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REVIEW

Firefly luciferase: an adenylate-forming enzyme for multicatalytic functions

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Abstract Firefly luciferase is a member of the acyl-adenylate/thioester-forming superfamily of enzymes and catalyzes the oxidation of firefly luciferin with molecular oxygen to emit light. Knowledge of the luminescence mechanism catalyzed by firefly luciferase has been gathered, leading to the discovery of a novel catalytic function of luciferase. Recently, we demonstrated that firefly luciferase has a catalytic function of fatty acyl-CoA synthesis from fatty acids in the presence of ATP, Mg^{2+} and coenzyme A. Based on identification of fatty acyl-CoA genes in firefly, *Drosophila*, and non-luminous click beetles, we then proposed that the evolutionary origin of firefly luciferase is a fatty acyl-CoA synthetase in insects. Further, we succeeded in converting the fatty acyl-CoA synthetase of non-luminous insects into functional luciferase showing luminescence activity by site-directed mutagenesis.

Keywords Bioluminescence · Luciferin analogue · Inhibitors · Fatty acyl-CoA synthetase · Molecular evolution

Introduction

The phenomenon of bioluminescence has been observed in many different organisms including bacteria, fungi, algae, fish, squid, shrimp and insects [1–5]. Luminous organisms produce light by an enzymatic reaction of a luciferase (=enzyme) with a luciferin (=substrate). Light-emitting reactions are quite distinct among luminous organisms, but in each case the reaction is an oxidation process with molecular oxygen and is a conversion of chemical energy into light [6–9]. The various luciferase genes have been isolated and adapted as reporter genes [10, 11]. In insects, the luminous species are mainly found in three families: fireflies (Lampyridae), railroad worms (Phengodidae) and click beetles (Elateridae) [12–14]. The bioluminescence systems of these insects are essentially the same with an identical luciferin, ATP, and Mg^{2+}, and with similar luciferases. Firefly luciferase has been studied for the last 50 years and proven to be a useful enzyme. It has been extensively used in molecular and cell biology, in particular for the quantification of ATP and as a reporter enzyme of gene expression [10, 11]. The purpose of this review is to reevaluate the basic information on firefly luciferase and luciferin including the luminescence reaction, and to reconsider the catalytic function of firefly luciferase and other unresolved problems, based on recent investigations. Further, the molecular evolution of firefly luciferase from a fatty acyl CoA synthetase in insects is described on the basis of our recent reports.

Chemical basis of firefly bioluminescence

Bioluminescence reaction of firefly luciferase

Firefly luciferase (EC. 1.13.12.7) is classified as a monooxygenase and catalyzes the emission of yellow-green light from the substrate D-luciferin, Mg^{2+}-ATP, and oxygen, according to the following equations:

$$\mathrm{Luc} + \mathrm{LH_2} + \mathrm{ATP} \overset{\mathrm{Mg^{2+}}}{\rightleftharpoons} \mathrm{Luc:LH_2\text{-}AMP} + \mathrm{PP_i} \quad (1)$$

$$\mathrm{Luc{:}LH_2\text{-}AMP} + \mathrm{O_2} \rightarrow \mathrm{Luc{:}Oxyluciferin^*} + \mathrm{AMP} + \mathrm{CO_2} \quad (2)$$

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$$\text{Luc:Oxyluciferin}^{*} \rightarrow \text{Luc:Oxyluciferin} + h\nu \quad (3)$$

The initial reaction catalyzed by firefly luciferase (Luc) is the formation of luciferase-bound luciferyl adenylate (Luc:LH_2-AMP) in the presence of Mg^{2+} and ATP by the release of inorganic pyrophosphate (PP_i) Eq. 1. The carboxyl group of D-luciferin (LH_2) is adenylated. The second step involves the oxygenation of LH_2-AMP with molecular oxygen (O_2) to produce the excited state of oxyluciferin (Oxyluciferin*), adenosine monophosphate (AMP) and carbon dioxide (CO_2) (Eq. 2). The emission of light is produced from the relaxation of excited state oxyluciferin to the corresponding ground state (Eq. 3). The luminescence reaction of firefly luciferin with other luciferases from luminous beetles and railroad worms is identical to that of firefly luciferase [13, 14].

On the other hand, firefly luciferase also catalyzes the formation of luciferyl-CoA (LH_2-CoA) from LH_2-AMP in the presence of coenzyme A (CoA) under anaerobic conditions (Eq. 4).

$$\text{Luc:}LH_2\text{-AMP} + \text{CoA} \rightarrow \text{Luc} + LH_2\text{-CoA} + \text{AMP} \quad (4)$$

The thiol-ester of CoA is formed at the carboxyl group of luciferin. L-Luciferyl-CoA is formed from L-luciferin in the same process as LH_2-AMP formation [15].

Dehydroluciferyl-CoA (L-CoA) is obtained from dehydroluciferin (L) in the presence of ATP, Mg^{2+} and CoA (Eqs. 5 and 6).

$$\text{Luc:L} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{Luc:L-AMP} + PP_i \quad (5)$$

$$\text{Luc:L-AMP} + \text{CoA} \rightarrow \text{Luc} + \text{L-CoA} + \text{AMP} \quad (6)$$

In 1967, on the basis of above results, McElroy et al. proposed that firefly luciferase is one of the family of acyl-CoA synthetases, similar to a fatty acid acyl-CoA synthetase and an amino-acyl tRNA synthetase [16]. However, nobody knew whether firefly luciferase can synthesize the fatty acyl-CoA from free fatty acids until we demonstrated it in 2003 [17].

The catalytic reaction of acyl-CoA synthetase is as follows:

$$\text{R-COOH} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{R-CO-AMP} + PP_i \quad (7)$$

$$\text{R-CO-AMP} + \text{CoA} \rightarrow \text{R-CO-CoA} + \text{AMP} \quad (8)$$

Thus, firefly luciferase is a member of the acyl-adenylate/thioester-forming superfamily of enzymes [16].

Chemical structures of related compounds involved in the firefly luciferin–luciferase reaction

To understand the catalytic mechanism of firefly luciferase, it is necessary to know the chemical structures of ATP, AMP and coenzyme A (CoA) (Fig. 1). The chemical structures of firefly luciferin including its related compounds are shown in Fig. 2 and their properties are summarized as follows.

Firefly luciferin = D(−)-luciferin (D-LH_2)

In 1957, approximately 9 mg of crystalline firefly luciferin was isolated from 15,000 firefly lanterns (70 g of acetone powder) of the North American firefly *Photinus pyralis* [18]. From the Japanese firefly, *Luciola cruciata*, approximately 5.5 mg of luciferin was crystallized from 12,000 adult abdomens (233 g of acetone powder) [19]. The structure of firefly luciferin, D(−)-Luciferin, (*S*)-2-(6′-hydroxy-2′-benzothiazolyl)-2-thiazoline-4-carboxylic acid, was determined by chemical synthesis [20, 21]. The absolute configuration of the carboxyl group at the C4 position was also determined to be the *S* form. This chirality of luciferin is crucial to the luminescence reaction with luciferase. Thus, L(+)-luciferin is practically inactive for the luminescence reaction. The molecular configuration and confirmation of synthetic luciferin including intra-atomic bond distances and angles were determined by X-ray structure analysis and were confirmed to be identical to the structure by chemical synthesis [22]. The K_m value of recombinant *P. pyralis* luciferase for luciferin and ATP were 15 and 160 μM, respectively [23]. Recently, the contents of firefly luciferin in luminous and non-luminous beetles were re-examined and confirmed by the methods of HPLC with fluorescence detection and the luminescence reaction of luciferin–firefly luciferase (L–L reaction) [24]. In Japanese fireflies, the amount of luciferin is at the range of 0.5–3.5 nmol (140–980 ng) per specimen, and these

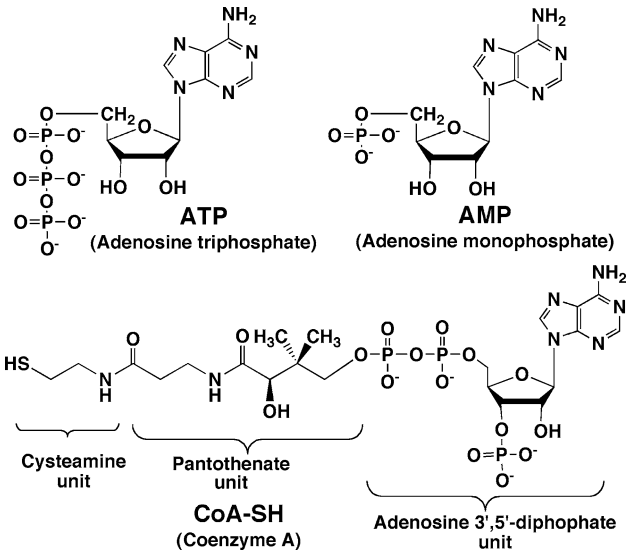

Fig. 1 Chemical structures of *ATP*, *AMP* and coenzyme A (*CoA*)

