CHEMPHYSCHEM

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The Chemistry of Bioluminescence: An Analysis of Chemical Functionalities

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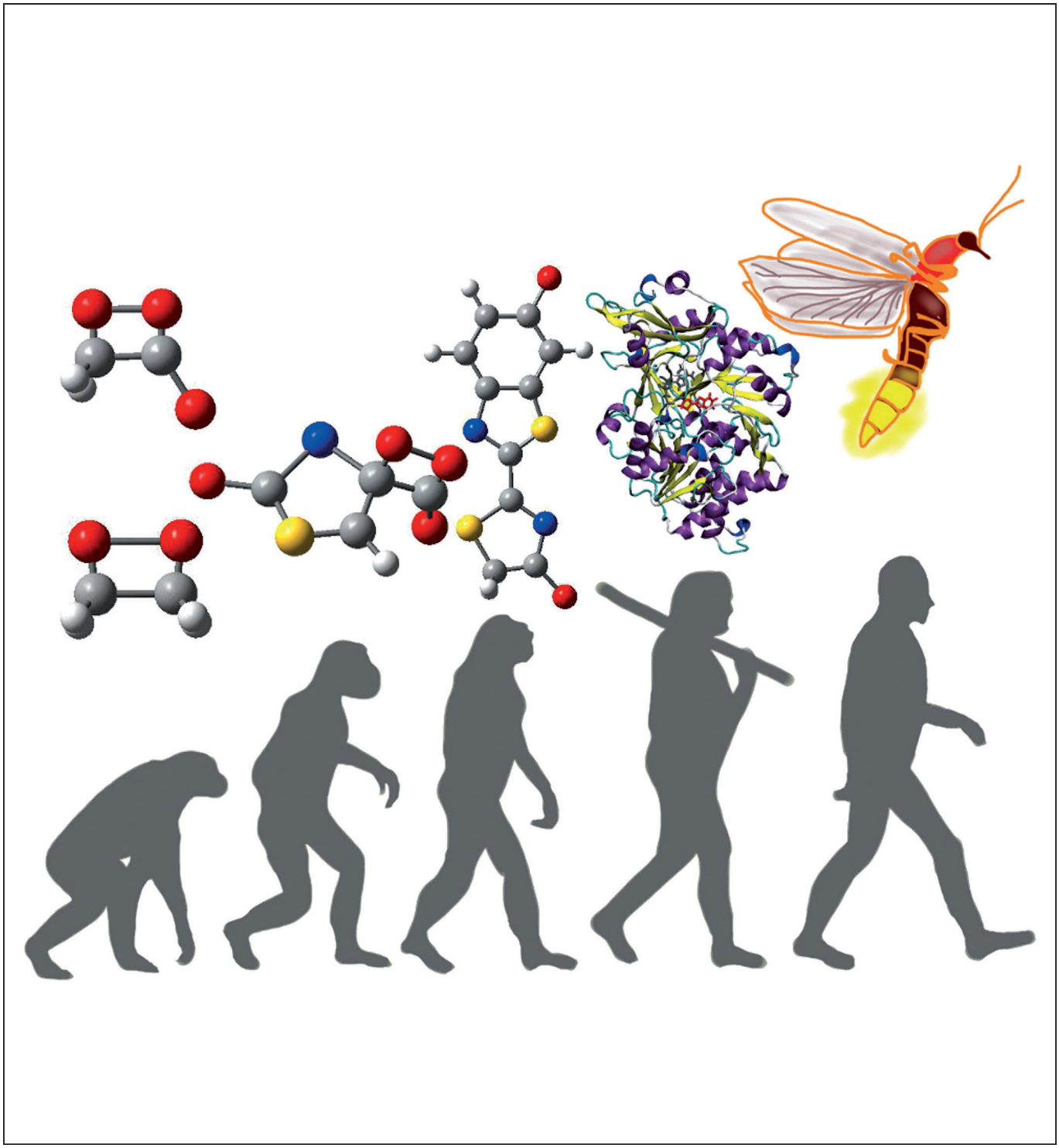

Firefly luciferase is one of the most studied bioluminescent systems, both theoretically and experimentally. Herein we review the current understanding of the bioluminescent process from a chemical functionality perspective based on those investigations. Three key components are emphasized: the chemiluminophore, the electron-donating fragment, and how these are affected by the substrate–enzyme interaction. The understanding is based on details of how the peroxide –O–O– bond supports the production of electronically excited products and how the charge-transfer (CT) mechanism, with the aid of an electron-donating group, lowers the activation barrier to support a reaction occurs in living organisms. For the substrate–enzyme complex it is demonstrated that the enzyme can affect the hydrogen-bonding around the CT-controlling group, resulting in a mechanism for color modulation. Finally, we analyse other luciferin–luciferase systems and compare them to the key chemical functionalities of the fragments of the luciferin–luciferase complex with respect to similarities and differences.

1. Introduction

Bioluminescence is the emission of light in living organisms resulting from a chemical reaction (chemiluminescence). The best known examples are the firefly beetles, which produce flashes of visible light in their abdomen for communication. The study of bioluminescence in practice and theory has attracted more attention in the last decades. The Nobel Prize in Chemistry of 2008 was awarded to Osamu Shimomura, Martin Chalfie and Roger Tsien as a recognition of their research on the green fluorescent protein (GFP). Bioluminescence has already found successful applications in different fields, such as gene expression,[1] biosensors for environmental pollutants,[2] and cancer monitoring.[3] Among the bioluminescence systems, the firefly luciferin–luciferase system is one of the most widely studied. Oxyluciferin is composed of two functional groups of hydroxybenzothiazolyl and oxythiazolyl connected by a carbon–carbon bond (Figure 1). These two planar moieties

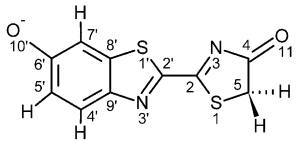

Figure 1. Numbering of oxyluciferin. The hydroxybenzothiazolyl or benzothiazol moiety (primed numbers) and oxythiazolyl or thiazolone moiety (plain numbers). Moieties are connected through a $C_{2'}$–C_2 bond.

form a conjugated system. Luciferase catalyzes the reaction between luciferin and adenosine triphosphate (ATP) in the presence of an oxygen molecule and Mg^{2+}, leading to the first singlet excited state of oxyluciferin (Figure 2). The relaxation of the excited oxyluciferin molecule to its ground state (GS) produces visible light with great efficiency. The firefly luciferin–luciferase system is also found in organisms other than fireflies, for example, in click beetles and railroad worms.[4,5] The color of the emitted light varies from green to red within the insect species whose luciferase structures are slightly different.[5] The understanding of the color modulation has been the subject of most recent studies on the luciferin–luciferase system.[6–12] Indeed, the color variation of such systems can be used in biotechnology and bioscientific research, such as bioluminescence imaging.

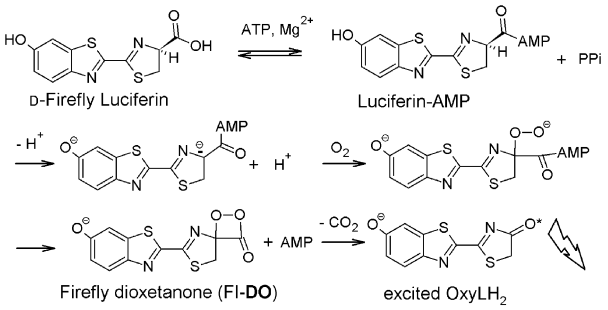

Figure 2. Mechanism of firefly bioluminescence.

This contribution does not include an exhaustive list of all the experimental and theoretical pieces of research on the oxyluciferin–luciferase system as recent reviews already did that.[13,14] We neither repeat all the different hypotheses proposed to explain the color modulation (see citations in refs. [14,15]). Our purpose is rather to review the theoretical studies up to date and to highlight the functional moieties of the molecules that contribute to the bioluminescent phenolmenon.

