Firefly Bioluminescence

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Introduction

Bioluminescence is an enchanting process in which living organisms convert chemical energy into light. With the interesting exception of the photoproteins（see "Spectroscopy and Structure"）, in most bioluminescence systems light results from the oxidation of an organic substrate, a luciferin, catalyzed by an enzyme called a luciferase. In nature, there is an amazing diversity of organisms that emit light including bacteria, fungi, crustaceans, mollusks, fishes and insects. While the specific biochemistries of bioluminescence are diverse, all include an enzyme-mediated reaction between molecular oxygen and an organic substrate. It is likely too that all bioluminescence processes involve the formation and breakdown of a four-member ring peroxide or a linear hydroperoxide. An overview of the chemical and mechanistic aspects of a major bioluminescence process, that of the bioluminescent beetles, will be presented here.
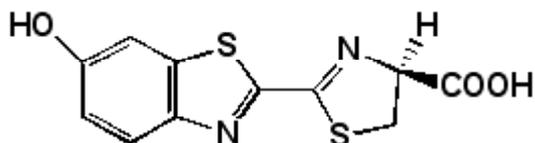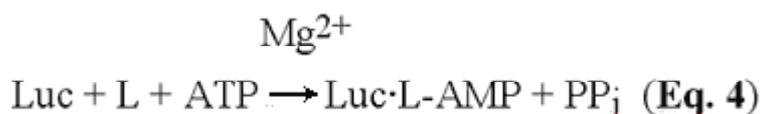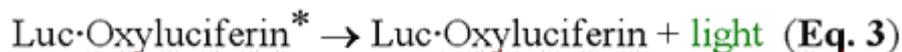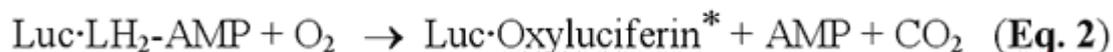

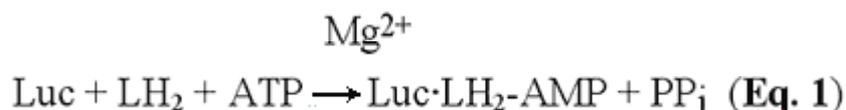
Figure 1. The North American firefly Photinus pyralis.

Representing an estimated 3,000 species of luminous beetles（Coeleoptera）, are three families: the true fireflies, click beetles, and glow-worms. Beginning approximately 50 years ago with the pioneering work of Johns Hopkins University scientists William McElroy, Emil White and Howard Seliger, basic research, mainly focused on the common North American firefly Photinus pyralis（Figure 1）, has progressed toward a very good understanding of how light is produced by fireflies. It is anticipated that the availability of two Photinus pyralis luciferase crystal structures will advance the present understanding of the key structure-function relationships that account for the efficient enzyme-catalyzed emission of light in the firefly. In turn, the prospects are bright for the continued application of firefly bioluminescence to the already impressive list of medical and pharmaceutical methods, including in vivo luminescence monitoring and monitoring gene expression and regulation
Biochemical Reactions of Bioluminescence

Firefly bioluminescence is a multi-step process that is outlined in Equations 1-3（shown in Figure 3）. Luc represents firefly luciferase, ATP is the universal biochemical energy source adenosine triphosphate, PPi is inorganic pyrophosphate, and the structures that correspond to the other abbreviations are shown below. In the first step（Eq. 1）, luciferase converts firefly D-luciferin into the corresponding enzyme-bound luciferyl adenylate. Firefly luciferase has extraordinary specificity for this nucleotide triphosphate. The adenylate is the true substrate of the subsequent oxidative chemistry. In fact, D-LH2-AMP produced synthetically reacts with oxygen in the presence of luciferase to produce light emission identical to that obtained with the natural substrates D-luciferin and Mg-ATP.

As Equations 2 and 3 indicate, the luciferase enzyme functions as a mono-oxygenase, although it does so in a very unusual manner without the apparent involvement of a metal or cofactor. In some way that has not been yet determined, luciferase amino acid residues are recruited to promote the addition of molecular oxygen to luciferin, which is then transformed to an electronic excited state oxyluciferin molecule and carbon dioxide, each containing one oxygen atom from molecular oxygen. Visible light emission results from the rapid loss of energy of the excited state oxyluciferin molecule via a fluorescence pathway. The very high quantum yield for this process（in alkaline solution, nearly each reacted LH2 molecule emits a photon）reflects not only efficient catalytic machinery, but also a highly favorable environment for the radiative decay of an electronic excited state.