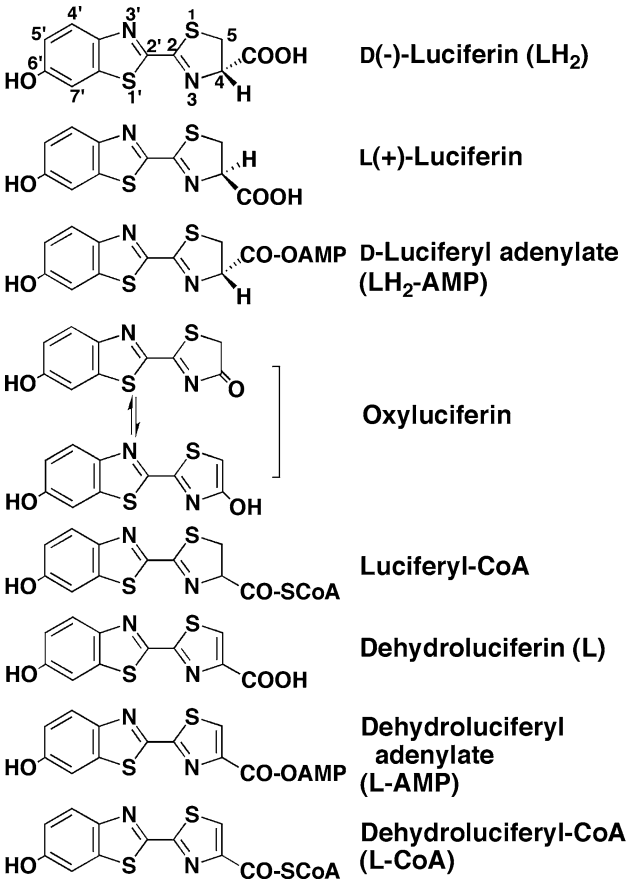

Fig. 2 Chemical structures of firefly luciferin and its related compounds

values were similar to those previously reported [19]. On the other hand, no D-luciferin was detected in the non-luminous beetles, as far as was examined [24].

L(+)-Luciferin (L-LH$_2$)

L-Luciferin is an enantiomer of D-luciferin and was synthesized in order to identify the chemical structure of natural D-luciferin [25]. In the standard reaction mixtures, L-luciferin is not used in the luminescence reaction [25]. L-Luciferin is a potent competitive inhibitor of luciferase and the K_i value is between 3 and 4 μM [26]. In 1996, weak light production from L-luciferin with luciferase was reported, and the mechanism of racemization from L-luciferin to D-luciferin through luciferyl adenylate formation was proposed [26]. In larvae of the Japanese firefly *Luciola cruciata*, approximately 1:1 mixtures of D- and L-luciferin were detected by an HPLC analysis using a chiral column [27]. Further, the extracts of light organs of *L. cruciata* showed a catalytic activity to convert L-luciferin to D-luciferin in the presence of ATP, Mg^{2+} and CoA [27]. However, these results did not explain the biosynthetic pathway of firefly luciferin and the synthesis process of luciferin was still unclear (see "Biosynthesis of firefly luciferin in vivo").

Dehydroluciferin (L)

Dehydroluciferin was firstly identified as one of the products formed during the biological oxidation of luciferin. Dehydroluciferin is present in small quantities in the firefly and can be prepared from luciferin by chemical oxidation. L-Luciferin and D-luciferin can be converted to dehydroluciferin by oxidation with ferricyanide or heating in alkaline solution. Dehydroluciferin is known to be potent inhibitor of the luminescence reaction [18].

Oxyluciferin

In the early 1960s, dehydroluciferin referred to firefly oxyluciferin [25, 28, 29]. The chemical structure of oxyluciferin was proposed by the fluorescence spectral data of the synthetic compounds [30–33]. Oxyluciferin from the reaction mixture of the luciferin–luciferase reaction was only detected by HPLC analysis [34], because oxyluciferin is an unstable compound in solution. Oxyluciferin is a carbonyl compound with a hydrogen atom on its α carbon and shows a keto–enol tautomerism that is catalyzed by both acids and bases. The keto form and enol form of oxyluciferin in solution show red fluorescence and yellow-green fluorescence, respectively. The K_i value of oxyluciferin for light production is 0.5–2 μM [35].

Luciferyl adenylate (LH$_2$-AMP)

Luciferyl adenylates are formed from D-luciferin and L-luciferin by luciferase in the presence of ATP and Mg^{2+}. The reversibility of this reaction has been demonstrated [28]. The K_m values of native luciferase and recombinant luciferase for light production are 2.4 and 4.7 μM, respectively [36]. Luciferyl adenylate has been chemically synthesized and characterized. Luciferyl adenylate is extremely unstable in aqueous solution [25, 29, 37] and is hydrolyzed to luciferin and adenylic acid very rapidly at pH 8 [28, 37]. Luciferyl adenylate racemized easily in neutral to basic buffer [15, 37, 38]. On the other hand, D- and L-luciferyl adenylates emit a red chemiluminescence in an aprotic solvent upon addition of strong base [29]. D-Luciferyl adenylate is used for the luciferase reaction, but not L-luciferyl adenylate.

Dehydroluciferyl adenylate (L-AMP)

Dehydroluciferyl adenylate is an inhibitor of the luminescence reaction [28]. The K_i value for light production is 0.0025–1.25 μM [35].

Mechanism of firefly luminescence reaction

The mechanism of the bioluminescence reaction of firefly luciferin catalyzed by firefly luciferase is shown in Fig. 3. The luminescence reaction involves the formation of a dioxetanone intermediate [38]. The formation of dioxetanone was confirmed by ^{18}O-labeling experiments [39]. Thus, one oxygen atom of molecular oxygen was incorporated into oxyluciferin and one into CO_2. The decomposition of dioxetanone results in light emission from the excited state of oxyluciferin by the chemically initiated electron-exchange luminescence (CIEEL) mechanism [40, 41].

Light emitter and color variation in firefly bioluminescence

In *P. pyralis* luciferase in vitro, the luminescence color at pH 7.6 is yellow-green (λ_{max} 565 nm), but a red luminescence (λ_{max} 620 nm) is observed under acidic conditions at pH 5.4 [42]. To explain the multiple colors of firefly bioluminescence, the light emitter species of oxyluciferin have been proposed. Until early 1990, the relationship between the light emitter of oxyluciferin and the color of luminescence was explained by the keto–enol tautomerism (Fig. 4a). Under acidic and basic conditions, the fluorescence spectra showed red and yellow-green colors that corresponded to the keto form and the enol form of oxyluciferin, respectively [30–33]. To understand the species of light emitter, the fluorescence properties of the *5*-methyl and *O*-methyl derivatives of oxyluciferin were investigated [43]. In 1991, a single amino acid mutant of *L. cruciata* luciferase, Ser286 to Asn286 (S286N), was prepared, which showed red bioluminescence (λ_{max} 607 nm) with natural firefly luciferin. In addition, the different colors including red, orange-yellow and yellow-green were also produced by other luciferase mutants [44]. These results suggested that the light emitter could not be explained by the keto–enol tautomerism of oxyluciferin. Further, in 2002, D-5, 5-dimethylluciferin and its adenylate were

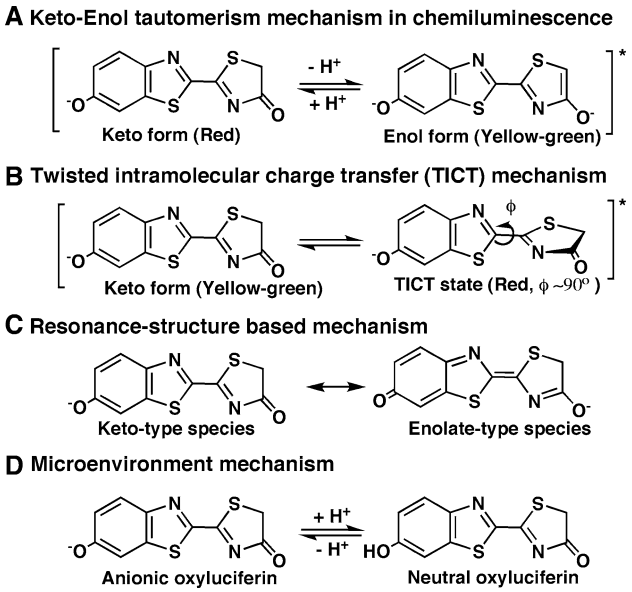

Fig. 4 Proposed mechanisms for light emission in the firefly luciferase reaction

chemically synthesized and examined as a substrate for the luciferase reaction. With the adenylate of D-5,5-dimethylluciferin as a substrate, the luciferases of the firefly (*P. pyralis*) and click beetle (*Pyrophorus plagiophthalamus*) gave yellow-green and red colors of luminescence, respectively [45]. This result suggested keto–enol tautomerism could not explain two colors of luminescence, since D-5,5-dimethyloxyluciferin has no hydrogen atom at the C5 position and cannot take the keto–enol tautomerism. Consequently, the twisted intramolecular charge transfer (TICT) mechanism was proposed [46, 47]. The benzothiazole and thiazolone rings in the oxyluciferin molecule are rotated around of the C2–C2′ bond (Fig. 4b), suggesting that different conformations of excited oxyluciferin give multicolor emission. Further, other mechanisms including the resonance-structure mechanism [48] (Fig. 4c) and microenvironment mechanism (Fig. 4d) have been proposed [48]. The discrepancy of emission color in the