This review is organized as follows: 1) In Section 2 we present the chemical understanding of the chemiluminescent mechanism as understood from smaller molecules, such as 1,2-dioxetane and 1,2-dioxetanone (dioxetanone hereafter), all the way up to oxyluciferin. This section especially summarizes our understanding of the chemiluminophore and the principles of the charge transfer (CT) mechanism from a molecular-orbital

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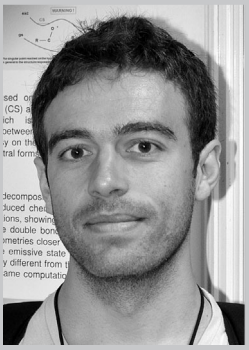
Isabelle Navizet received her Ph.D. in Physical Chemistry in 2004 from the University of Paris VI, France, for her research on molecular modeling (MM) and analysis of mechanical properties of proteins. She then joined the group of Prof. Pavel Rosmus at the University East-Paris Marne-la-Vallee, where she was appointed assistant professor, and performed quantum mechanics (QM) on small systems. In 2007 she moved to Beijing Normal University, China, where she started with her ongoing work of performing QMMM calculations to clarify the relationship between the structure of bio-luminescent systems and the color of the emitted light. Since September 2010, she has been working at the School of Chemistry of the University of the Witwatersrand, South Africa.

Ya-Jun Liu received his Ph.D. degree in physical chemistry at the University of Science and Technology of China, in 2002. After a postdoctoral period at Uppsala University (Sweden) with Prof. Sten Lunnel and at Lund University (Sweden) with Prof. Björn Roos, he returned to China in 2006 as an associate professor at Beijing Normal University where he has remained ever since. Most of his scientific work has been devoted to the theoretical study of photochemistry. In recent years his research has focused on bioluminescence and chemiluminescence.



Daniel Roca-Sanjuán is currently working as a postdoctoral fellow at the Department of Chemistry Ångström, Uppsala University (Sweden). In 2003 he graduated in chemistry at the University of Valencia (Spain), where he received his Ph.D. degree in 2009 for his studies on quantum chemistry DNA photoreactivity and charge transfer, done in the Quantum Chemistry of the Excited State (QCEXVAL) group co-led by Manuela Merchán and Luis Serrano-Andrés (1966–2011). In 2010 he obtained a Marie Curie grant to do postdoctoral research at the University of Uppsala (Sweden), within the group of Roland Lindh, focusing on the development and application of quantum-chemical methods to the bioluminescence and chemiluminescence phenomena, and their implementation into the MOLCAS program.

point of view. 2) In Section 3 we discuss the significance of protonation/deprotonation and the keto–enol tautomerization of the firefly luciferin. 3) In Section 4 we report on the understanding of the significance of the luciferase enzyme in the light-emitting process and how it can induce color modulation. 4) Section 5 discusses to what extent the current understanding of the chemical functionality of bioluminescence in the firefly luciferin–luciferase system can be applied to similar systems. Up to this point we avoid any discussion with respect to the computational details. However, right before the summary we spend some words on the computational challenge that the study of chemiluminescence entails and what type of methods have to be employed.

2. Chemiluminescence by Chemical Functionalities

The chromophore oxyluciferin is the product of a series of chemical reactions inside luciferase. The last step of these reactions is the decomposition of a dioxetanone-like compound,

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Roland Lindh has been professor in theoretical chemistry and chair of the theoretical chemistry programme at the Chemistry Department–Ångström, Uppsala University (Sweden) since 2010. He obtained his Ph.D. in 1988 with Prof. Björn O. Roos at Lund University, Lund, Sweden, and did his post-doc 1988–91 with Dr. Bowen Liu at the IBM Almaden Research Center, San Jose, California (USA). Between 1991 and 2010 he was active at the Department of Theoretical Chemistry, Lund University as assistant professor before he became a full professor in 2003. His research interest lie in the development of ab initio methods, chemical reactivity, photo chemistry and chemiluminescence. He is the chairman of the MOLCAS quantum chemistry program project.

namely firefly-dioxetanone (**Fl-DO**) (Figure 2). The decomposition partly leads to the first singlet excited state of oxyluciferin ($OxyLH_2$), which decays to the GS while emitting light. The decomposition mechanism of the dioxetanone compounds is the key point to understand how the ground-state reactant leads to the excited-state product. Therefore we have studied the mechanism starting from the small model compounds, 1,2-dioxetane,[16] dioxetanone,[17] and thiazol-substituted dioxetanone[18] (Figure 3). In this series of theoretical studies we

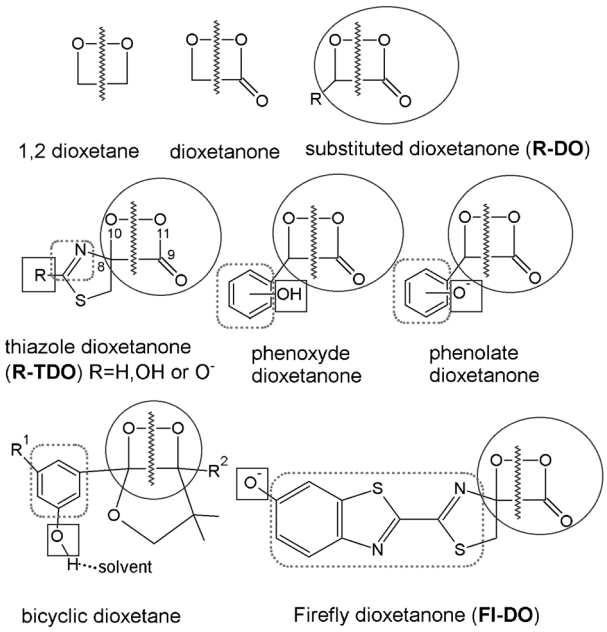

Figure 3. Structures of the model compounds. The bond break is symbolized by a zigzag line. The chemiluminophore is surrounded with a circle, the electron reservoir is emphasized by a dotted rectangle and the CT controlling group by a square.

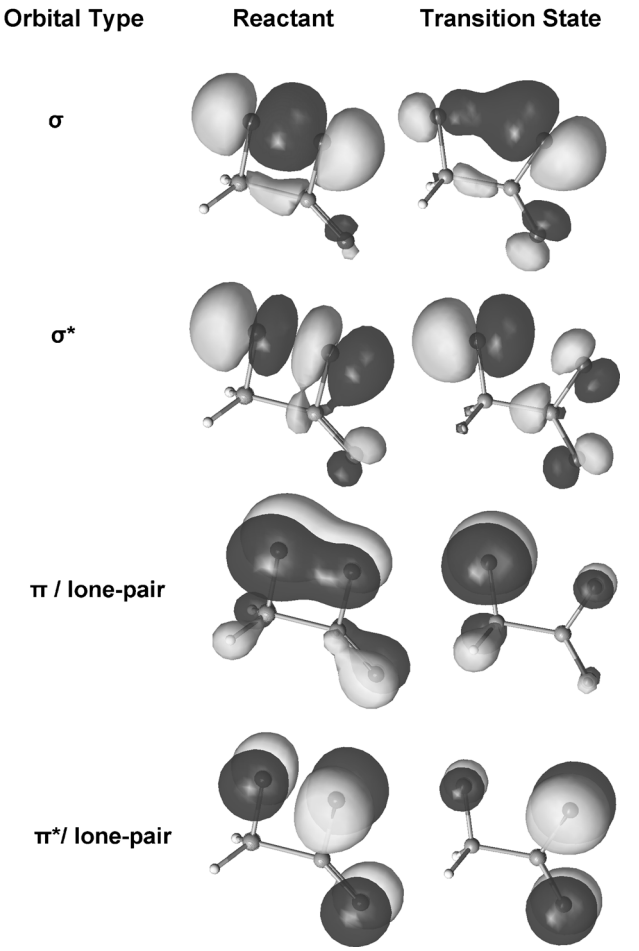

Figure 4. Evolution of the oxygen lone-pairs and the σ/σ* orbitals around the peroxide bond of the dioxetanone molecule. As the peroxide σ bond breaks the peroxide oxygen 2p orbitals, perpendicular to the C–O direction, become (near-)degenerate, and a single or double occupation of the orbitals does not make much difference in terms of energy. Hence, at the TS or close to this point, a conical intersection appears between the S_0 and the S_1 state.

hoped to rationalize the importance of each individual part of the firefly dioxetanone system. As we improve our understanding of the properties of the substrate in the gas phase we will be able to proceed with further studies of the luciferin–luciferase complex to shed some light on the significance of the enzyme.

