R. Branchini, Modification of firefly luciferase with a luciferin analog - red light producing enzyme, *J. Am. Chem. Soc.*, 1975, **97**, 1243–1245.
63 F. McCapra, D. J. Gilfoyle, D. W. Young, N. J. Church and P. Spencer, in *Bioluminescence and Chemiluminescence: Fundamentals and Applied Aspects*, Wiley, Chichester, UK, 1994.
64 G. Orlova, J. D. Goddard and Y. Brovko, Theoretical study of the amazing firefly bioluminescence: the formation and structures of the light emitters, *J. Am. Chem. Soc.*, 2003, **125**, 6962–6971.
65 N. Nakatani, J. Hasegawa and H. Nakatsuji, Red light in chemiluminescence and yellow-green light in bioluminescence: color-tuning mechanism of firefly, Photinus pyralis, studied by the symmetry-adapted cluster-configuration interaction method, *J. Am. Chem. Soc.*, 2007, **129**, 8756–8765.
66 O. A. Gandelman, L. Y. Brovko, N. N. Ugarova, A. Y. Chikishev and A. P. Shkurimov, Oxyluciferin fluorescence is a model of native bioluminescence in the firefly luciferin luciferase system, *J. Photochem. Photobiol.*, 1993, **19**, 187–191.
67 H. H. Seliger and W. D. McElroy, Quantum yield in the oxidation of firefly luciferin, *Biochem. Biophys. Res. Commun.*, 1959, **1**, 21–24.
68 Y. Ando, K. Niwa, N. Yamada, T. Enomoto, T. Irie, H. Kubota, Y. Ohmiya and H. Akiyama, Firefly bioluminescence quantum yield and color change by pH-sensitive green emission, *Nat. Photonics*, 2008, **2**, 44–47.
69 K. R. Kopecky and C. Munford, Luminescence in thermal decomposition of 3,3,4-trimethyl-1,2-dioxetane, *Can. J. Chem.*, 1969, **47**, 709.

70 T. Wilson, Chemiluminescence in the liquid phase: thermal cleavage of dioxetanes, *Int. Rev. Sci. Phys. Chem. Ser. Two*, 1976, **9**, 265–311.
71 F. McCapra, Chemical generation of excited states: the basis of chemiluminescence and bioluminescence, *Methods Enzymol.*, 2000, **305**, 3–47.
72 G. B. Schuster, Chemiluminescence of organic peroxides - conversion of ground state reactants to excited state products by the chemically initiated electron-exchange luminescence mechanism, *Acc. Chem. Res.*, 1979, **12**, 366–373.
73 F. McCapra, I. Beheshti, A. Burford, R. A. Hann and K. A. Zaklika, Singlet excited states from dioxetane decomposition, *Chem. Commun.*, 1977, **9**, 44–946.
74 M. Matsumoto, dvanced chemistry of dioxetane-based chemiluminescent substrates originating from bioluminescence, *J. Photochem. Photobiol., C*, 2004, **5**, 27–53.
75 J. Y. Koo, S. P. Schmidt and G. B. Schuster, Bioluminescence of firefly - key steps in formation of electronically excited state for model systems, *Proc. Natl. Acad. Sci. USA*, 1978, **75**, 30–33.
76 E. H. White, H. Worther, G. F. Field and W. D. McElroy, Analogs of firefly luciferin, *J. Org. Chem.*, 1965, **30**, 2344–2348.
77 W. Adam, D. Reinhardt and C. R. Saha-Moller, From the firefly bioluminescence to the dioxetane-based (AMPPD) chemiluminescence immunoassay: a retroanalysis, *Analyst*, 1996, **121**, 1527–1536.
78 L. H. Catalani and T. Wilson, Electron transfer and chemiluminescence. Two inefficient systems: 1,4-dimethoxy-9,10-diphenylanthracene peroxide and diphenoyl peroxide, *J. Am. Chem. Soc.*, 1989, **111**, 2633–2639.
79 J. Y. Koo and G. B. Schuster, Chemiluminescence of diphenoyl peroxide - chemically initiated electron exchange luminescence - new general mechanism for chemical production of electronically excited states, *J. Am. Chem. Soc.*, 1978, **100**, 4496–4503.
80 B. R. Branchini, M. M. Hayward, S. Bamford, P. M. Brennan and E. J. Lajiness, Naphthyl- and quinolylluciferin: green and red light emitting firefly luciferin analogues, *Photochem. Photobiol.*, 1989, **49**, 689–695.
81 R. A. Morton, T. A. Hopkins and H. H. Seliger, The spectroscopic properties of firefly luciferin and related compounds. An approach to product emission, *Biochemistry*, 1969, **8**, 1598–1607.