Biochemical Reactions of Bioluminescence

$$\text{Luc} + \text{LH}_2 + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{Luc·LH}_2\text{-AMP} + \text{PP}_i \quad \textbf{(Eq. 1)}$$

$$\text{Luc·LH}_2\text{-AMP} + \text{O}_2 \rightarrow \text{Luc·Oxyluciferin}^* + \text{AMP} + \text{CO}_2 \quad \textbf{(Eq. 2)}$$

$$\text{Luc·Oxyluciferin}^* \rightarrow \text{Luc·Oxyluciferin} + \text{light} \quad \textbf{(Eq. 3)}$$

$$\text{Luc} + \text{L} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{Luc·L-AMP} + \text{PP}_i \quad \textbf{(Eq. 4)}$$

D-Firefly Luciferin (LH$_2$)

Beetle Luciferin

D-Luciferyl-O-AMP(LH$_2$-AMP)

Oxyluciferin

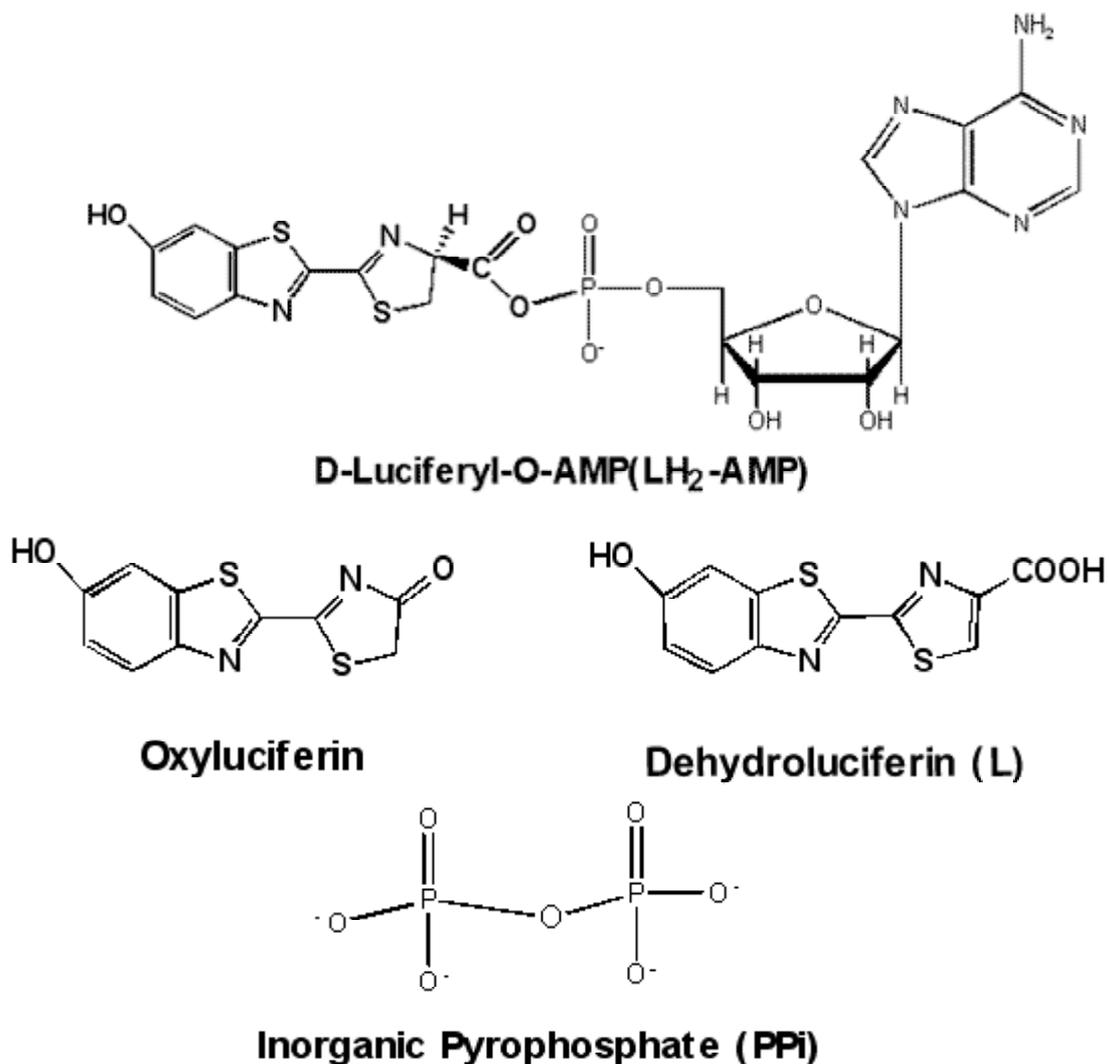**Dehydroluciferin (L)**

Inorganic Pyrophosphate (PPi)