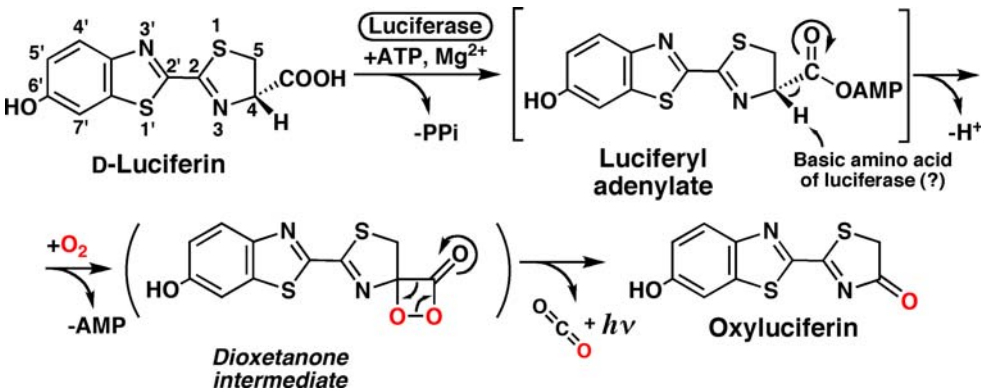

Fig. 3 Mechanism of the bioluminescence reaction of firefly luciferin by firefly luciferase

emitter structures of the keto form of oxyluciferin is preserved between Fig. 4a (red) and b (yellow-green). Several theoretical studies on the light emitter species of oxyluciferin combined with fluorescence spectral data of analogues have been reported [49–52]. However, the actual light emitter species in firefly bioluminescence is not yet conclusively known.

Luminescence pattern and emission spectrum of firefly luciferase

The kinetics of the firefly luciferase reaction in vitro are complicated, due to the fact that luciferin, ATP, Mg^{2+} and molecular oxygen are essential for the luminescence reaction [53]. Also, the luminescence pattern and the luminescence emission spectrum are affected by the reaction conditions such as pH, metal ion and temperature [54–57]. Further, the luminescent products including oxyluciferin and dehydroluciferin inhibit the luminescence reaction of firefly luciferase. As shown in Fig. 5, in the presence of saturating amounts of ATP, light emission by firefly luciferase rises quickly followed by a rapid decay, and then continuous weak-glow luminescence is observed. The rapid decay of light emission can be prevented by the addition of coenzyme A [58]. This luminescence enhancement may be explained by the formation of dehydroluciferyl-CoA, resulting in release of dehydroluciferin from the luciferase. This result suggests that firefly luciferase has CoA synthetic activity of dehydroluciferin through to dehydroluciferyl adenylate.

Emission spectra with λ_{max} between 546 and 618 nm were observed in various luciferases and their mutants [14, 59]. Some pH-sensitive and pH-insensitive luciferases have been reported, and several mutants of pH-sensitive luciferases were converted to pH-insensitive forms with a different color of light emission [14]. There are no clear explanations for these phenomena, because the emission spectrum of luciferase is dependent on the excitation species of oxyluciferin. Further, the quantum yield of the firefly luciferase reaction is dependent on the emission spectrum. In an early report, the quantum yield was estimated to be 0.88 ± 0.25 at pH 7.6 in Tris-buffer [42, 60]. Recently, the quantum yield was revised to 0.41 ± 0.074 at pH 8.5 in Tris-buffer [61]. To avoid similar historical confusion, it is necessary to confirm these values by other methods, and the quantum yields of other luciferases should be re-examined by the same procedures.

Firefly luciferase reaction with firefly luciferin

Under atmospheric conditions, the reaction scheme of firefly luciferin is summarized in Fig. 6, but the reaction catalyzed by firefly luciferase is still not completely

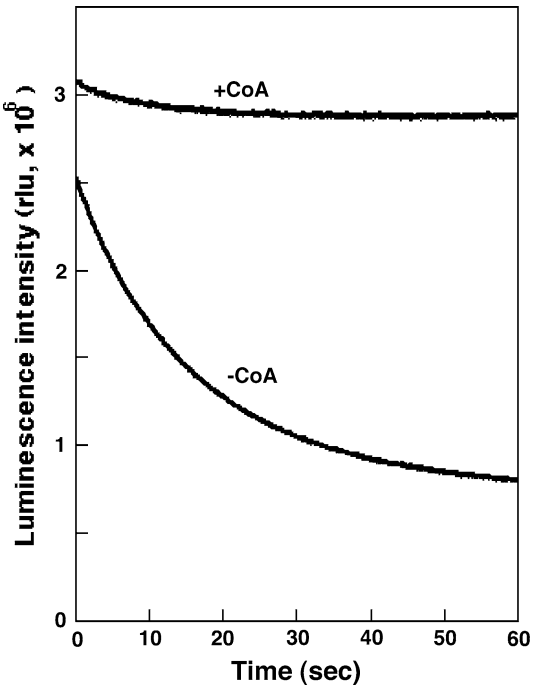

Fig. 5 Luminescence pattern of firefly luciferase in the presence and absence of coenzyme A. The reaction mixture (100 µl) contained firefly luciferin (10 µM), ATP (250 µM) and $MgCl_2$ (5 mM) in 100 mM Tris–HCl (pH 7.8). In the presence and absence of CoA (250 µM), the reaction was started by the addition of recombinant *P. pyralis* luciferase (1 pmol: Promega) and the light intensity of luminescence was measured at 22–23°C

described. In the firefly luciferase reaction (Fig. 3), the deprotonation and O_2 addition at the C4 position of D-luciferyl adenylate is the limiting step to light emission. The racemization between D-LH_2-AMP and L-LH_2-AMP might not occur in the luciferase molecule, but in the solution non-enzymatically after releasing luciferyl adenylate from the luciferase molecule. D-Luciferyl adenylate bonded within luciferase reacts with O_2 to emit light. Recently, the formation of dehydroluciferin from D-luciferin was proposed by reacting D-luciferyl adenylate with O_2 to give H_2O_2 as a side reaction [62]. This reaction seems to be competitive with the luminescence reaction, and it is necessary to confirm the conversion from D-/L-luciferyl adenylate to dehydroluciferin in the presence and absence of firefly luciferase.

Biosynthesis of firefly luciferin in vivo

Regarding the biosynthesis of firefly luciferin in vivo, the synthetic pathway and precursor(s) are not solved. In the 1970s, studies on the biosynthesis of firefly luciferin were carried out by incorporation experiments using ^{14}C-labeled compounds such as cysteine and quinone (Fig. 7). Biosynthetic pathways have been proposed, but based on insufficient information with inadequate experiments

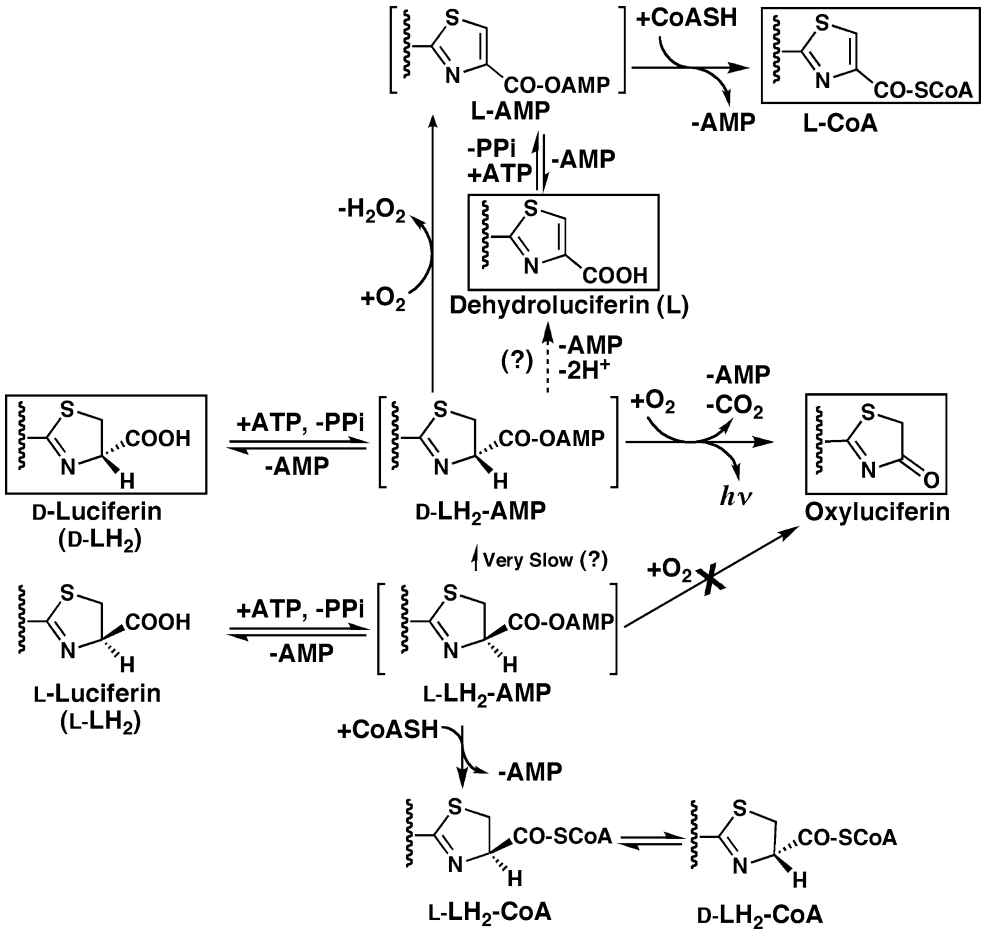

Fig. 6 Formation of dehydroluciferin, dehydroluciferyl CoA and D-luciferyl CoA from D- and L-luciferin during the luminescence reaction by firefly luciferase

[63–66]. The only clear evidence is that cysteine is a synthetic unit of firefly luciferin [63, 66]. In 2001, a luciferin-regeneration enzyme of firefly luciferin from oxyluciferin was reported [67, 68]. Later, it was found that the protein has high amino acid sequence similarity to the senescence marker protein-30, that is a hydrolase, gluconolactonase (EC. 3.1.1.17). It is necessary to reconsider the previous results and re-examine these experiments [69, 70]. In some cases, mass spectral analysis using a stable isotope labeled compound could be useful, as reported in *Cypridina* luciferin biosynthesis [71, 72].