2.1. The Chemiluminophore

Almost all currently known chemiluminescent substrates have the peroxide bond, –O–O–, in common as a *chemiluminophore*. This chemical system facilitates the essential mechanism of chemiluminescence—providing a route for a thermally activated chemical ground-state reaction to produce a product in an electronically excited state. The basics of this process can be understood from studies of 1,2-dioxetane and dioxetanone. Whereas both systems contain a peroxide bond, the latter also fragments like the firefly luciferin system to carbon dioxide. While the two molecules fragment through slightly different reaction paths they share the same origin of chemiluminescence—the degeneracy of the peroxide oxygen non-bonding 2p orbitals after the rupture of the peroxide bond (Figure 4). In Figure 4 we note that for the transition state (TS) the lone-pairs and the σ orbitals develop into essentially two in-plane and two out-of-plane lone-pair orbitals. Before the rupture there are two electrons localized in the O–O σ bond and two electrons in each of the lone-pair orbitals of the same oxygen atoms. The GS is labelled $^1(\sigma,\sigma^*)$ state as it mainly corresponds to a closed-shell configuration (all orbitals are either doubly occupied or empty) or to a σ-to-σ* excitation during the elongation. As the peroxide bond is broken, the σ and σ* orbitals become degenerate and mix into two atomic in-plane 2p orbitals, with shapes similar to those of the lone-pairs. At the bond-breaking step these two orbitals are singly occupied—forming a biradical singlet.

However, small geometrical perturbations and the anisotropic influence of the environment cause occupations of other orbitals than the original σ and lone-pair orbitals which are transformed into oxygen 2p orbitals at the TS, to be energetically degenerate with the original occupation. Therefore electronic

states other than the $^1(\sigma,\sigma^*)$ state exist around the TS (especially a $^1(n,\sigma^*)$ state, a singlet excited state which results from an n-to-σ^* excitation), which are degenerate or close to degenerate with the GS. This facilitates the molecular system to reach products in an excited state.

The computational results showed that the thermal decomposition of 1,2-dioxetane takes place through stretching of the O–O bond induced by an O–C–C–O torsional motion, leading to a TS where $^3(n,\sigma^*)$ and $^1(n,\sigma^*)$, the first triplet and singlet excited states, respectively, are almost degenerate with the GS $^1(\sigma,\sigma^*)$. At the TS the reaction coordinate changes along the GS surface curves from a mainly torsional motion to a pure separation of the fragments. Following the minimum-energy path along the GS surface, the degeneracy of the GS and the first excited states disappears at the moment when fragments start to separate. However, if the reaction continues along the torsional mode, the degeneracy is preserved. An entropic trap prevents the molecule from quickly decaying to the dissociated GS and allows the formation of a species in an excited state before the dissociation to formaldehyde is completed (Figure 5).[16] The excited formaldehyde will then eventually decay, releasing the excess energy as a photon. The activation energy of the fragmentation reaction is found to be in the range of 22–24 kcal mol^{-1}. For a bioluminescence reaction this is too high in energy.

The decomposition of the dioxetanone proceeds in a two-step-like mechanism. The first step consists in the formation of a biradical intermediate through a TS, which is characterized by O–O stretching while the structure of the whole molecule remains planar. Around the TS the GS reaches a conical intersection (CI) with the lowest singlet excited state and an intersystem crossing (ISC) with the lowest triplet states. The reaction path for $^1(\sigma,\sigma^*)$ bifurcates, into the O–C–C–O torsional mode, or into the stretching mode. Following the stretching mode causes the $^1(n,\sigma^*)$ state to become the most stable. These two states then cross again near the second TS, which corresponds to the breaking of the C–C bond, leading to a fragmented excited formaldehyde and a carbon dioxide in its ground state. Between the two CIs, the region exhibits large multireference-correlation effects because of the near-degeneracy of the states. The resulting excited state decays to the ground state and emits light (Figure 6).[17] Again we find the

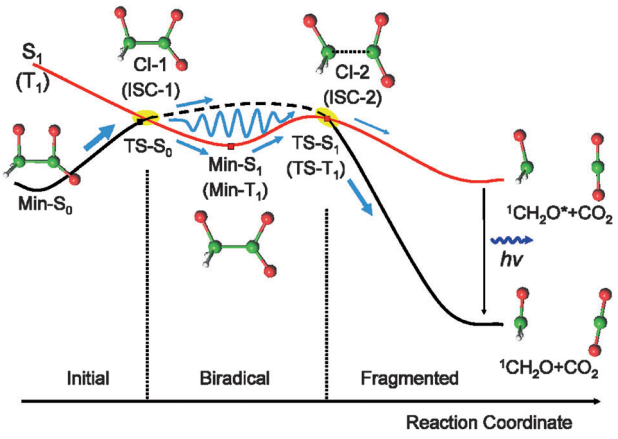

Figure 6. Energy profile along the ground-state minimum energy path (MEP) [predominately $^1(\sigma, \sigma^*)$ in character] of dioxetanone. The dashed line in the biradical region illustrates the energy variation along the MEP while the $^1(n,\sigma^*)$ state is the lowest-energy state. Reprinted from ref. [17] with permission from American Chemical Society (ACS).

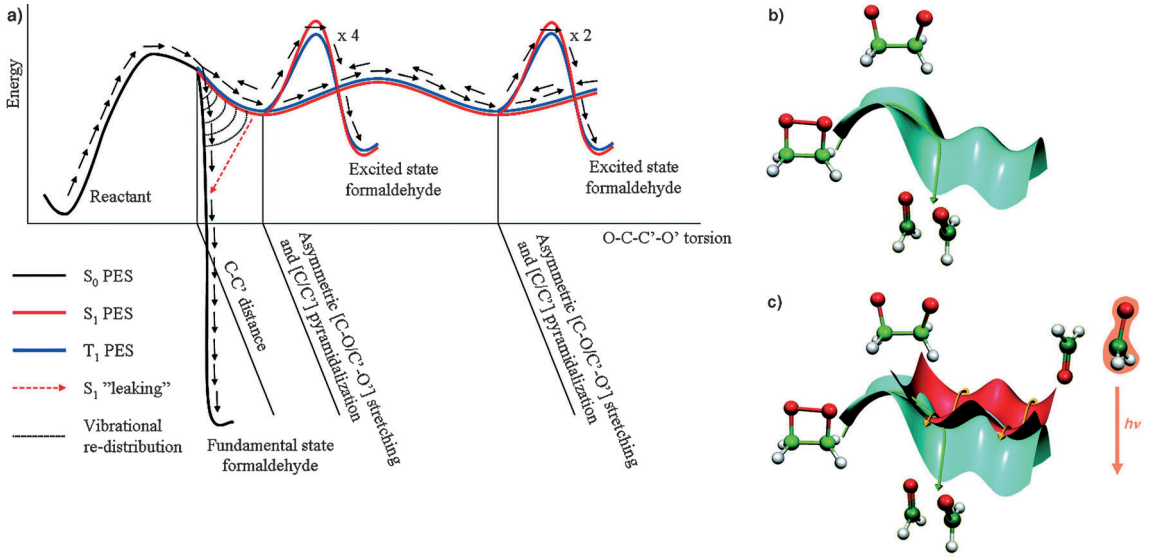

Figure 5. a) Possible routes for 1,2-dioxetane to produce ground- or excited-state formaldehyde molecules. The main reaction coordinate is represented by the torsion around the O–C–C–O dihedral angle. The routes to reach the final products are also shown (from left to right): The C–C bond stretching to reach the ground-state formaldehyde and the asymmetric [CO/CO] bond stretching and [C/C] pyramidalization to produce excited-state formaldehyde. The dashed curves stand for the redistribution of vibrational energy following the first transition-state structure. The dotted arrow represents the production of ground-state formaldehyde from the S_1 potential energy surface (PES) through an intersection with S_0. Because of the tentative 3D representation, energies are not scaled. b) The dissociation process on S_0 and c) on the excited states S_1 and T_1, along with their corresponding dissociation products. Reprinted from ref. [16] with permission from American Chemical Society (ACS).

activation energy of this fragmentation process to be too large to support bioluminescence.