Figure 3. Equations and structures illustrating the reactions catalyzed by firefly luciferase（Luc）. The symbol * denotes an electronic excited state.

In addition to the reactions leading to light emission, firefly luciferase also catalyzes the in vitro formation of the adenylate of dehydroluciferin（L-AMP）（Eq. 4）, which cannot react further and potently inhibits enzyme activity. Thus, luciferase exhibits two distinct enzymatic functions: as a synthetase in the formation of an acyl adenylate, and as a mono-oxygenase.

Also, under certain conditions LH2-AMP, formed according to Eq. 1, may be oxidized by luciferase to produce low levels of L-AMP. Subsequently, luciferase catalyzes the transfer of the AMP moiety from L-AMP to ATP producing diadenosine tetraphosphate. These chemical transformations indicate that firefly luciferase can also function as a ligase. Furthermore, Coenzyme A can inhibit this ligase function and modulate the usual light emission kinetics, although the cofactor is not a required substrate. Coenzyme A is known to stimulate light production possibly by promoting the release of product oxyluciferin from luciferase allowing the enzyme to react again.

Generally Accepted Mechanism of Bioluminescence

The generally accepted mechanistic details of the overall process of firefly bioluminescence are presented in greater detail below (Figure 4).
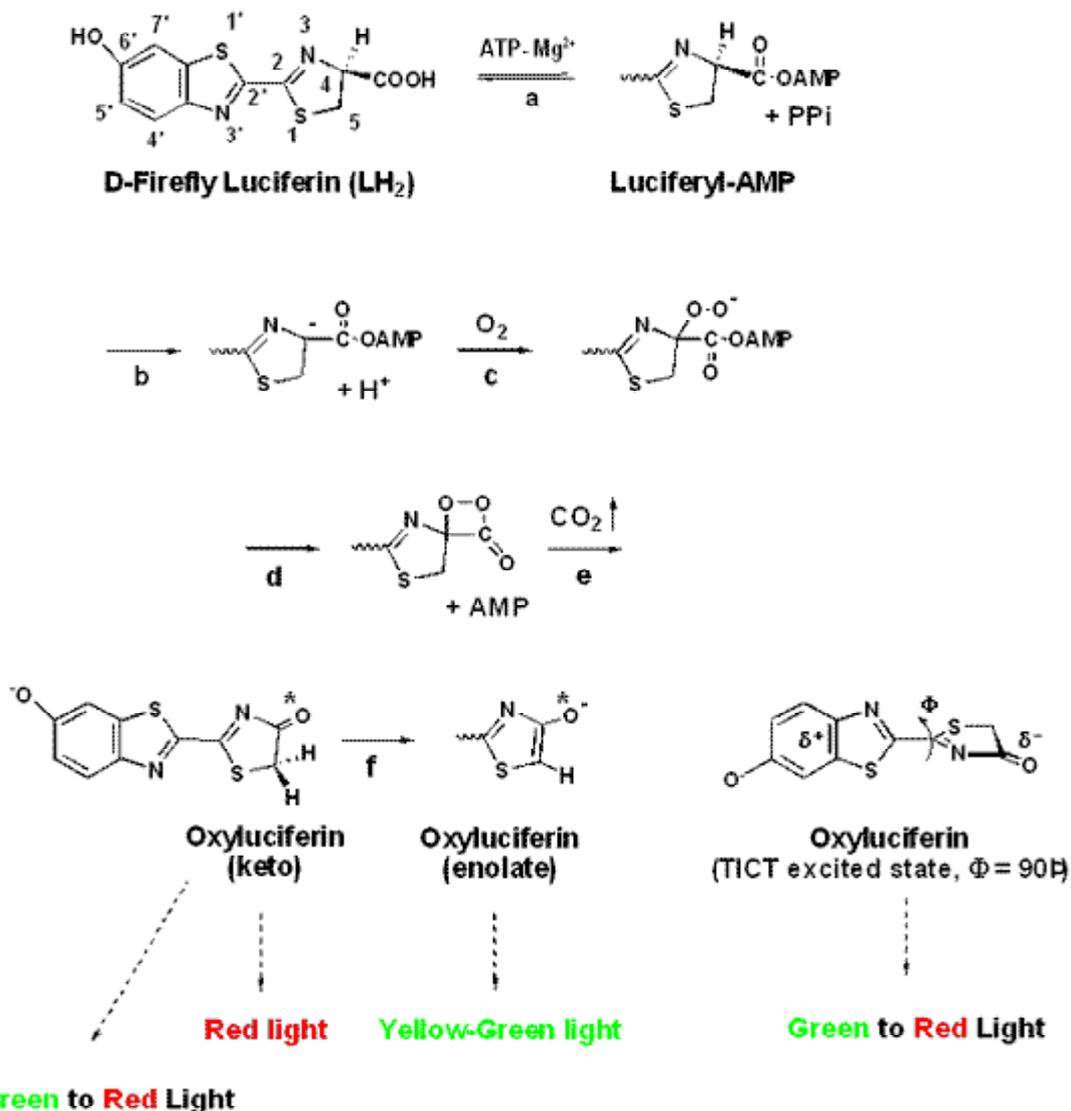