Luciferin analogues for luciferase substrate

White et al. first synthesized the luciferin analogues in the 1960s [73–76] and showed narrow substrate specificity for luciferase. A convenient method of firefly luciferin synthesis was reported by Seto et al. [77]. The main structures of luciferin analogues and its luminescence activity are shown in Fig. 8. The luciferin esters of the hydroxyl group at the 6′ position of benzothiazole ring and the carboxyl group at the 4 position of thiazoline ring were not utilized for the luciferase reaction. Thus, the phenolic hydroxyl group of benzothiazole ring might interact with luciferase for the luminescence reaction. 6′-Amino-D-luciferin, a substituent of the hydroxyl group, showed lower luminescence activity (∼10%) with red luminescence [74]. 4′-Methyl-D-luciferin showed ∼5% activity [78]. *N*-Alkylated luciferins of 6′-aminoluciferin were synthesized and the luminescence activity was characterized [79]. D-Quinolylluciferin and D-naphthylluciferin, with substitutes of the benzothiazole ring with quinoly- and naphthyl-groups, showed 7 and 1.5% luminescence activities, respectively [80]. 5-Methylluciferin can be used for the luciferase reaction, but not 5,5-dimethylluciferin [76]. However, 5,5-dimethylluciferyl adenylate showed ∼16% activity [45]. These findings by Branchini et al. gave insight into the light emitter of oxyluciferin (see "Light emitter and color variation in firefly bioluminescence", above).

Luciferin derivatives for bioluminescent assays

To develop bioluminescence enhanced enzyme assays and immunoassays, derivatives of luciferin ester and amide have been synthesized. These enzymes hydrolyze the luciferin derivatives and the released D-luciferin is utilized

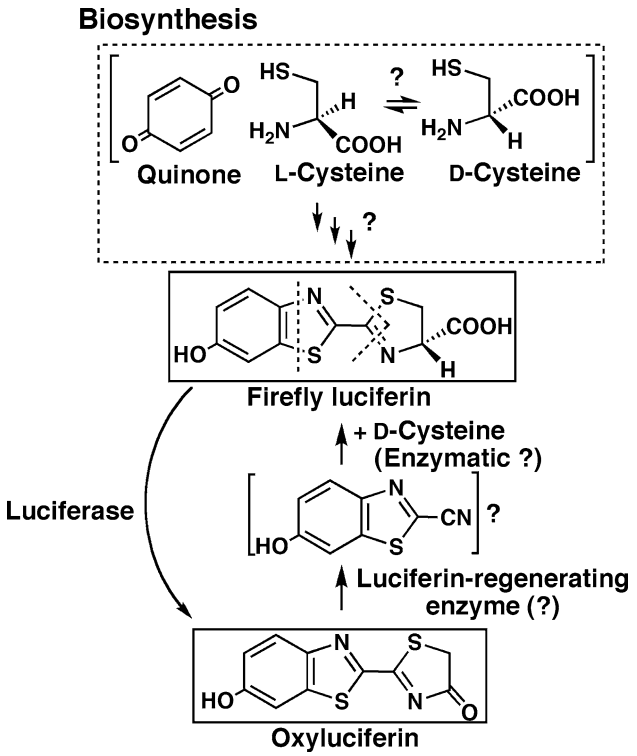

Fig. 7 Biosynthesis of firefly luciferin and conversion to firefly luciferin form oxyluciferin

for the firefly luciferase reaction. The derivatives are shown in Fig. 9. The sulfate and phosphate of D-luciferin were used as a substrate for acylsulphatase and alkaline phosphatase, respectively [81–85]. Also, D-luciferin glycosides were used to determine the glycohydrolase activity [86]. Further, the esters and amide at the carboxyl group of D-luciferin were used for carboxylic esterase and carboxypeptidase, respectively [81–83]. The amide of 6′-amino-D-luciferin was used for α-chymotrypsin assay [87]. A bifunctional luminogenic substrate (ALPDO) for both firefly luciferase and horseradish peroxidase was also reported [88]. To determine monoamine oxidase activity in a homogeneous assay system, acylic amine derivatives of luciferin were synthesized and applied [89, 90]. For bioluminescence imaging in mammalian cells using firefly luciferase as a reporter gene, membrane-permeable luciferin esters were reported [79, 91, 92], and 6′-amino-D-luciferin amides including glycine [93] and the transporter conjugate [94] were synthesized and applied in mice [95]. To improve the circulatory half-life time of luciferin for in vivo tumor imaging, 6′-amino-D-luciferin modified with polyethylene glycol (PEG-luciferin) was prepared [96].

Inhibitors of firefly luciferase

Firefly luciferase has been used as a reporter protein in various assay systems including gene expression and was applied in the process of high-throughput screening for drug discovery. To avoid false positives in the chemical screening assays, it is valuable to know the inhibitors of the luciferin–luciferase reaction. Substrate-related compounds

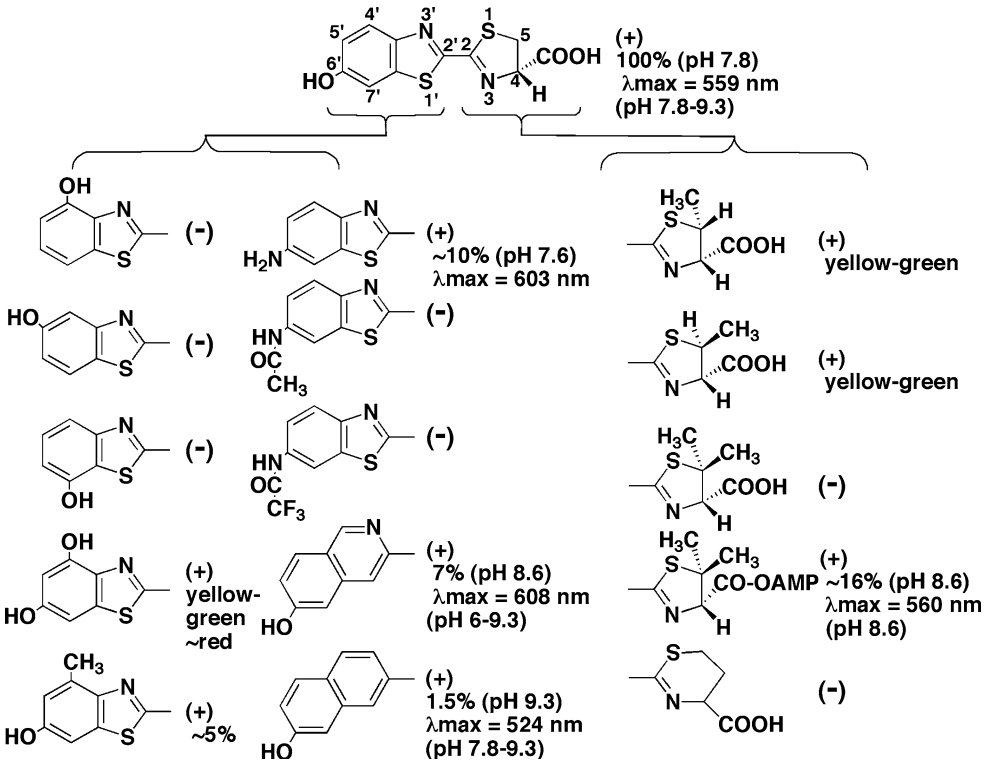

Fig. 8 Firefly luciferin analogues for the luciferase reaction and its luminescence activity. *Plus* and *minus* in *parentheses* show the positive and negative activities of luminescence as a substrate