2.2. The Electron-Donating Fragment

The study on the decomposition of the thiazole-substituted dioxetanone (Figure 3), represents a more realistic model of the luciferin reaction scheme's last step. The larger model now also has an adjacent conjugated system in common with the firefly luciferin. Below we describe its importance to the bioluminescent reaction. In particular we identify the importance of aromatic systems as an *electron reservoir* as well as a *CT controlling group*. Together these two characteristics define the *electron-donating fragment*.

Two types of CT mechanisms are possible: stepwise chemically initiated electron-exchange luminescence (CIEEL) and concerted CT-induced luminescence (CTIL). Both are well explained in the theoretical study by Isobe et al. performed on phenoxide-substituted dioxetanone (Figure 3).[19] The authors compare the stepwise CIEEL to the concerted CTIL processes. In the CIEEL mechanism electron transfer (ET) from the electron-donating fragment to the dioxetane moiety forms a radical ion pair which is then annihilated by a back electron transfer (back ET) yielding an excited carbonyl compound (Figure 9). On the other hand, no intermediate radical ion pair is formed in the concerted CTIL. The advantage of the concerted CTIL over the stepwise CIEEL is to generate high-luminescence reactivity. The advantage in a biochemical environment of the concerted CTIL over the stepwise CIEEL reaction is that the former, in avoiding the formation of any intermediate radical species, suppresses a number of harmful side reactions as proton transfer from the environment and intersystem crossings to non-luminescent triplet states. The theoretical study on phenoxide-substituted dioxetanones shows that the preference of one over the other mechanism depends on different factors. A concerted mechanism is observed for the calculated pathway in vacuo, whereas the stepwise one is favored when the solvent effects are added. The *meta*-phenolate compound shows a concerted mechanism while the *para*-phenolate shows a stepwise mechanism.[19]

The comparative analysis on the decomposition of 1,2-dioxetane, dioxetanone, and thiazole-substituted dioxetanone reveals that the process for neutral thiazole-substituted dioxetanone compounds (**H-TDO** and **OH-TDO**) resemble the mechanisms of the two former species (1,2-dioxetane and dioxetanone). As seen for diaxetanone, the non-CT stepwise mechanism for the decomposition involves two discrete TSs and one excited-state intermediate. Although no CIs were found, the two states are sufficiently close in energy to allow population of the excited state. Similar to 1,2-dioxetane the torsional mode of $O_{10}C_8C_9O_{11}$ increases along with the $C_8C_9/O_{10}O_{11}$ stretching mode. Whereas the substitution of the thiazole by hydroxyde (**O$^-$-TDO**) decreases the barrier height by about 7 kcal mol^{-1} compared to **H-TDO**, the neutral **OH-TDO** has a barrier height of around 1 kcal mol^{-1} higher than **H-TDO**. The mechanism which is non-CT stepwise for the neutral species is for **O$^-$-TDO** a CT mechanism (either CIEEL or CTIL), where an electron is transferred from the electron-donating fragment, formed by a charged hetero-atom and an electron reservoir π aromatic system, to the dioxetanone moiety (Figure 7).[18] While the reaction for the **H-TDO** and **HO-TDO** species can strictly be associated with the $^1(\sigma,\sigma^*)$ and $^1(n,\sigma^*)$ electronic structures, the **O$^-$-TDO** carries significant additional $^1(\pi,\sigma^*)$ and $^1(\pi,n)$ character (Figure 8) near the TS. In principle this leads to

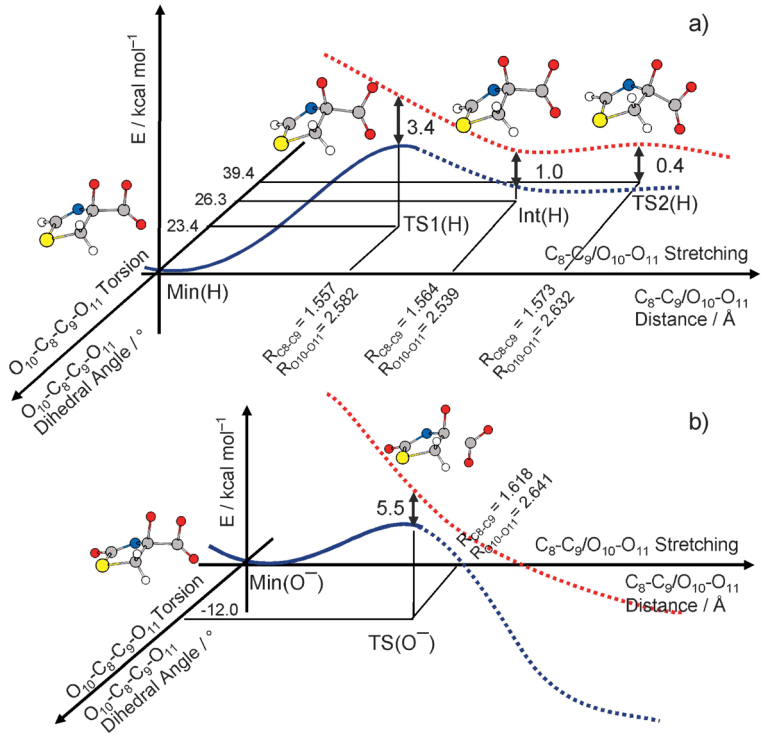

Figure 7. Reaction paths for the neutral **H-TDO** a) and anionic **O$^-$-TDO** b) with respect to the $C_8C_9/O_{10}O_{11}$ stretching and $O_{10}C_8C_9O_{11}$ torsional modes (see atoms' number Figure 3). The solid and dashed lines represent the potential energy curves determined by two computational strategies commonly employed in the theoretical studies on chemical reactivity (see ref. [18] for details). Reprinted from ref. [18] with permission from Elsevier.

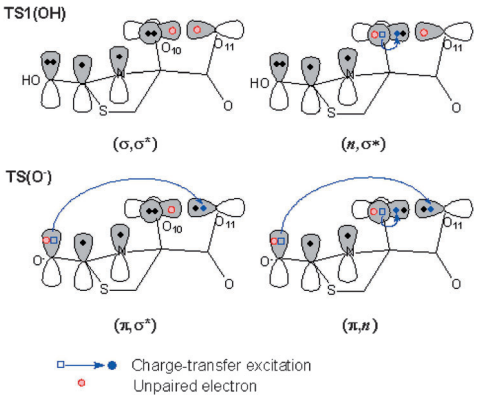

Figure 8. Important electronic configurations of the ground state and excited state of **HO-TDO** and **O$^-$-TDO** around the transition state. Reprinted from ref. [18] with permission from Elsevier.