Figure 4. Detailed mechanism of firefly bioluminescence including various proposals to account for the variation in color. The symbol * denotes an electronic excited state.

Following the formation of the enzyme-bound luciferyl adenylate (step a), a proton is abstracted from the C-4 carbon of the adenylate by a basic side chain amino acid residue of luciferase (step b). Next, molecular oxygen adds to the newly formed anion (step c); and an electronically excited state oxyluciferin molecule and carbon dioxide are produced (step e) from a highly reactive dioxetanone intermediate (step d). According to the original mechanism based predominantly on model studies of the P. pyralis enzyme, red light emission (max 615 nm), which is observed at pH 6.0, results from the keto form of the emitter. At pH 8, the familiar yellow-green light emission (max 560 nm) is produced from the enolate dianion form of the excited state oxyluciferin by a presumed enzymatic assisted tautomerization (step f). In nature,

beetle luciferases display various colors of light from green (max ~540 nm) to red (max ~635 nm). Recent experimental data obtained with a firefly luciferin analog is consistent with the keto form of oxyluciferin alone being capable of producing all of the colors of firefly bioluminescence. Possibly, luciferase modulates emission color by altering the resonance-based charge delocalization of the excited state as shown in Eq. 5.
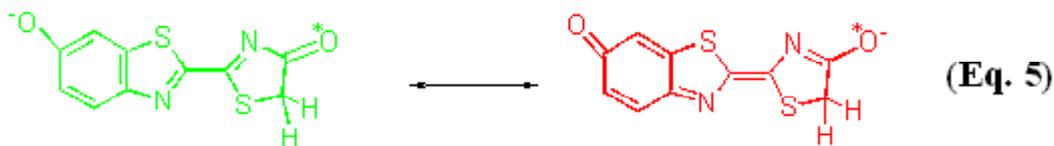
Alternatively, using molecular orbital calculations as the basis, McCapra has proposed that all of the luminescent colors ranging from green to red are generated from twisted intramolecular charge transfer excited states (TICT) of the keto form of oxyluciferin (Figure 4). Possibly, changes in luciferase tertiary structure might modulate color by affecting the stabilization of oxyluciferin conformers formed by rotation about the C2-C2' bond.