82 W. Adam, I. Bronstein and A. V. Trofimov, Solvatochromic effects on the electron exchange chemiluminescence (CIEEL) of spiroadamantyl-substituted dioxetanes and the fluorescence of relevant oxyanions, *J. Phys. Chem.*, 1998, **102**, 5406–5414.
83 W. D. McElroy, M. DeLuca and J. Travis, Molecular uniformity in biological catalyses. The enzymes concerned with firefly luciferin, amino acid, and fatty acid utilization are compared, *Science*, 1967, **157**, 150–160.
84 R. L. Airth, W. C. Rhodes and W. D. McElroy, The function of coenzyme A in luminescence, *Biochim. Biophys. Acta*, 1958, **27**, 519–532.
85 K. Chang, H. Xiang and D. Dunaway-Mariano, Acyl-adenylate motif of the acyl-adenylate/thioester-forming enzyme superfamily: a site-directed mutagenesis study with the Pseudomonas sp. strain CBS3 4-chlorobenzoate:coenzyme A ligase, *Biochemistry*, 1997, **36**, 15650–15659.
86 Y. Oba, M. Ojika and S. Inouye, Firefly luciferase is a bifunctional enzyme: ATP-dependent monooxygenase and a long chain fatty acyl-CoA synthetase, *FEBS Lett.*, 2003, **540**, 251–254.
87 Y. Oba, M. Sato, M. Ojika and S. Inouye, Enzymatic and genetic characterization of firefly luciferase and Drosophila CG6178 as a fatty acyl-CoA synthetase, *Biosci., Biotechnol., Biochem.*, 2005, **69**, 819–828.
88 Y. Oba, M. Qjika and S. Inouye, Characterization of CG6178 gene product with high sequence similarity to firefly luciferase in Drosophila melanogaster, *Gene*, 2004, **329**, 137–145.
89 Y. Oba, M. Sato and S. Inouye, Cloning and characterization of the homologous genes of firefly luciferase in the mealworm beetle *Tenebrio molitor*, *Insect Mol. Biol.*, 2006, **15**, 293–299.
90 V. R. Viviani and E. J. H. Bechara, Larval Tenebrio molitor (Coleoptera: Tenebrionidae) fat body extracts catalyze firefly D-Luciferin- and ATP-dependent chemiluminescence: A luciferase-like enzyme, *Photochem. Photobiol.*, 1996, **63**, 713–718.
91 J. C. Day, L. C. Tisi and M. J. Bailey, Evolution of beetle bioluminescence: the origin of beetle luciferin, *Luminescence*, 2004, **19**, 8–20.
92 K. Cho, J. S. Lee, Y. D. Choi and K. S. Boo, Structural polymorphism of the luciferase gene on the firefly *Luciola lateralis*, *Insect Mol. Biol.*, 1999, **8**, 193–200.
93 V. Viviani and Y. Ohmiya, Bovine serum albumin displays luciferase-like activity in presence of luciferyl–adenylate: insights on the origin of protoluciferase activity and bioluminescence colours, *Luminescence*, 2006, **21**, 262–267.
94 B. R. Branchini, R. A. Magyar, M. H. Murtiashaw, S. M. Anderson and M. Zimmer, Site-directed mutagenesis of histidine 245 in firefly luciferase: a proposed model of the active site, *Biochemistry*, 1998, **37**, 15311–15319.
95 H. Fraga, R. Fontes and J. E. da Silva, Synthesis of luciferyl coenzyme A: A bioluminescent substrate for firefly luciferase in the presence of AMP, *Angew. Chem., Int. Ed.*, 2005, **44**, 3427–3429.
96 M. Nakamura, K. Niwa, S. Maki, T. Hirano, Y. Ohmiya and H. Niwa, Construction of a new firefly bioluminescence system using L-Luciferin as substrate, *Tetrahedron Lett.*, 2006, **47**, 1197–1200.
97 K. Niwa, M. Nakamura and Y. Ohmiya, Stereoisomeric bio-inversion key to biosynthesis of firefly D-luciferin, *FEBS Lett.*, 2006, **580**, 5283–5287.
98 V. Wsol, L. Skálová and B. Szotáková, Chiral inversion of drugs: coincidence or principle?, *Curr. Drug Met.*, 2004, **5**, 517–533.
99 D. Kato, K. Teruya, H. Yoshida, M. Takeo, S. Negoro and H. Otha, New application of firefly luciferase - it can catalyse the enantioselective thioester formation of 2-arylpropanoic acid, *FEBS J.*, 2007, **274**, 3877–3885.
100 A. G. McLennan, Dinucleoside polyphosphates - friend or foe?, *Pharmacol. Ther.*, 2000, **87**, 73–89.
101 M. A. G. Sillero, A. Guranowski and A. Sillero, Synthesis of dinucleoside polyphosphates catalyzed by firefly luciferase, *Eur. J. Biochem.*, 1991, **202**, 507–513.