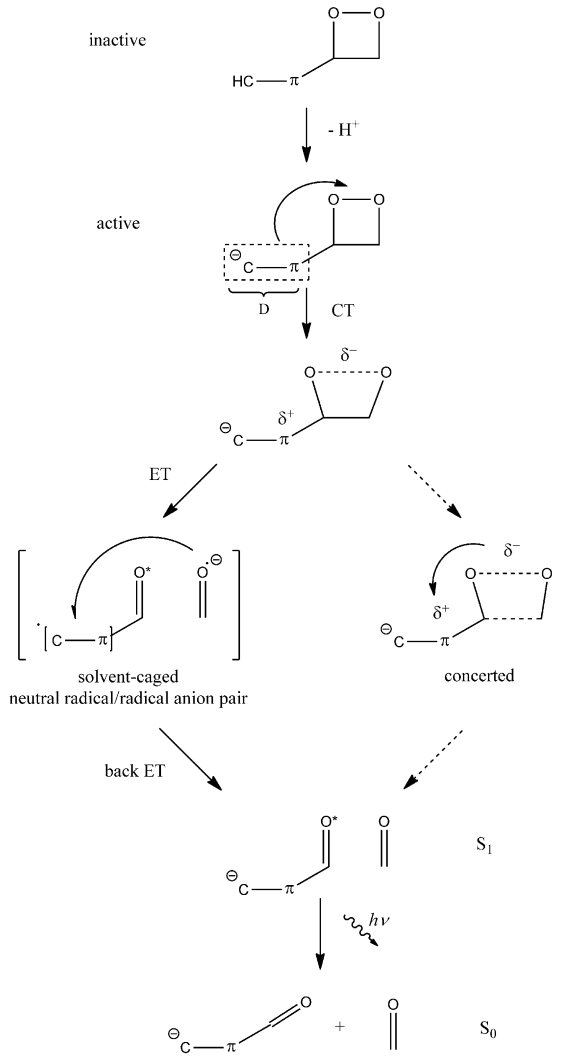

Figure 9. Reaction mechanism for the stepwise chemically initiated electron exchange luminescence CIEEL (left) and concerted charge transfer induced luminescence CTIL (right). Both mechanisms start with a partial charge transfer δ^- from the electron-donating fragment D to the chemiluminophore dioxetane moiety. The CT controlling group C activates the electron donation from the aromatic electron reservoir π. In the stepwise mechanism a radical ion pair is formed through a single electron transfer (ET) followed by a back electron transfer (back ET). In the CTIL, the rupture of the C–C bond is concerted with the back charge transfer.

a configuration in which the σ and σ^* orbitals are occupied by three electrons, thus destabilizing the bond and lowering the activation barrier for the fragmentation process. This is an important mechanism in making the reaction energetically feasible in a living species. The significant similarity between the **HO-TDO** and the **O^--TDO** species is therefore that both carry an electron reservoir, and they differ in that the deprotonation of the hydroxyl group leads to an effectively lower ionization potential of the π electrons, rendering them accessible to the CT mechanism.

This theoretical study is in line with the recent experimental results on bicyclic dioxetanes (Figure 3), where deprotonation of the phenolic hydroxyl side group induced by the solvent leads to chemiluminescence.[20] Chemiluminescence of these compounds appeared to be effective only in aprotic polar solvents (strong proton acceptor and a weak proton donor) or in the presence of a base. An H-bond between the solvent and the phenolic hydroxyl group, or deprotonation to the phenolate facititates electron transfer to the dioxetane moiety and therefore decomposition with emission of light. As seen for the thiazole-substituted dioxetanone compound, the chemiluminophore dioxetane moiety, an electron-acceptor functionality, is linked to an aromatic electron-donating fragment. The CT mechanism implies the presence of three key features: an electron-accepting chemiluminophore, an aromatic electron reservoir, and a CT controlling group in the form of a heteroatom as part of the aromatic π system.

Some theoretical studies on the mechanism of the decomposition of the dioxetanone intermediate of $OxyLH_2$ (**FI-DO**) have also been reported. Chung et al. found that the pathway which provides the excited state for the firefly bioluminescence proceeds through a TS and a CI as seen earlier for the model molecules.[21] Another recent study on the formation and the decomposition of the **FI-DO** intermediate proposed two decomposition reaction pathways, based on calculations of the geometries of stationary points in the gas phase and in solution.[22] The authors concluded that the concerted reaction path involving an O–O bond cleavage is more favorable than the reaction path predicting an intermediate compound. This conclusion is in favor of a concerted CTIL mechanism for the decomposition of **FI-DO**.

The type of mechanism occurring in vivo is still unknown and therefore further studies of the reaction in the protein must be carried out in order to get an answer. However, one would anticipate a CTIL process, as unwanted reactions caused by radicals generated in the case of the CIEEL mechanism would be excluded.

2.3. The Bioheterojunction Functionality

Oxyluciferin's structure consists of two planar moieties connected by a C–C bound. In 1994, McCapra et al.[23] proposed that color variation originates from the different conformations of the excited state obtained by rotation around the C_2–$C_{2'}$ bond. But later calculations have shown that the twisted structures are saddle points on both the S_0 and S_1 potential energy surface.[24,25] The rotation barriers in vacuo between the *cis*- and *trans*-structures are about 30 kcal mol^{-1}, which renders the isomerization difficult, let alone the steric constraints from the protein cavity in vivo. The almost planar *trans*-structures are therefore considered as light-emitters. Cai and coworkers explained the high efficiency for visible-light emission of oxyluciferin with the concept of a bio-heterojunction, in analogy to semiconductors. They concluded that the electronegative nitrogen atoms on the heterocyclic rings and the π–π conjugation play a key role in the chemiluminescence of luciferin.[26]

3. The State of the Substrate

To fully utilize the potential of color modulation in the luciferin–luciferase system, an exact identification of the light-emitter

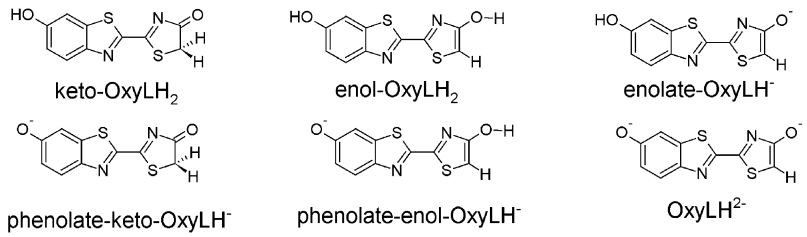

Figure 10. Different isomers of Oxyluciferin. In the first line are listed the protonated benzothiazole compounds and in the second line the anionic benzothiazolate. The first column list the keto forms, the second column the enol forms and the last column the enolate forms of the thiazolone moiety.

is important. This is a non-trivial issue for the firefly luciferin molecule. Luciferin molecules in vivo can be present in different forms, depending on 1) the protonated/deprotonated state of the CT controlling group (the hydroxyl group on the benzothiazole fragment), 2) a keto–enol tautomerization, and 3) a possible deprotonation of the hydroxyl group of the enol-form. The six possible chemical forms of oxyluciferin are shown in Figure 10. Herein we first discuss the protonation state of the CT controlling group, followed by some remarks on the keto–enol tautomerization. However, the overall conclusion is that the light-emitter of the firefly is a singly deprotonated luciferin in a keto-form.