Electronic Excited State Formation

A relatively large amount of excitation energy is required to produce visible light, on the order of 40-70 kcal. The key dioxetanone intermediate shown in the reaction sequence above and in Figure 5, below, contains both a strained four member ring and a weak peroxide bond (O-O). The cleavage of the high energy dioxetanone ring is capable of releasing sufficient energy as a result of the low energy of activation required to cleave the peroxide bond and the relief of the ring strain inherent in the structure.
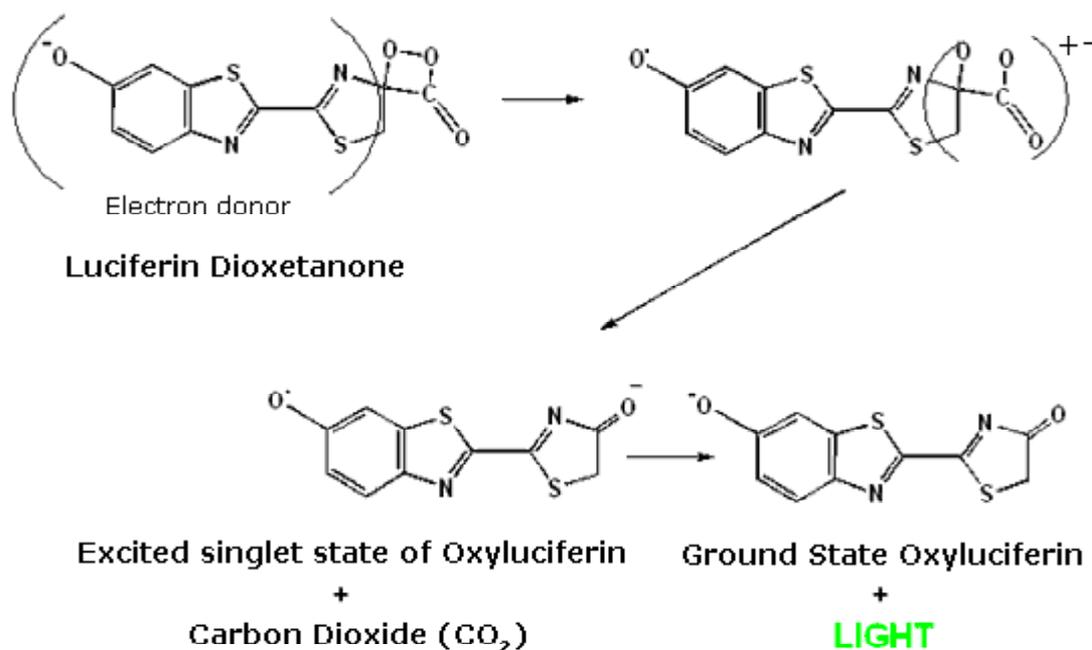

Figure 5. Chemically initiated electron exchange (CIEEL) mechanism for the formation of excited state oxyluciferin in firefly bioluminescence.

In the firefly, the energy released is very efficiently directed into the production of an electronically excited state of the bioluminescence product oxyluciferin. Subsequent rapid relaxation of the excited state to the ground state is then accompanied by the emission of a photon of light. One detailed mechanistic view of this process is termed the CIEEL（Chemically Initiated Electron Exchange Luminescence）mechanism （Figure 5）. In firefly bioluminescence, intramolecular electron transfer from the heterocylic portion of the molecule to the dioxetanone produces a radical ion pair and the radical anion of carbon dioxide. Next, the back transfer of an electron from the radical anion of carbon dioxide to the radical form of oxyluciferin results in the formation of electronically excited oxyluciferin and carbon dioxide. The CIEEL mechanism may occur in other bioluminescent systems as well as the firefly.

Firefly Luciferase Structure and Mechanistic Functions

The cloning and sequencing of P. pyralis luciferase and similar enzymes from approximately fifteen other beetle species has revealed that these luciferases are closely related to a large family of non-bioluminescent proteins that catalyze reactions of ATP with carboxylate substrates to form acyl-adenylates. The formation of enzyme-bound LH2-AMP and L-AMP （Eqs. 1 and 4） illustrates the chemistry common to this large group of enzymes. This group of proteins shares an identifying motif（198SerSerGlySerThrGlyLeuProLysGly207 in luciferase） and has been termed the "acyl-adenylate/thioester-forming" enzyme family. The superfamily of enzymes includes: a variety of acyl:CoA ligases; the acyl-adenylate-forming domains of enzyme complexes involved in the non-ribosomal synthesis of antibiotics, peptides and polyketides; the luciferases; and several other types of enzymes. Most of these enzymes generate thioester （e.g., of Coenzyme A） intermediates or products from the initially formed corresponding acyl-adenylates, and these reactions are similar to one suggested to account for the stimulatory effect of Coenzyme A on luciferase activity.