102 H. Fraga, J. da Silva and R. Fontes, pH opposite effects on synthesis of dinucleoside polyphosphates and on oxidation reactions catalyzed by firefly luciferase, *FEBS Lett.*, 2003, **543**, 37–41.
103 A. G. McLennan, E. Mayers, I. Walker-Smith and H. Chen, Lanterns of the firefly Photinus pyralis contain abundant diadenosine 5′,5‴-P1,P4-tetraphosphate pyrophosphohydrolase activity, *J. Biol. Chem.*, 1995, **270**, 3706–3709.
104 G. A. Murphy and A. G. McLennan, Synthesis of dinucleoside tetraphosphates in transfected cells by a firefly luciferase reporter gene, *Cell. Mol. Life Sci.*, 2004, **61**, 497–501.
105 Y. Ohmiya and F. I. Tsuji, Mutagenesis of firefly luciferase shows that cysteine residues are not required for bioluminescence activity, *FEBS Lett.*, 1997, **404**, 115–117.
106 S. C. Alter and M. Deluca, The sulfhydryls of firefly luciferase are not essential for activity, *Biochemistry*, 1986, **25**, 1599–1605.
107 K. V. Wood, J. R. De Wet, N. Dewji and M. Deluca, Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns, *Biochem. Biophys. Res. Commun.*, 1984, **124**, 592–596.
108 J. R. De Wet, K. V. Wood, D. R. Helinski and M. Deluca, Cloning firefly luciferase, *Methods Enzymol.*, 1986, **133**, 3–14.
109 S. Gould, G. Keller, N. Hosken, J. Wilkinson and S. Subranami, A conserved tripeptide sorts proteins to peroxisomes, *J. Cell Biol.*, 1989, **108**, 1657–1664.
110 E. Conti, N. P. Franks and P. Brick, Crystal structure of firefly luciferase throws light on a superfamily of adenylate-forming enzymes, *Structure*, 1996, **4**, 287–298.
111 E. Conti, T. Stachelhaus, M. A. Marahiel and P. Brick, Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S, *EMBO J.*, 1997, **16**, 4174–4183.
112 J. J. May, N. Kessler, M. A. Marahiel and M. T. Stubbs, Crystal structure of DhbE, an archetype for aryl acid activating domains of modular nonribosomal peptide synthetases, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 12120–12125.
113 J. Gerwald and T. Liang, Crystal structure of yeast acetyl-coenzyme A synthetase in complex with AMP, *Biochemistry*, 2004, **43**, 1425–1431.
114 A. M. Gulick, X. Lu and D. Dunaway-Mariano, Crystal strucuture of 4-chlorobenzoate:CoA ligase/synthetase in the unliganded and aryl substrate bound states, *Biochemistry*, 2004, **43**, 8670–8679.
115 N. Kajiyama and E. Nakano, Enhancement of thermostability of firefly Luciferase from *Luciola-Lateralis* by a single amino-acid substitution, *Biosci., Biotechnol., Biochem.*, 1994, **58**, 1170–1171.
116 N. Kajiyama and E. Nakano, Thermostabilization of firefly luciferase by a single amino acid substitution at position-217, *Biochemistry*, 1993, **32**, 13795–13799.

117 T. P. Sandalova and N. N. Ugarova, Model of the active site of firefly luciferase, *Biochemistry (Moscow)*, 1999, **64**, 962–967.
118 B. R. Branchini, R. A. Magyar, M. H. Murtiashaw and N. C. Portier, The role of active site residue arginine 218 in firefly luciferase bioluminescence, *Biochemistry*, 2001, **40**, 2410–2418.
119 B. R. Branchini, R. A. Magyar, M. H. Murtiashaw, S. M. Anderson, L. C. Helgerson and M. Zimmer, ite-directed mutagenesis of firefly luciferase active site amino acids: a proposed model for bioluminescence color, *Biochemistry*, 1999, **38**, 13223–13230.
120 B. R. Branchini, M. H. Murtiashaw, R. A. Magyar and S. M. Anderson, The role of lysine 529, a conserved residue of the acyl adenylate forming enzyme superfamily, in firefly luciferase, *Biochemistry*, 2000, **39**, 5433–5440.
121 B. R. Branchini, T. L. Southworth, M. H. Murtiashaw, H. Boije and S. E. Fleet, A mutagenesis study of the putative luciferin binding site residues of firefly luciferase, *Biochemistry*, 2003, **42**, 10429–10436.
122 B. R. Branchini, T. L. Southworth, M. H. Murtiashaw, R. A. Magyar, S. A. Gonzalez, M. C. Ruggiero and J. G. Stroh, An alternative mechanism of bioluminescence color determination in firefly luciferase, *Biochemistry*, 2004, **43**, 7255–7162.