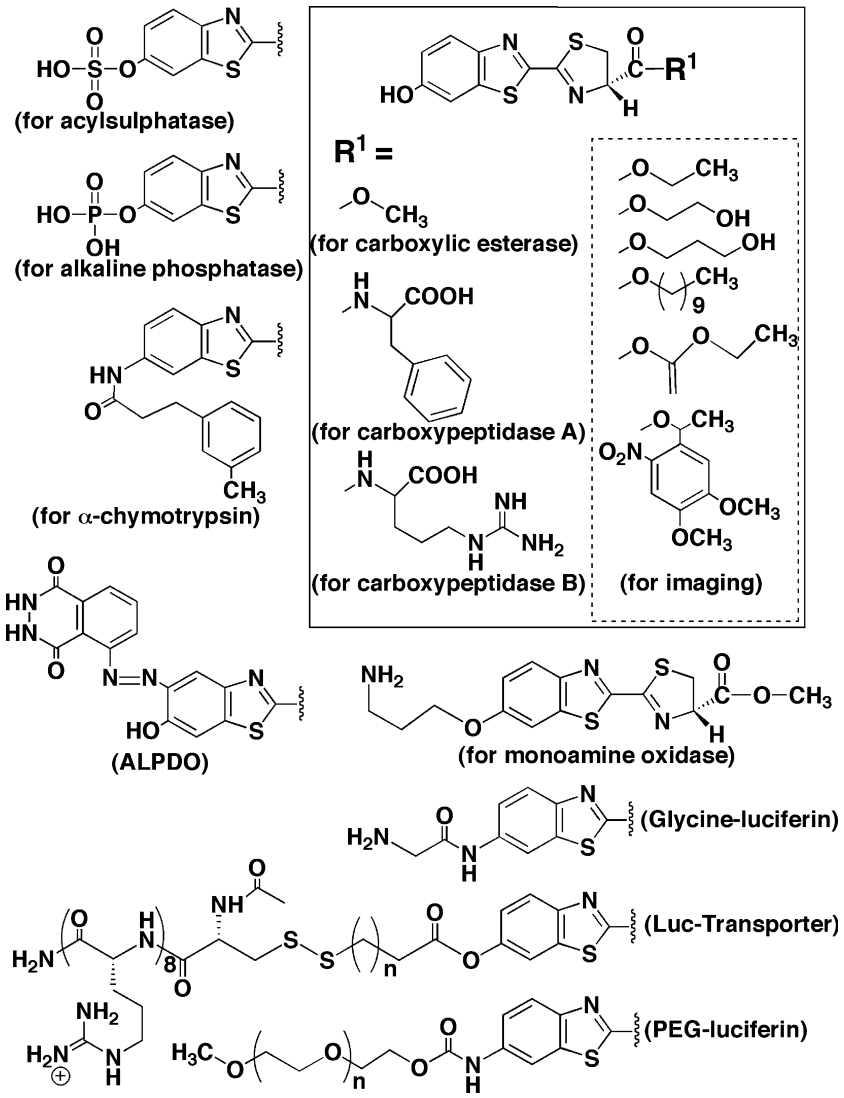

Fig. 9 Luciferin derivatives for bioluminescence assays using firefly luciferase

including dehydroluciferin [18], L-luciferin [26], oxyluciferin [97], pyrophosphate [98], AMP [99] and ATP analogues [100] were known as inhibitors. In contrast, after initiating the luminescence reaction with ATP, the addition of pyrophosphate and triphosphate stimulates light production under some conditions [57, 98]. As shown in Fig. 10, structurally unrelated compounds such as halothane [101], fatty acids [102, 103], pifithrin-α [104], lipoic acid [105], *N*-pyridin-2-ylbenzamide analogues [106], and 2-phenylbenzothiazole analogue [107] also inhibit luciferase activity. In cell-based luciferase reporter assays, the stabilization of luciferase by inhibitors results in the counterintuitive phenomenon of signal stimulation [108, 109]. Among competitive inhibitors, the luminescence inhibition of fatty acids reported by Ueda and Suzuki [102, 103] suggested to me that firefly luciferase has a catalytic function of fatty acyl-CoA synthesis.

Protein chemistry of firefly luciferase

Purification of firefly luciferase

In 1956, firefly luciferase was isolated from firefly lanterns of *P. pyralis* by gel filtration and was crystallized yielding 30 mg protein from ~6,000 specimens [53, 110]. Later, to purify native firefly luciferase more conveniently, several affinity chromatographies were performed using benzylamine [111] and Cibachoron Blue F3GA [112] as a ligand. The benzylamine group functions through hydrophobic interaction with luciferase, and 6.2 mg of luciferase was obtained from 2.2 g of acetone powder of lanterns. Cibachoron Blue F3GA (Blue Dextran, Blue Sepharose), which binds enzymes requiring adenylyl-type cofactors such as ATP, NADH and FAD, was applied for an affinity purification of luciferase. To remove adenylate kinases and

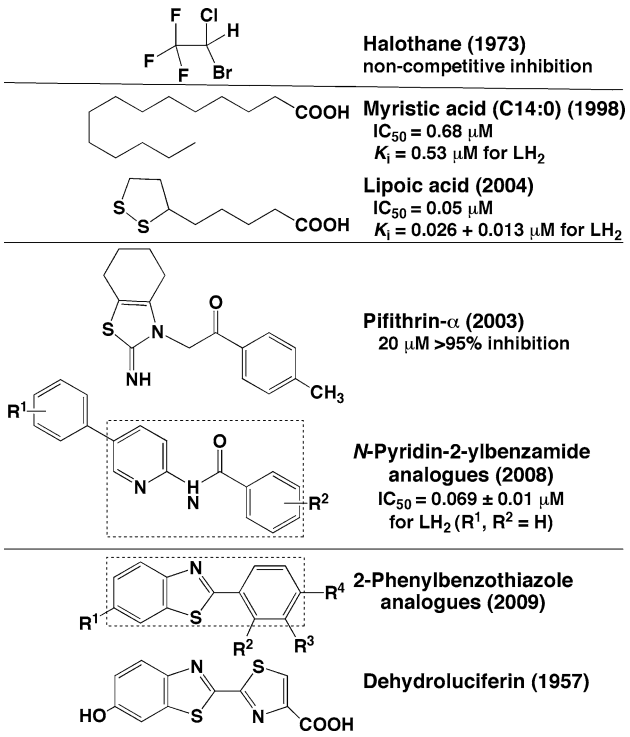

Fig. 10 Inhibitors of the firefly luciferase–luciferin reaction

prevent interference with ATP assays by firefly luciferase, Dyematrex Orange gel was used [113]. The Japanese firefly luciferases, *L. cruciata* and *L. lateralis*, were purified by ammonium sulfate precipitation, gel filtration, followed by hydroxyapatite HPLC [114] to afford ~0.2 mg protein from 1.5 g lanterns (wet weight). In a complementary approach, firefly luciferase gene was expressed in prokaryotic and eukaryotic cells. To express recombinant firefly luciferase in *E. coli* cells, the induction temperature is most effective when kept between 20 and 25°C to refold the active luciferase [115, 116]. Recently, firefly luciferase fused to IgG binding-domain of protein A was efficiently expressed in *E. coli* cells under the control of cold shock protein A promoter at 15°C [117].

Primary structure of firefly luciferase

The gene for *P. pyralis* luciferase was cloned [118] and the nucleotide sequences of cDNA and genomic DNA were determined in 1987 [119]. The primary structure of *P. pyralis* luciferase consists of 550 amino acid residues in a single polypeptide chain with a peroxisome targeting signal sequence of -Ser-Lys-Leu (-SKL) at C-terminus [120]. The calculated average molecular weight and theoretical *pI* were 60,745.17 and 6.42, respectively. Genomic sequence analysis of *P. pyralis* luciferase revealed that six short introns are present [119]. Interestingly, the identical positions of the introns were found in *L. lateralis* luciferase [121]. In 1989, the sequence similarity of firefly luciferase to plant 4-coumarate:CoA ligase (EC 6.2.1.12) was firstly reported [122] and the significant homology to rat long-chain acyl-CoA synthetase (EC 6.2.1.3) was also reported [123]. Further, the conserved motif sequences for the characteristic adenylate formation in the phosphate-binding sites of the phosphoproteins and the nucleotide-binding proteins were found by a homology search and it was proposed that acetyl-CoA synthetases were identified as members of firefly luciferase family [124]. The putative AMP-binding domain motif of [S/T/S]–[S/T/G]–G–[S/T]–[T/S/E]–[G/S]–x–[P/A/L/I/V/M]–K was found as the sequence of [^{198}S–S–G–S–T–G–L–P–K^{206}] [124]. After the cDNA clone of *P. pyralis* luciferase was isolated, many other firefly luciferase (beetle luciferase) genes including click beetle (*Pyrophorus* sp.) and railroad warm (*Phrixothrix* sp.) were readily cloned, sequenced, and characterized (Table 1). All luciferases are a single polypeptide chain and show high sequence identities among them with 48–99% identity [125, 126].