3.1. Protonation State of the Benzothiazole Moiety

Different experimental and theoretical studies on the effect of pH, solvent and counter-cation have been performed.[9,15,27,28] The effects on color modulation have been studied at different levels: by the protonation state of the CT controlling group,[29] by the global polarization of the surroundings done by the calculation of the absorption and emission spectra in solvents of different polarity,[28,30] and by the local interaction of the CT controlling group with a protonated moiety or a cation.[15,27,28,30] The calculated emission energy of keto-OxyLH_2 is 3.53 eV with a value of 0.32 for the oscillator strength (*f*), while the corresponding energy for phenolate–keto-OxyLH^- is 2.10 eV with an *f* value of 0.71.[29] The value of 1.78–3.10 eV for the neutral form is out of range for visible light. These results show that the emitter form is the phenolate form. Calculations on the neutral and phenolate forms were performed in different solvents. The absorption and emission spectra of the neutral keto-OxyLH_2 form were calculated in benzene, dimethyl sulfoxide DMSO, CH_3CN and H_2O to analyze the effects of these solvents on the spectroscopical properties.[28] The calculations show that *f* and the red-shift of absorption and emission of the neutral form increase with the polarity of the solvent. On the other hand, for the phenolate species, a blue-shift is observed with the increase of the dielectric constant of the solvent.[9,30] The trend of the color modulation with the global polarity of the solvent depends on the species (neutral, phenolate or with a counter-ion) studied. The results from the theoretical investigation on the effect of the micro-environment close to the phenol moiety of firefly oxyluciferin roughly explain the color modulation.[15] A scanning of the simple model of the complex of keto-OxyLH_2 and NH_3 increasing the $O_{10'}$—$H_{12'}$ bond length shows that light-emission is modulated by two aspects, namely how covalently the hydrogen between the phenolate form and a protonated basic moiety is bonded, and by how polar the surrounding of the micro-environment is.[28] This is in line with the previous experimental results from Hirano et al. on the C_5-dimethylated phenolate–oxyluciferin in different solvents and with different counter-cations.[27] This conclusion on the importance of the polarity of the micro-environment and effects of different interactions with side molecules were confirmed by the calculations in the presence of Na^+ and NH_3 on phenolate–keto-OxyLH^-.[9,31] The authors emphasized the relevance of knowing the different interactions of the chromophore with the surrounding residues, adenosine monophosphate (AMP) and water molecules. To summarize, the deprotonation of the CT controlling group turns on the CT mechanism and this produces a red-shift in the emitted color as compared to the emission from the neutral species. In addition, subtle differences in the local interactions of the anionic CT controlling group with the surrounding residues or water, through hydrogen bonds or polarized complexes, allow for fine-tuning of the color modulation.

3.2. Keto–Enol

The oxythiazolyl moiety of oxyluciferin has also been the topic of studies and discussions.[6,7,9,29,32,33] Two tautomers are possible, the keto- and the enol-form. White et al. proposed that the color modulation would result from the keto–enol equilibrium as the color of bioluminescence systems changes with pH. They showed that enol-form emits yellow-green light while the keto-form emits red light.[34] Since this proposition, the tautomeric nature of the emitting chromophore has been discussed extensively. Naumov and coworkers turned their attention toward the electronic and environmental effects on the enol–keto–enolate equilibrium.[6,7] The most polar solvents do not necessarily cause the strongest red-shifts in a model where the hydroxybenzothiazol moiety is dehydroxylated and the relative order in the UV/VIS spectrum of emission of the series enol-, keto- and enolate-forms depends on the nature of the CT controlling group. Branchini et al. were the first to demonstrate that the color modulation can depend only on the keto-form of oxyluciferin.[32] Later, Hirano et al. showed that the color modulation could also be obtained by 5,5-dimethyloxyluciferin, a mutated compound forced into its keto-form.[27] Calculations on keto-OxyLH_2 and phenolate-keto-OxyLH^- in differently polarized micro-environments reproduced the range of multicolor emission.[15] All these results, as well as those from the studies including the protein effects lead to the conclusion that the color modulation can be explained only with the

keto-form of the phenolate anion and depends on the micro-environment in the cavity.

4. The Significance of the Enzyme

In the first part of this review, it is pointed out that the calculations performed on the isolated light-emitter leads to significant insights in the intrinsic mechanisms responsible for the luminescence phenomena. However, such simple models cannot explain how the bioluminescence takes place effectively, such as how the protein favors a particular reaction path over all the other ones possible. Since bioluminescence reactions take place in the cavity of the enzyme, the residues defining this active site, such as other ligands and crystallographic water molecules, may contribute to the chemical mechanism (Figure 11). The cavity is like a channel straight through the enzyme (Figure 12). When the substrate put into place, there remain two different pockets. The cavity on the thiazolone side is where dioxetanone is formed and the fragmentation process takes place, while the cavity on the benzothiazole side is mainly where the color is modulated. To continue the quest for the light-emitter beyond the studies done in vacuo and in the solvent model (see Section 3) the effects of the enzyme have been included. The calculations of the emission energies of phenolate–keto-OxyLH$^-$ and phenolate–enol-OxyLH$^-$ in vacuo or in DMSO do not provide a final answer to which of the two forms is the light-emitter. Actually, the first singlet excited state of the keto-form is more stable by 16.6 kcal mol^{-1} than the enol-form in vacuo and by 10.9 kcal mol^{-1} in DMSO,[29] which is in agreement with the results from Yang and Goddard[24] and Song and Rhee.[35] However, considering the interaction between the substrate and the protein, the phenolate–enol-OxyLH$^-$ S_1 state is 3.35 kcal mol^{-1} more stable than the phenolate–keto-OxyLH$^-$ S_1 state.[29] According to a standard Boltzmann population distribution, this result could be in favor of an enol-form emission. However, the reaction pathway leading to the S_1 excited state is the main factor that controls the form of the light-emitter and the loss of CO_2 (Figure 2) leads to a keto-like form. Hence the enol-form would result from a tautomerization taking place inside the protein. The corresponding energy barrier to overcome may be too high to end in a significant amount of the enol-form. This hypothesis has been confirmed by a recent study by Song et al., showing that an excited-state proton transfer, needed for the tautomerization reaction, from an adjacent lysine residue to the keto-form, optionally mediated by a water molecule is unlikely to take place.[36]

In order to test the interpretation given by Nakatsu et al. concerning the color modulation of the luciferin–luciferase system and based on experiments and crystal structures of the protein,[37] we did calculations on some luciferase–luciferin models matching the experimental hypothesis.[38] Nakatsu et al. obtained three crystal structures of *Luciola cruciata*: the wild-type protein including 5′-O-[N-(dehydroluciferyl)-sulfamoyl]adenosine (DLSA) (Figure 13) in its active site, the red-emitting S286N mutant with DLSA, and the wild-type protein including oxyluciferin and AMP. They found that the cavity of the wild-type + oxyluciferin + AMP system as well as the mutant one are much bigger than the one in the wild-type protein with DLSA. As DLSA is a good analog of the intermediate state of the bioluminescence reaction, the authors concluded that the color

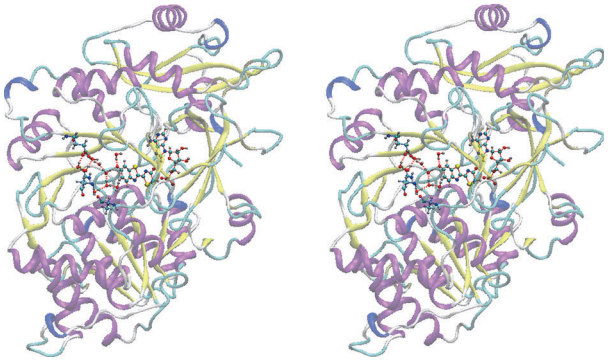

Figure 11. Stereo view of luciferase. Shown in ball and stick are oxyluciferin, AMP, and some residues and water molecules involved in the H-bonding network of the cavity. H-bonds are represented in dashed lines. The rest of the protein is represented in ribbon. The picture was prepared with VMD software.[48]

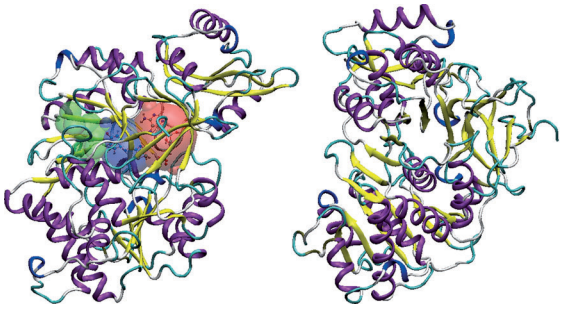

Figure 12. Two different views of the luciferase protein. On the left the cavity where the reaction is taking place is drawn. Blue: oxyluciferin region, green: benzothiazole side pocket, red: thiazolone side pocket. The cavity looks like a channel which crosses the protein. On the right the ribbon representation is rotated by 90 degrees on the vertical axis to show the hole formed by the cavity. The picture was prepared with VMD software.[48]