The P. pyralis luciferase crystal structure （Figure 6）, the first structure of a member of the " acyl-adenylate/thioester-forming" enzyme family, revealed a unique molecular architecture consisting of a large N-terminal domain （residues 1-436） and a small C-terminal domain （residues 440-550）. The structure was solved without substrates or products present so it was not possible to determine which amino acid residues participated in the bioluminescence process. However, based on an analysis of the positions of several strictly conserved residues among a group of enzymes sharing the adenylation function, a general location of the luciferase active site was proposed.
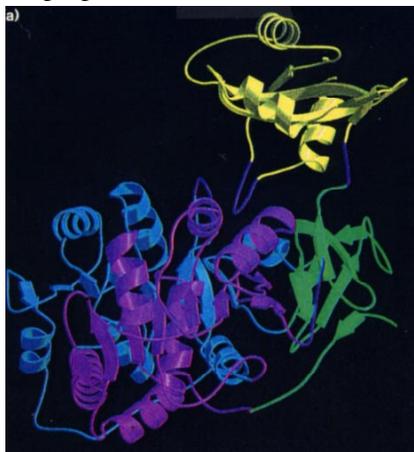

Figure 6. Ribbon diagram of the firefly luciferase （Luc） structure. The large N-terminal domain （amino acids 1-436） is connected to the smaller C-terminal domain （amino acids 440-550 shown in yellow） through a short hinge peptide （from Conti, Franks and Brick.（1996） Structure 4, 287-298

Next, the crystal structure of a second member of the adenylate-forming family, the phenylalanine-activating subunit of gramicidin synthetase 1 （PheA）in a complex with phenylalanine, Mg ion and AMP, was reported. The active site of PheA was determined to be at the interface of the two domains, which were remarkably similar in size and shape to the corresponding domains of luciferase. In the PheA structure, however, the C-terminal domain was rotated 94° and was 5Å closer to the N-terminal domain than in the luciferase structure. Starting with the two available crystal structures, molecular modeling techniques were used to produce a potential working model of the luciferase active site containing substrates luciferin and Mg-ATP. The model produced in our laboratory is shown in Figure 7.

This model has been quite useful in the rational design of site-directed mutagenesis-based luciferase structure-function studies, including several related to the determination of bioluminescence color and the characterization of the luciferin binding site.
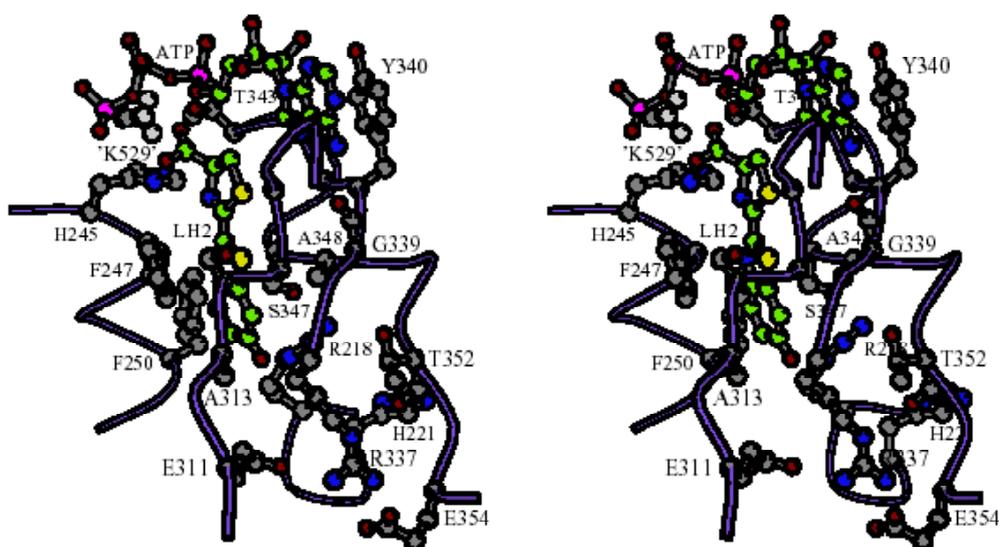

Figure 7. Stereo diagram showing the substrate binding sites suggested by molecular modeling of luciferase with LH2 and ATP（carbon atoms are green in both）and Mg2+ ion（not shown）. The model was created starting with the luciferase x-ray structure 1LCI, and methylammonium ion （labeled 'K529'）was used to represent possible interactions of the Lys529 side chain. Traces through the -carbons of regions Val217-His221, His244-Thr252, His310-Leu319, and Arg337-Gly355 are shown as purple coils. The -carbons of Gly246, Ser314（and side chain group）, Gly315, Gly316 and Gly341 are shown（gray）but are not labeled. The main chain carbonyl groups（oxygen atoms are red）of Gly339 and Thr352 also are shown. This diagram was generated using the program MOLSCRIPT.