Three-dimensional structure of firefly luciferase

In 1996, the three-dimensional structure of *P. pyralis* luciferase was firstly determined as an unbounded form without any substrate or analogue [127]. The luciferase molecule folds two distinct domains, a large N-terminal domain (1–436 aa) and a small C-terminal domain (440–550 aa), joined by a flexible linker peptide (PDB ID: 1LC1). The N-terminal domain consists of an antiparallel β-barrel and two β-sheets. The two β-sheets are assembled to form a five-layered $\alpha\beta\alpha\beta$ tertiary structure. Bromoform ($CHBr_3$) is known as an anesthetic compound that inhibits the luminescence reaction of firefly luciferase. The crystal structure of bromoform-bounded firefly luciferase was determined, suggesting that two bromoform molecules might bind in the possible substrate interacting area (PDB ID: 1BA3) [128]. Later, to elucidate the conformational changes of the enzyme–substrate complex in the firefly luciferase reaction, an intermediate analogue of the natural luciferyl adenylate, 5′-*O*-[*N*-(dehydroluciferyl)sulfamoyl]adenosine (DLSA), was synthesized (Fig. 11) [129]. The stable adenylate analogue DLSA is a potent competitive inhibitor for the luminescence reaction; $K_i = 34 \pm 5$ nM for LH_2, $K_i = 41 \pm 3$ nM for Mg^{2+}-ATP, and $K_i = 340 \pm 50$ nM for LH_2-AMP. In 2006, DLSA was synthesized by another synthetic route and used for determining the crystal structure of *L. cruciata* luciferase in complex with DLSA (PDB ID: 2D1S) [130] (Fig. 12a). The results showed that two domain structures of *L. cruciata* luciferase are essentially the same as *P. pyralis* luciferase [127, 128], and a relationship between color variation and luciferase structure was proposed [130]. It is interesting that many water

Table 1 List of cDNA sequences of beetle luciferases from insects

Insect species name	GenBank (Nucleotide) Accession no.	References
Photinus pyralis	M15077	[119]
Luciola cruciata	M26194	[139]
Luciola lateralis	X66919	[140]
Luciola lateralis	Z49891	[121]
Luciola mingrelica	S61961	[115]
Photuris pennsylvanica	D25415	[141]
Photuris pennsylvanica	U31240	[125]
Pyrocoelia miyako	L39928	[142]
Pyrocoelia rufa	AF328553	[143]
Hotaria parvula	L39929	[142]
Hotaria unmunsana	AF420006	[144]
Lampyris noctiluca	X89479	[145]
Lampyris turkestanicus	AY742225	[146]
Cratomorphus distinctus	AY633557	[147]
Lampyroidea maculata	DQ137139	[148]
Pyrophorus plagiophthalamus	–	[149]
Pyrearinus termitilluminans	AF116843	[150]
Pyrophorus plagiophthalamus	AF543368	[151]
Pyrophorus mellifluus	AF545854	[151]
Phrixothrix viviani	AF139644	[152]
Phrixothrix hirtus	AF139645	[152]

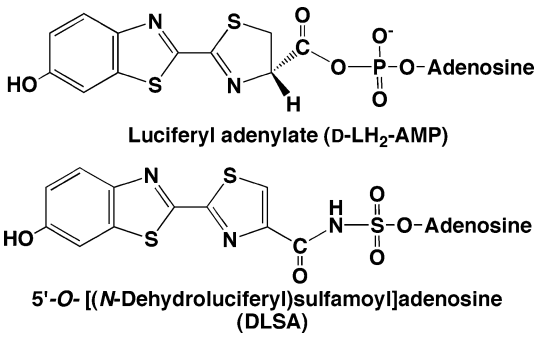

Fig. 11 Comparison of chemical structures between luciferyl adenylate and its analogue DLSA

molecules could interact with the luciferyl adenylate analogue (DLSA) and oxyluciferin (PDB ID: 2D1Q) (Fig. 12b). However, the color variations were not explained by these structural analyses including the oxyluciferin–luciferase complex. The attack of molecular oxygen (O_2) to D-luciferyl adenylate might be from a specific direction, because L-luciferyl adenylate could not react with O_2 to produce light. An important point is how molecular oxygen accesses to the luciferyl adenylate to emit light from outside the luciferase molecule [Fig. 6]. An open space for O_2 to reach luciferyl adenylate has been found in the structure of the luciferase–DLSA complex (A. Nakagawa, personal communication).

Multiple functions of luciferase as an adenylate-forming enzyme

Firefly luciferase is a fatty acyl-CoA synthetase

We predicted that firefly luciferase has the catalytic function of fatty acyl-CoA synthesis from free fatty acids, based on the following background information: (1) the luminescence reaction involves in an adenylation step [28]; (2) dehydroluciferin, a potent inhibitor for luciferase, might be released from luciferase molecule as dehydroluciferyl-CoA, suggesting that luciferase has the catalytic function of a acyl-CoA synthesis [58]; (3) the primary structure of firefly luciferase showed high sequence similarity to a long-chain acyl-CoA synthetase [123]; and (4) the long fatty acids (C_{12}–C_{20}) inhibited the luminescence reaction in competition with firefly luciferin [102, 103]. As shown in Fig. 13, the catalytic function of fatty acyl-CoA synthesis by firefly luciferase was determined [17]. The initial step of the acyl-adenylate formation was examined by monitoring the formation of [α-^{32}P]AMP from [α-^{32}P]ATP in the presence of Mg^{2+}, CoA and various carboxylic acids; acetic acid (C_2), propionic acid (C_3), palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$), arachidonic acid ($C_{20:4}$), and *p*-coumaric acid [17]. Based on these results, long-chain unsaturated fatty acids (C_{16}–C_{20}) are suitable substrates for the adenylation reaction by *P. pyralis* luciferase, but other substrates of aromatic acids for plant *p*-coumarate CoA:ligase and short chain acids were not utilized efficiently by *P. pyralis* firefly luciferase. The identification of fatty acyl-CoA formation via fatty acyl-adenylate was performed using [1-^{14}C]oleic acid as a substrate (Fig. 13). The formation of [1-^{14}C]oleoyl-CoA was confirmed by TLC analysis, and the essential cofactors for the fatty acyl-CoA synthesis are ATP, Mg^{2+} and CoA. Other nucleotides including GTP, CTP, TTP, UTP, and ITP did not stimulate the formation of fatty acyl-CoA significantly. Further, fatty acyl-CoA synthesized from fatty acid ($C_{18:1}$, $C_{18:2}$, $C_{18:3}$,$C_{20:4}$) by *P. pyralis* and *L. cruciata* luciferase was isolated by reversed-phase HPLC and identified by MALDI-TOF–MS. Finally, we demonstrated that firefly luciferase is a bifunctional enzyme that catalyzes not only the luminescence reaction but also long-chain fatty acyl-CoA synthesis [17]. The substrate specificity of *P. pyralis* and *L. cruciata* luciferase with a series of carboxylic acids including amino acid, aromatic acid and monohydroxyl fatty acids was determined [131]. Very weak activities of adenylation were