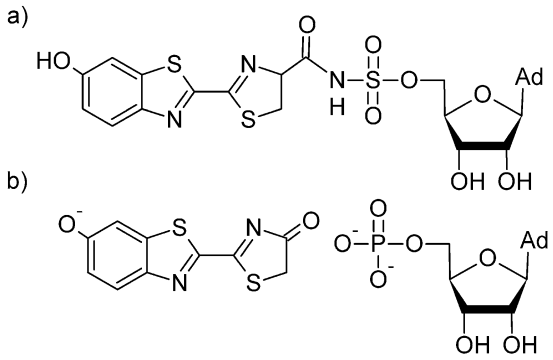

Figure 13. a) 5′-*O*-[*N*-(dehydroluciferyl)-sulfomoyl]adenosine DLSA, an analogue of the intermediate structure of oxyluciferin and AMP. b) Oxyluciferin and AMP.

modulation originates from the rigidity and tightness of the protein cavity. Accordingly, the S286N mutation would prevent the structure from breathing and the reaction would occur in an OPEN structure, similar to the one characterizing the final product. On the other hand, in the wild-type protein the emission would occur in a CLOSED structure, constraining the oxyluciferin substrate. Based on the crystal data of Nakatsu et al. we constructed six models according to a given relaxation protocol, namely three OPEN and three CLOSED ones. Each vertical transition from the geometry-optimized phenolate–keto-OxyLH$^-$ structure into its first singlet excited state was then calculated. It was found that the emission energies were not correlated with the OPEN or CLOSED nature of the protein cavity but rather with the hydrogen-bond network connecting the water molecules, the substrate, and the residues involved in the protein cavity (Figure 11). If therefore this micro-hydration pattern is responsible for an external electrostatic potential able to stabilize the formal charge carried by the benzothiazole moiety of phenolate–keto-OxyLH$^-$ in its electronic ground state, the emission energy increases. In simpler words, a blue-shifted energy is observed with the increase in the number of water molecules H-bonded to the benzothiazole oxygen. Accordingly, the S286N mutation located at the entrance of the cavity on the phenolate side modifies the hydrogen-bond network and ultimately shifts the emitted light color from yellow to red.

The H-bonding in the benzothiazole side pocket might not be the only reason for the color modulation. Others residues of the cavity, which may be involved in the H-bonding network but not necessarily are, might also play a role in the color modulation.[30, 33, 39]

To summarize, the enzyme functionality allows one 1) to favor the reaction between D-Firefly luciferin and ATP, 2) to modulate the distance to the nearest proton(s) of the CT controlling group by the orientation of the water molecules in the cavity and the arrangement of the residues of the cavity, and 3) to apply on the chromophore an external electrostatic potential. The two last points are important in the color modulation of the light emission. As the transition from S_1 to S_0 leads to an internal negative charge transfer from the thiazolone ring to the benzothiazole ring, an increase in the stabilization of the charge on the CT controlling group will result in a higher energy difference between the ground and excited states and therefore in strong blue-shifts.

5. Bioluminescence in Other Organisms

To conclude this review let us expand on our understanding of the bioluminescent process of the firefly luciferin to that of luciferin systems of other species, but first the various mechanisms in action for the bioluminescent reaction to take place need to be summarized. In our study of systems ranging from the 1,2-dioxetane to the firefly luciferin–luciferase system we have identified three key functionalities. 1) The chemiluminophore allows the route from the ground-state to the excited-state surface. In the case of the firefly luciferin molecule a peroxide fragment offers this functionality. As the –O–O– bond breaks the former oxygen 2p orbitals, which formed the σ bond, and the oxygen lone-pair orbitals become degenerate. This will be the origin of either a CI or a tight avoided crossing between the S_0 and S_1 states. The presence of this feature along the thermal dissociation channel offers a way to generate a product in the excited state. 2) The electron-donating fragment tunes the activation energy of the carbon dioxide fragmentation. The activation energy of dioxetanone is by far too high for the reaction to proceed with any significant speed in a living organism. To reduce this, the substrate uses a mechanism (CTIL or CIEEL) in which an extra electron is borrowed to change the fragmentation process from a non-CT reaction to a CT reaction. The activation energy of the latter is then 5-8 kcal mol^{-1}. The CT is facilitated by the presence of an electron-donating moiety in the form of an aromatic system with an electronic π system that can deliver a loosely bound electron during the reaction. Cai and coworkers have argued that bioheterojunctions can be of importance in this process. 3) The CT controlling group controlled and fine-tuned the CT mechanism as its protonation state and H-bonding implication depend on the micro-environment (pH) created by the enzyme. Our research has demonstrated that the hydroxyl group on the benzothiazole fragment can turn the CT on and off, it can also be used as a tool to fine-tune the color emission.

Can we identify these functionalities in other luciferin systems? The chemiluminophore, in the form of a peroxide –O–O– bond, is common to all the different systems depicted in Figure 14. Small variations are found, in particular both the bacterial and dinoflagellate luciferins fragment through a single bond-breaking procedure and a different ejected fragment. Further research will tell if this changes the importance and the mechanism of the CT (CTIL or CIEEL) for these specific luciferin systems. One may also wonder if the periodic table could support a chemiluminophore which is not based on oxygen chemistry.

The electron-donating fragment, the aromatic system in close vicinity to the chemiluminophore, can be found in all cases but one. The latia luciferin substrate has no such functionality. It can be speculated that this missing functionality in the substrate has to be compensated by the enzyme providing this by an aromatic amino acid group (phenylalanine, tryptophan, histidine or tyrosine) in the vicinity of the substrate. Future X-ray studies of the latia luciferin–luciferase system should clarify this question. Moreover, comparing the coelenterazine and vargula/cypridina luciferin molecules we note that the electron-donating fragment can consist of quite different types of aromatic systems—the functionality is of greater importance than a specific chemical composition. Concerning the CT controlling group, we note that all of the examples depicted have either an –OH or –NH group on the electron-donating fragment. These groups could be used to turn the CT off or on and to modulate the emitted color.

Finally, comparing the coelenterazine and vargula/cypridina luciferin we find fragments attached to the molecular framework through sp^3 hybridized carbons. These fragments are clearly not involved in the bioluminescent process but are