Systematic mutation of fifteen luciferase amino acid residues and biochemical studies on the resulting mutant proteins has produced data substantiating the view of the firefly luciferin binding site shown in the stereo diagram below in Figure 8.
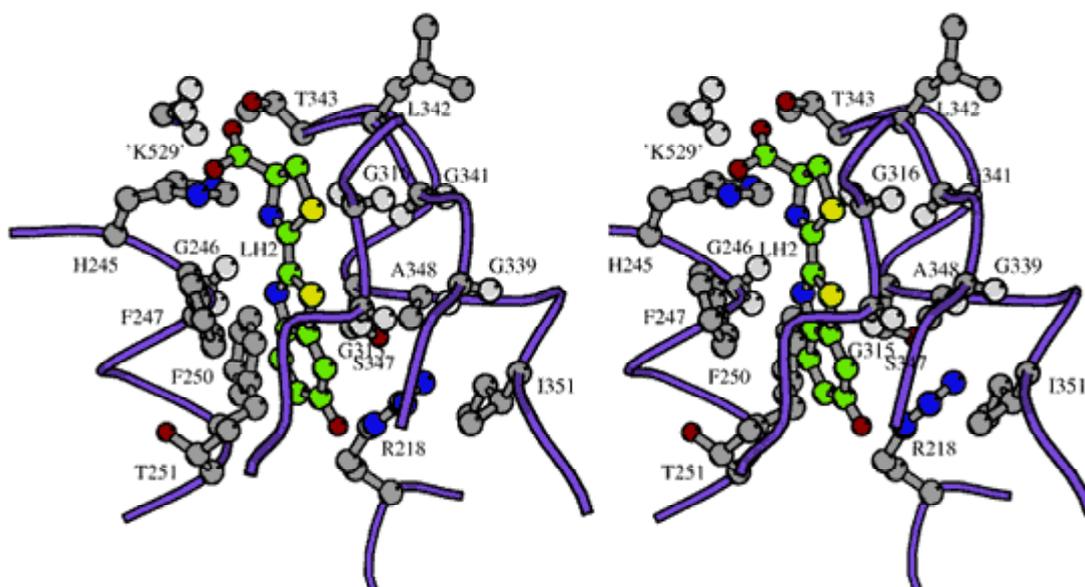
Figure 8. Stereo diagram showing the residues within 5 Å of LH2 at the putative luciferase substrate binding site suggested by molecular modeling of Luc with LH2 and ATP（not shown）and Mg2+ ion（not shown）. The model was created starting with the Luc X-ray structure 1LCI, and methylammonium ion（labeled 'K529'）was used to represent possible interactions of the Lys529 side-chain. Traces through the -carbons of regions V217-F219, H244-T252, I312-P318, and R337-P353 are shown as purple coils. This diagram was generated using the program MOLSCRIPT.

Based on mutational studies of the luciferases and enzymes in the related acyl adenylate-forming superfamily, a possible mechanism for the luciferase–catalyzed formation of adenylates（Eqs. 1 and 4）is presented in Figure 9.
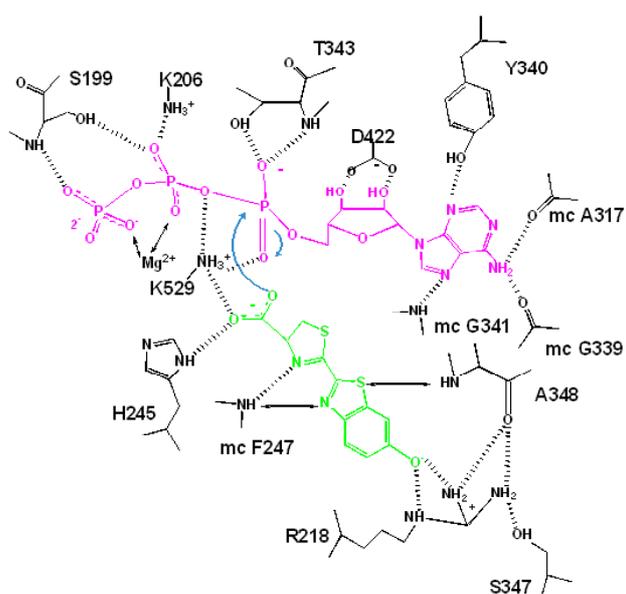