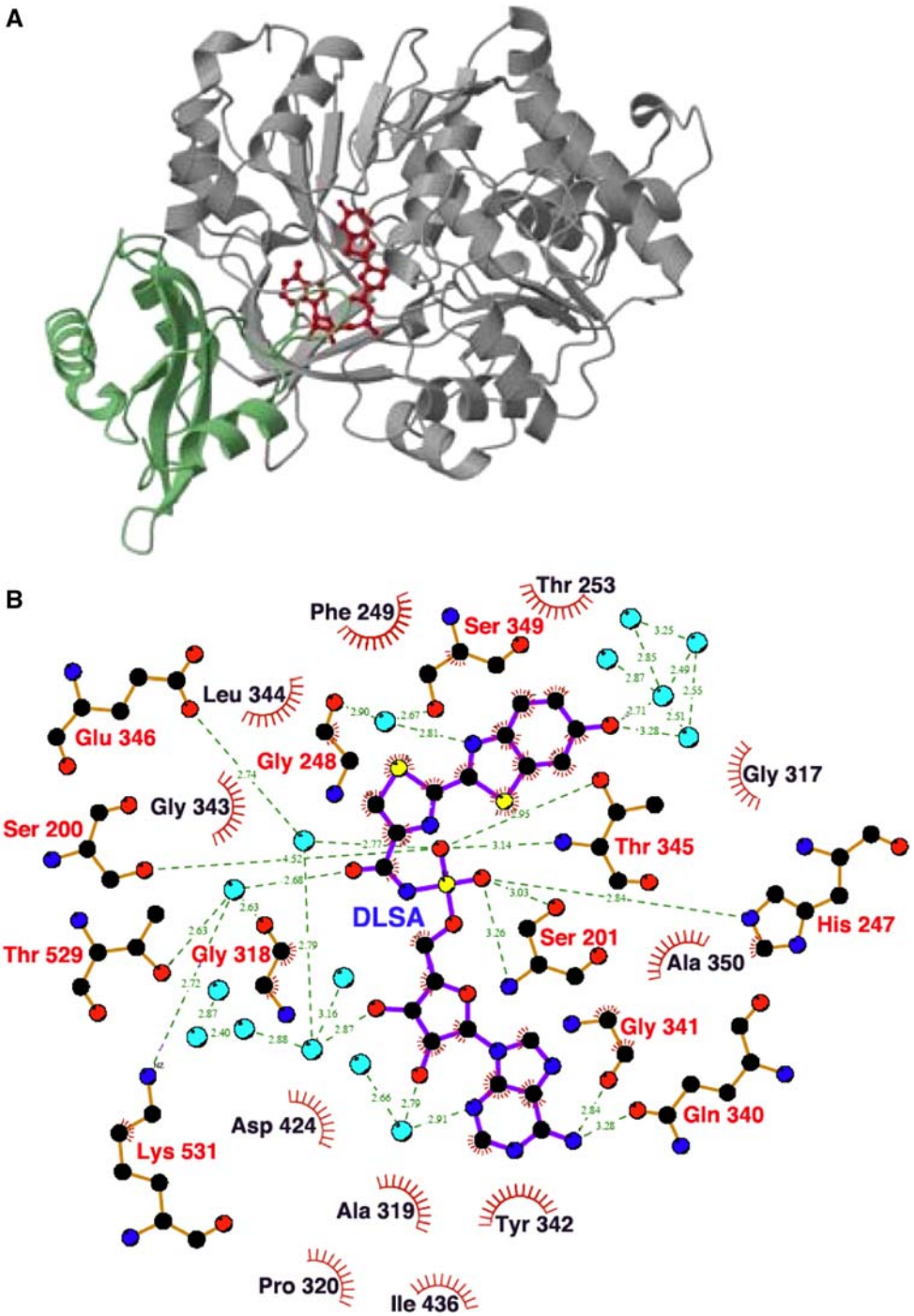

Fig. 12 Structure of the firefly *L. cruciata* luciferase–DLSA complex. **a** Ribbon diagram of firefly luciferase in complex with DLSA (*red*). The N-terminal large and C-terminal small domains are drawn in *gray* and *green*, respectively, **b** A schematic representation of the DLSA binding area in firefly luciferase. The possible hydrogen bonds are shown by a *green dashed line*, and *light blue circles* show water molecules. The amino acid residues labeled with *red* and *black* show the hydrophobic contacts with DLSA

observed for amino and imino acids (L-proline, D-proline, L-cysteine, D-cysteine, L-phenylalanine, L-thiaproline, and thiazolidine-2-carboxylic acid). No significant activity was observed for short chain acids (acetic acid, propionic acid, *n*-hexanoic acid, and lipoic acid) and aromatic acids (*p*-coumaric acid, caffeic acid, ferulic acid, 1-naphthylacetic acid, 2-naphthylacetic acid, 2-quinolinecarboxylic acid, and benzoic acid) [132].

Homologous gene of firefly luciferase in Drosophila melanogaster

To understand the genetic origin and biological function of firefly luciferase, a homology search has been performed in FlyBase for *Drosophila.* The homology search showed that the gene product of *CG6178* (GC6178) was referred to as a "firefly luciferase", due to the highest similarity in amino

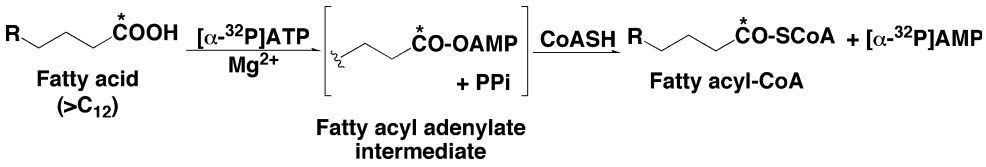

Fig. 13 Determination of fatty acyl-CoA synthesis through fatty acyl adenylate from fatty acid, catalyzed by firefly luciferase. *Asterisks* indicate ^{14}C-labeled position

acid sequence with firefly luciferase. Also, several other homologous genes including *CG4830*, *CG8834*, *CG9390*, *CG11407*, and *CG18586* were detected. However, there was no experimental evidence for the luminescence activity. The cDNA of GC6178 was isolated and expressed in *E. coli* cells. The recombinant CG6178 was purified and characterized [132]. As expected, the purified CG6178 showed a long fatty acyl-CoA synthetic activity, but no luminescence activity. Crude extracts of *D. melanogaster* adults did not show detectable luminescence activity with firefly luciferin (S. Zenno, personal communication). The long-chain fatty acids (C_{18}–C_{20}) are a good substrate for CG6178. The optimum pH is at 7.5–8.5, similar to firefly luciferase. High concentrations (>10 μM) of oleic acid ($C_{18:1}$), linolenic acid ($C_{18:3}$) and arachidonic acid ($C_{20:4}$) inhibit the fatty acyl-CoA synthetic activity of CG6178 and *P. pyralis* luciferase, and moderate inhibition was observed in linoleic acid. The K_m values of CG6178 and *P. pyralis* luciferase for linoleic acid are 1.88 and 13.6 μM, respectively. On the other hand, no inhibition by lauric acid ($C_{14:0}$) was observed at 40 μM in CG6178 and *P. pyralis* luciferase. The K_m values of CG6178, *P. pyralis* luciferase and *L. cruciata* luciferase for lauric acid were 1.68, 7.41, and 16.3 μM, respectively [131]. Thus, CG6178 is the first protein that was identified as a long-chain fatty acyl-CoA synthetase in *Drosophila*. To understand the catalytic properties, the chimeric proteins of *P. pyralis* luciferase and CG6178 were prepared, and only the chimeric protein of the N-terminal domain of *P. pyralis* luciferase and C-terminal domain of CG6178 showed the luminescence activity with ~4% activity of *P. pyralis* luciferase [133].

Homologous genes of firefly luciferase in luminous and non-luminous beetles are fatty acyl-CoA synthetases

Two homologous genes of firefly luciferase, *LcLL1* and *LcLL2*, were cloned from the Japanese firefly, *L. cruciata*, and characterized [134]. The gene product of *LcLL1* had long-chain fatty acyl-CoA synthetic activity, but not luciferase activity. The other gene product of *LcLL2* did not show enzymatic activities of acyl-CoA synthetase or luciferase. The gene expression levels of *LcLL1*, *LcLL2* and *L. cruciata* luciferase in larva and adults of *L. cruciata* were analyzed by RT-PCR, indicating that three genes were abundant in larvae and *LcLL2* was only abundant in adults. Thus, *LcLL1* and *LcLL2* are paralogous genes of firefly luciferase and may have been derived from gene duplication before the luminous and non-luminous insects diverged. From the non-luminous mealworm beetle, *Tenebrio molitor,* three orthologous genes of firefly luciferase, *TmLL-1*, *TmLL-2* and *TmLL-3*, were isolated, and the recombinant proteins were purified. The gene products were identified as a fatty acyl-CoA synthetase [135], and they did not show detectable luminescence activity. Further, a homologous gene of firefly luciferase was isolated from the Japanese non-luminous click beetle, *A. binodulus*, and assigned *AbLL*

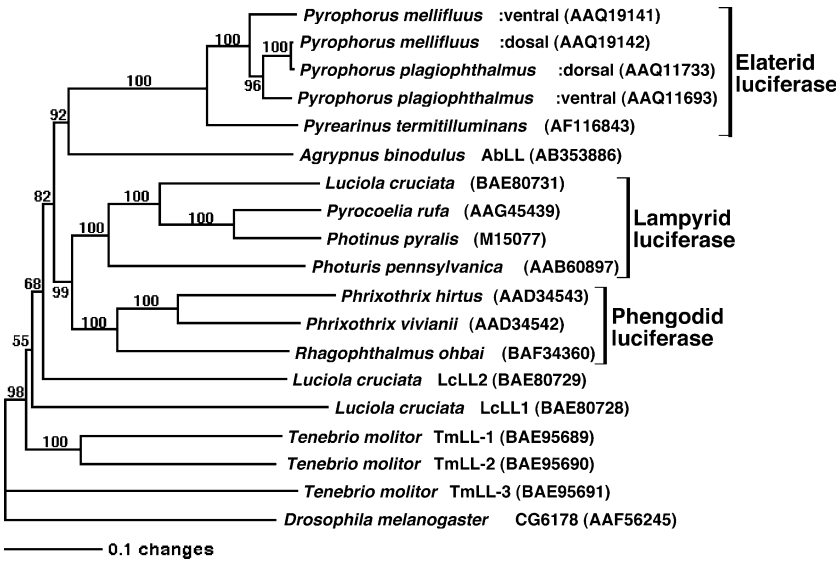

Fig. 14 A phylogenetic reconstruction of luciferases and their similar genes in insects. *Numbers* on the nodes indicate bootstrap values % from 10,000 replicates, and only the values over 50% are shown. Horizontal branch lengths indicate the genetic distances. GenBank accession number (Protein/Nucleotide) is shown in *parentheses*

(*Agrypnus binodulus* luciferase-like gene) [136]. The identity of the amino acid sequence deduced from *AbLL* with the click beetle luciferase from *P. plagiophthalmus* was 55%, which is higher than that between click beetle luciferase and firefly luciferase (~48%). The gene product of *AbLL* (AbLL) had medium- and long-chain fatty acyl-CoA synthetase activity, but not luciferase activity [136]. Thus, all homologous genes of firefly luciferase from luminous and non-luminous insects did not show the significant activity of luminescence [131–136]. As shown in Fig. 14, phylogenic analysis was performed using homologous genes from luminous and non-luminous insects. Taken together with the fatty acyl-CoA synthetic activity in these homologues, firefly luciferase (beetle luciferase) originated from a fatty acyl-CoA synthetase by gene duplication in insects [136]. The gene duplications might occur before luminous insects within Lampyridae, Phengodidae and Elateridae diverged [132–137].