123 B. R. Branchini, T. L. Southworth, M. H. Murtiashaw, S. R. Wilkinson, N. F. Khattak, J. C. Rosenberg and M. Zimmer, Mutagenesis evidence that the partial reactions of firefly bioluminescence are catalyzed by different conformations of the luciferase C-terminal domain, *Biochemistry*, 2005, **44**, 1385–1393.
124 M. Deluca and W. D. McElroy, Kinetics of firefly luciferase catalyzed reactions, *Biochemistry*, 1974, **13**, 921–925.
125 M. Deluca, J. Wannlund and W. D. McElroy, Factors affecting the kinetics of light emission from crude and purified firefly luciferase, *Anal. Biochem.*, 1979, **95**, 194–198.
126 S. R. Ford and F. R. Leach, in *Bioluminescence Methods & Protocols (Methods in Molecular Biology)*, Human Press, New Jersey, USA, 1998.
127 W. D. McElroy, J. W. Hastings, J. Coulombre and V. Sonnenfeld, The mechanism of action of pyrophosphate in firefly luminescence, *Arch. Biochem. Biophys.*, 1953, **46**, 399–416.
128 S. R. Ford, K. H. Chenault, L. S. Bunton, G. J. Hampton, J. McCarthy, M. S. Hall, S. J. Pangburn, L. M. Buck and F. R. Leach, Use of firefly luciferase for ATP measurement: other nucleotides enhance turnover, *J. Biolumin. Chemilumin.*, 1996, **11**, 149–167.
129 J. J. Lemasters and C. R. Hackenbrock, Kinetics of product inhibition during firefly luciferase luminescence, *Biochemistry*, 1977, **16**, 445–447.
130 N. N. Ugarova, Luciferase of Luciola mingrelica fireflies. Kinetics and regulation mechanism, *J. Biolumin. Chemilumin.*, 1989, **4**, 406–418.
131 T. Goto, I. Kubota, N. Suzuki and Y. Kishi, in *Bioluminescence*, Plenum Press, New York, USA, 1973.
132 J. L. Denburg, R. T. Lee and W. D. McElroy, Substrate binding properties of firefly luciferase. I. Luciferin, binding site, *Arch. Biochem. Biophys.*, 1969, **134**, 381–394.
133 G. W. J. Moss, N. P. Franks and W. R. Lieb, Modulation of the general anesthetic sensitivity of a protein - a transition between two forms of firefly luciferase, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 134–138.
134 N. P. Franks, A. Jenkins, E. Conti, W. R. Lieb and P. Brick, Structural basis for the inhibition of firefly luciferase by a general anesthetic, *Biophys. J.*, 1998, **75**, 2205–2211.
135 H. Fraga, D. Fernandes, J. Novotny, R. Fontes and J. C. Esteves da Silva, Firefly luciferase produces hydrogen peroxide as a coproduct in dehydroluciferyl adenylate formation, *ChemBioChem*, 2006, **7**, 929–935.
136 B. R. Branchini, M. H. Murtiashaw, J. N. Carmody, E. E. Mygatt and T. L. Southworth, Synthesis of an N-acyl sulfamate analog of luciferyl–AMP: a stable and potent inhibitor of firefly luciferase, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 3860–3864.
137 H. Fraga, D. Fernandes, R. Fontes and J. C. Esteves da Silva, Coenzyme A affects firefly luciferase luminescence because it acts as a substrate and not as an allosteric effector, *FEBS J.*, 2005, **272**, 5206–5216.
138 S. R. Ford, M. S. Hall and F. R. Leach, Enhancement of firefly luciferase activity by cytidine nucleotides, *Anal. Biochem.*, 1992, **204**, 283–291.
139 K. V. Wood, Novel assay of firefly luciferase providing greater sensitivity and ease of use, *J. Cell Biol.*, 1990, **111**, 380a.
140 S. R. Ford, L. M. Buck and F. R. Leach, Does the sulfhydryl or the adenine moiety of CoA enhance firefly luciferase activity, *Biochim. Biophys. Acta*, 1995, **1252**, 180–184.
141 H. Fraga, J. da Silva and R. Fontes, Chemical synthesis and firefly luciferase produced dehydroluciferyl-coenzyme A, *Tetrahedron Lett.*, 2004, **45**, 2117–2120.
142 H. Fraga, R. Fontes and J. E. da Silva, unpublished results.
143 O. Gandelman, I. Allue, K. Bowers and P. Cobbold, Cytoplasmic factors that affect the intensity and stability of bioluminescence from firefly luciferase in living mammalian cells, *J. Biolumin. Chemilumin.*, 1994, **9**, 363–371.