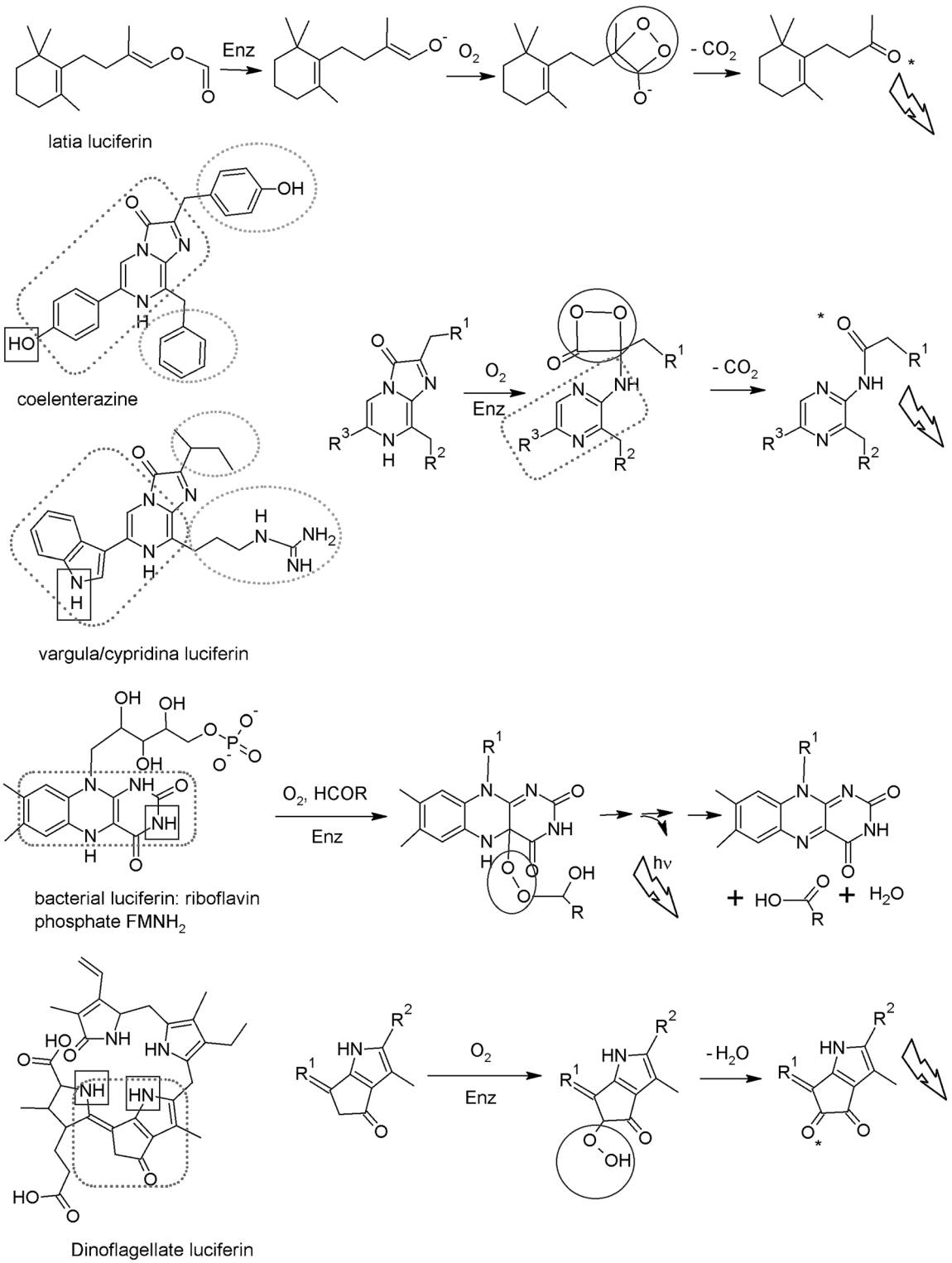

Figure 14. Bioluminescence of the luciferin–luciferase reaction in different compounds. Parts of the mechanism of the bioluminescence process are shown for different species: in mollusk (latia luciferin), ostracods (cypridina (vargula) hirgendorfii luciferin), jellyfish (coelenterazine), bacteria ($FMNH_2$) and plankton (dinoflagellate luciferin). The chemiluminophore is circled, the electron reservoir is emphasized by a dashed rectangle and the CT controlling group by a square, tethering groups are circled by dashed ovals.

most likely tethers to position the substrate at the correct place in the associated enzyme.

To summarize this comparison between different luciferin systems we clearly see that extensive research on the details on the bioluminescent mechanism for these systems will teach

us much about how Nature designed the optimal molecular systems to provide this luminescent property. Similarities and differences, in structures and mechanisms, will help us to understand and produce optimal man-designed luciferin–luciferase systems. Examples of artificial modifications of the electron reservoir and the CT controlling group[40,41] of the firefly luciferin have already been reported in the literature.

Computational Details

The theoretical study of chemiluminescence is a challenge. The phenomenon requires the study of two or several states at or close to a CI or seam. Excited states are both of valence and CT character. If spin–orbit effects are to be included, balanced treatment of singlet and triplet states are required. Furthermore, the phenomenon carries the hallmark of a strongly correlated system in which no single electronic configuration can be expected to dominate a priori. These constraints limit the number of methods that can be used. In general, only multiconfigurational methods can be used to study the whole process. Configurations that are strongly correlated and describe the CT mechanism have to be included. The selected active space has to include strongly correlating orbital pairs, orbitals that correctly describe the dissociation, and orbitals that are involved in the electronic excitations, in order to describe the excited states. However, single-reference methods and density functional theory can be employed to give qualitative results for some parts of the reaction. These methods should be used with caution to describe the key mechanism of the chemiluminophore—the CI and the fragmentation process. Here the multi-configurational character is well-developed and single-reference or DFT methods are not recommended due to the absence of a well-defined reference function and a qualitative incorrect description of the bond breaking.

In the studies conducted by our laboratories we have used the complete active space second-order perturbation theory/complete active space self-consistent field CASPT2/CASSCF method to describe the processes. This method associated with an appropriate active space, is both qualitatively and quantitatively correct. The selection of the active spaces have been far from easy and in most of the cases ended up in calculations which are on the edge of what is possible with standard implementations. For some parts of the studies, where it was appropriate to do so, we and others have used DFT methods.

The strong correlation present in the firefly luciferin is in itself a benchmark for methods of strong correlation and selection of actives spaces. Recently Greenman et al.[42,43] challenged the results of Chung and coworkers[21] with respect to studies of the chemiluminecent process of luciferin by using the variational calculations of the two-particle reduced-density-matrix. According to Greenman, rather large active spaces, beyond the capacity of standard methods, have to be employed to safeguard quantitative accuracy. However, the calculated results presented on 1,2-dioxetane,[16] dioxetanone[17] and thiazol-substituted dioxetanone[18] were done at the MS-CASPT2/SA-CASSCF level of theory in association with appropriately selected active spaces.

For the theoretical study of the luciferin–luciferase system further considerations and assumptions are required. From the theoretical point of view, different approaches can be chosen in order to take the surroundings of the active site into account. First, a structural model of the whole protein must be built. This is usually performed by inspection of the Protein Data Bank (PDB).[44] If the crystal structure of the protein is not available, a model can be built analogously to other similar biomolecules.[45,46] Second, no matter how the protein structure is elaborated, current computational resources cannot afford a quantum mechanical description of the whole protein. One approach is to select some of the important residues according to given criteria and to include them in the calculations. Examples of this approach in association with studies of the luciferin–luciferase system can be found in the literature.[30,33,39] Another possibility consists in the selection of a qualitative but less expensive method to describe the protein and other molecules, like water and AMP, while the description of $OxyLH_2$ is done with a more accurate method. An example of such a hybrid model is the quantum mechanics/molecular mechanics (QM/MM) approach. CASPT2/CASSCF//AMBER is one of the state-of-the-art methods to perform these kinds of calculations. This approach have been adopted in our investigation of the luciferin-luciferase system.[29,38]

Finally, special care must be also taken with respect to the difference between bioluminescence, chemiluminescence, and fluorescence, since these processes may involve different emissive states. The discrepancy between chemiluminescence and fluorescence has been recently analyzed in the preliminary study on a coelenterazine model.[47]

6. Summary

In the work presented herein we reviewed the theoretical studies performed on the bioluminescence of the firefly luciferin system, focusing entirely on the chemical aspects of the phenomenon, and using a systematic approach from studies on small molecules to investigations on large and complex systems. That allows us to rationalize the relevance of the chemical groups of the molecule in the bioluminescence process. Small models, such as 1,2-dioxetane, dioxetanone, and distinct substituted dioxetanone molecules, ease the understanding of the molecular basis of the reaction and the establishing of the mechanisms. Improvements to the description and the characterization of the light-emitting species can be obtained from studies with the entire molecule and systems that include luciferin and some explicit side molecule or solvent effects. Since the reaction takes place in a protein, the luciferase–luciferin system needs to be understood by means of effects derived from the protein–substrate interactions. Only then can the reaction mechanism be refined and an explanation be given for the bioluminescent properties of luciferin in its natural environment. Throughout this systematic analysis of the theoretical studies, three moieties of the luciferin–luciferase system were associated with the key components for the bioluminescence

process: the *chemiluminophore*, which opens the path to the excited-state surface, the *electron-donating fragment*, which lowers the activation energy of the reaction by means of a CT mechanism, and the *CT controlling group*, which turns the CT mechanism on or off and modulates the color emission, depending on the interactions between this moiety and the protein. The crucial role of these individual parts of the firefly luciferin–luciferase system and their presence in several molecular systems, responsible for light-emission in other organisms, allow us to understand them as the chemical functionalities of the bioluminescence phenomenon. Finally, some effort is spent on discussing the computational challenges one faces in the theoretical studies of the same phenomenon.

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