Functional conversion of fatty acyl-CoA synthetase to firefly luciferase

To demonstrate the evolutionary origin of luciferase in insects, we tried functional conversion to firefly luciferase

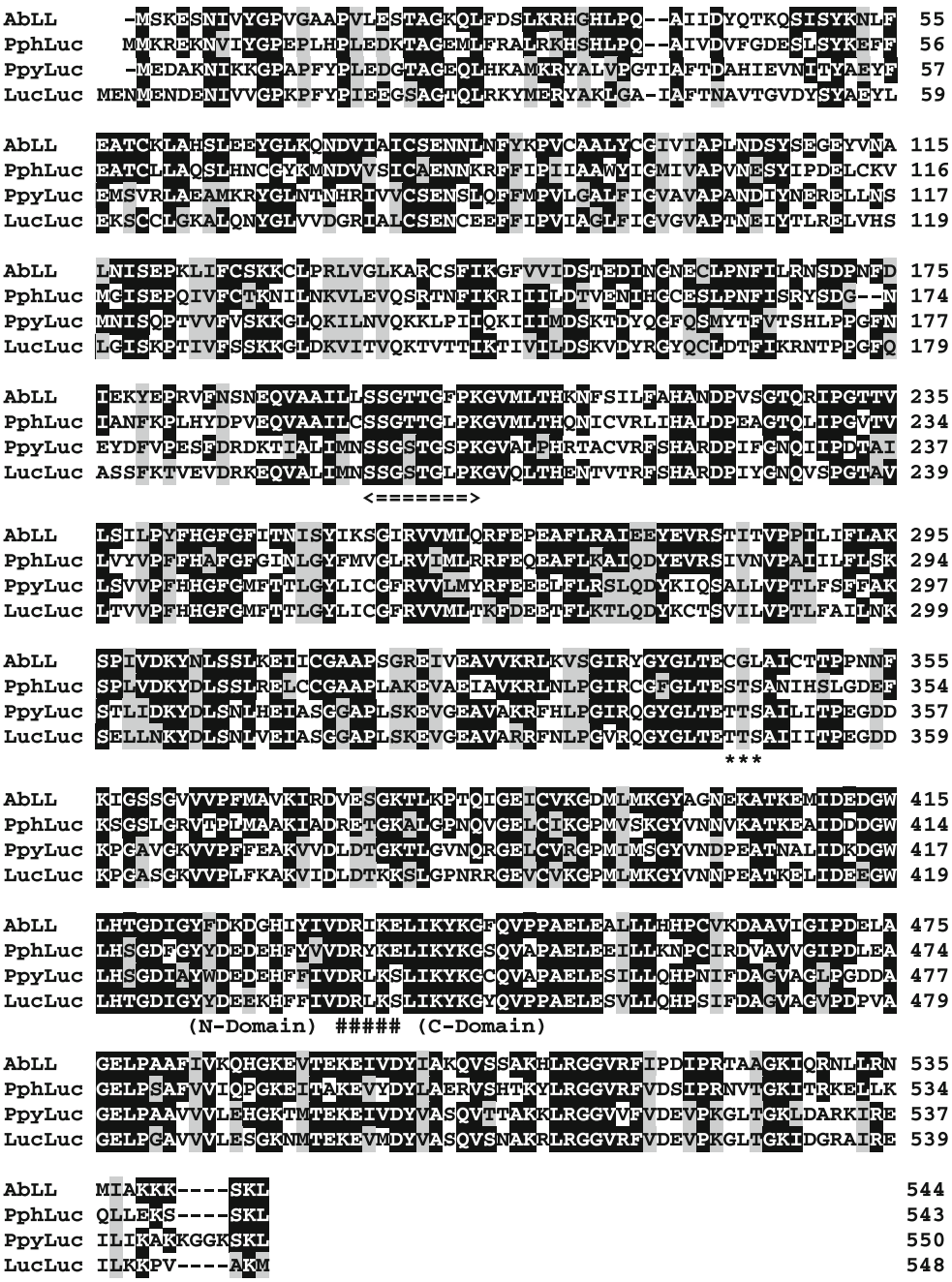

Fig. 15 Comparison of the amino acid sequences of AbLL (AbLL, AB353886), *P. plagiophthalmus* luciferase (PplLuc; AF543373), *P. pyralis* luciferase (PpyLuc; M15077) and *L. cruciata* luciferase (LcuLuc; M26194). *Letters* in the *black boxes* and *shaded boxes* indicate the identical amino acids and the similar amino acid groups with AbLL, respectively. The groups are defined as follows: A, S, T, P, and G; N, D, E, and Q; H, R, and K; M, L, I, and V; F, Y, and W. *Gaps* were inserted to assist in sequence alignment. The *numbers* on the *right margin* indicate the position of amino acid residues. *Double arrow* indicates the AMP-binding motif. Three *asterisks* indicate the positions of mutation in AbLL. The linker region between the N-terminal domain and C-terminal domain is shown by the *sharp* symbols

Table 2 Comparison of amino acid sequence of mutated AbLL and their activity [138]

Enzymes	Amino acid sequences[a]	Relative activity (%)
A. binodulus AbLL	(337)-GYGLTECGLA-(346)	0.2
AbLL-Mt14	(337)-GYGLTECG**S**A-(346)	5.9
AbLL-Mt17	(337)-GYGLTEC**TS**A-(346)	27.0
AbLL-Mt11	(337)-GYGLTE**STS**A-(346)	100.0
AbLL-Mt13	(337)-GYGLTEC**T**LA-(346)	0.2
AbLL-Mt12	(337)-GYGLTE**S**GLA-(346)	0.2
AbLL-Mt16	(337)-GYGLTE**SGS**A-(346)	3.9
AbLL-Mt15	(337)-GYGLTE**ST**LA-(346)	0.2
-Enzyme		0.1
L. cruciata luciferase	(341)-GYGLTETTSA-(350)	–
P. pyralis luciferase	(339)-GYGLTETTSA-(348)	–
P. mellifluus luciferase	(336)-GYGLTESTSA-(345)	–
P. termitilluminans luciferase	(336)-GYGLTESTSA-(345)	–

[a] The mutated amino acid residues in AbLL are shown in bold

from fatty acyl-CoA synthetase. For this purpose, we focused on AbLL, which possesses 55% identity in amino acid sequence with the luminous click beetle luciferase and is the most homologous gene to firefly luciferase among non-luminous insects [136] (Fig. 15). The functional conversion of AbLL to a luciferase-like AbLL possessing luminescence activity was performed by site-directed mutagenesis [138]. The crystal structure analysis of *L. cruciata* luciferase-bound with an adenylate analogue of DLSA suggested that the benzothiazole ring of DLSA is in van der Waals contact with several amino acid residues and water molecules [130] (Fig. 12b). The hydroxyl group of Ser349 forms a hydrogen bond with the benzothiazole ring through a water molecule. By comparison of the amino acid residues in several luciferases with AbLL, two AbLL mutants of AbLL-L216R (Mt2) and AbLL-L345S (Mt14) were constructed (Table 2). The mutant AbLL-Mt2 did not show luminescence enhancement but AbLL-Mt14 did. This result indicated that a single substitution to Ser345 is crucial for light emission in AbLL. The amino acid sequence from 337 to 346 is Gly-Tyr-Gly-Leu-Thr-Glu-Cys-Gly-Leu-Ala in AbLL, and the three amino acids italicized are different from the corresponding sequence in luciferases: Gly-Tyr-Gly-Leu-Thr-Glu-Ser(Thr)-Thr-Ser-Ala. The mutant AbLL-Mt11 was introduced by additional mutations of Ser343 and Thr344 in AbLL-Mt14 and showed a significant increase of luminescence activity (Table 2). The luminescence enhancement of AbLL-11 might be explained by the formation of a hydrogen-bonding network, similar to the case of Met398, Thr347, Thr348, and Ser349 in *L. cruciata* luciferase. The interaction of the hydroxyl group of Ser345 in AbLL mutants with the benzothiazole moiety of luciferin through a water molecule is key for functional conversion from a fatty acyl-CoA to a luciferase. However, the luminescence intensity of AbLL-Mt11 was only 0.001% of that of *L. cruciata* luciferase. It is not clear whether this mutation affects the adenylation step or oxygenation step in the luciferase reaction. To increase the conversion efficiency of the luminescence activity, X-ray structural analysis of AbLL might suggest the amino residues for mutations. The luminescence pattern of AbLL-Mt11 was different from firefly luciferases, and showed a steady-state luminescence reaction like a general enzyme, in the presence and absence of coenzyme A. The maxima of emission spectra of AbLL mutants, Mt14, Mt17, Mt11, and Mt16, were around 610 nm at pH 7.8, different from *P. pyralis* and *L. cruciata* luciferases. Further, the CG6178 mutant at the same positions enhanced the luminescence intensity, similar to the case of AbLL [138].

Concluding remarks

Many valuable books and reviews on bioluminescence have been published in the past [1–9], and firefly luciferase is one of the well-characterized enzymes in bioluminescence. However, there are still many unsolved problems, including the light-emitting species of oxyluciferin, the catalytic process of luminescence in a luciferase molecule, and the biosynthetic pathway of luciferin in the firefly.

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