Figure 9. Schematic representation of hydrogen bonding（ ）between Luc and substrates luciferin (green), ATP (violet) and Mg2+ predicted by molecular modeling. Potential interactions between substrates and atoms in close proximity（~2 to ~4 Å）are indicated（ ）. For main chain (mc) atoms, only those interacting with substrates are included. The methylammonium ion was used to represent possible interactions of the Lys529 side chain. The curved blue arrows represent the nucleophilic attack of the luciferin carboxylate at the -phosphorus of ATP and the corresponding formation of the pentavalent intermediate.

Luciferase residues Arg218, Phe247, Ser347 and Ala348 are shown making H-bond interactions with luciferin, fixing its position in the active site. The adenine ring of ATP is held in place by interactions to Gly339, Tyr340, Gly341 and Ala317, while the side chain carboxylate of Asp422 is H-bonded to the ribose hydroxyl groups. Residues Ser199 and Lys206, highly conserved throughout the acyl adenylate forming superfamily are shown chelating the ß- and -phosphate portion of ATP. The key residue in the catalytic process mainly responsible for lowering the energy of the transition state is Lys529, which is probably assisted by Thr343. The side chain ammonium group of Lys529 first is shown orienting luciferin and ATP for a productive bimolecular reaction. The curved arrows show a carboxylate ion oxygen atom of luciferin carrying out a nucleophilic attack at the phosphorus atom of the -phosphate of ATP. A pentavalent transition state is formed that is likely stabilized by electrostatic interactions with the ammonium ion of Lys529 and H-bonding interactions with the side chain hydroxyl group of Thr343. Protein stabilization of the transition state accounts for the catalysis of the adenylation reaction.

In addition, signature sequence residues Ser199 and Lys206 probably remove the PPi leaving group as the product adenylate is formed（Figure 10.）
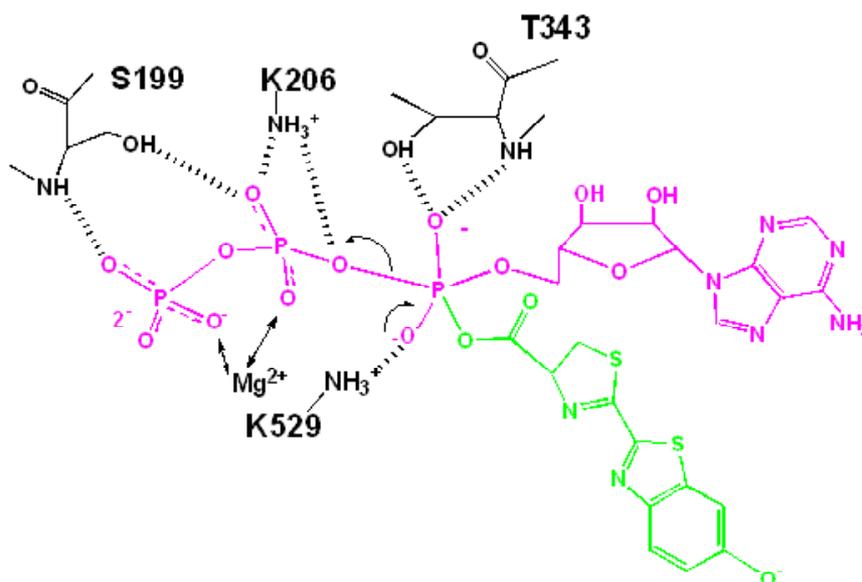

Figure 10. Schematic representation of the possible role of signature sequence residues Ser199 and Lys206 in the removal of inorganic pyrophosphate（PPi）during the formation of lucferyl-adenylate. The curved arrows represent the flow of electrons leading to reformation of the phosphorus-oxygen double bond and release of PPi.

Unfortunately, the mechanistic details relating the luciferase structure to its function as an oxidase（Eqs. 2 and 3）are still not well understood. It does appear, however, that a structural motif identified in the acyl-adenylate forming superfamily,（340YGLTE344 in luciferase）, plays a significant role in the catalysis of these reactions leading to the emission of light in the firefly. Research continues aimed at developing a detailed explanation of just how the firefly oxidizes its substrate to process the familiar lights many associate with beautiful warm summer evenings.

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