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Computational Studies of the Luciferase Light-Emitting Product: Oxyluciferin

Luís Pinto da Silva and Joaquim C.G. Esteves da Silva*

Centro de Investigação em Química (CIQ-UP), Departamento de Química e Bioquímica, Universidade do Porto, Campo Alegre 687, 4169-007 Porto, Portugal

ABSTRACT: Firefly luciferase is the most studied bioluminescence system, and its catalyzed reactions have been relatively well characterized. However, the color tuning mechanism that leads to firefly multicolor bioluminescence is still unknown, nor is consensual which is the yellow-green and red emitters. Computational studies have been essential in the study of oxyluciferin ($OxyLH_2$) chemi- and bioluminescence and are responsible for most of our knowledge of this natural phenomenon. The objective of this manuscript is the analysis of the benefits and the conclusions derived from the theoretical studies of the light emitter, $OxyLH_2$, and its applications on bioluminescence research.

INTRODUCTION: THE FIREFLY MULTICOLOR BIOLUMINESCENCE ENIGMA

The emission of light resulting from an enzyme catalyzed biochemical reaction is known as bioluminescence. This natural phenomenon is found in many types of organisms, including fungi, insects, bacteria, worms, dinoflagellates, and fish. Despite some structural differences, the majority of these bioluminescence reactions are catalyzed by an enzyme named luciferase, which reacts with different subtracts called luciferin.[1,2] In recent years, this bioluminescence system has gained numerous bioanalytical, biomedical, and pharmaceutical applications, among others. More specifically, it is involved in the analytical determination of adenosine 5′-triphosphate (ATP) in microbial detection, imunoassays, bioimaging, biosensing and is used as a gene reporter.[1−7]

The most studied bioluminescent reaction known is that of the North American firefly *Photinus pyralis*.[1,3] *Photinus pyralis* luciferase (EC 1.13.12.7, Luc) catalyzes a two-step reaction: The first is the condensation reaction between the luciferin subtract (LH_2), a derivative of benzothiazolyl-thiazole, and ATP in the presence of Mg^{2+}. The second step consists of the oxidation of the first step product, an adenylyl intermediate (LH_2−AMP) and the release of AMP, CO_2, and $OxyLH_2$. The light emitter is formed in an excited singlet state S_1, which decays to the ground state with the emission of visible light (Scheme 1). This system is known for its efficiency when compared with chemiluminescence. For many years the efficiency of the this reaction was thought to be 88%,[8,9] but a recent work performed by Ando et al. estimated it at 41%.[10] Albeit the sharp decrease, this new value still strongly supports the study and the development of practical applications for this bioluminescence system. Other firefly luciferase system commonly studied include *Luciola cruciata*, *Luciola lateralis*, *Luciola mingrelica*, *Phrixotrix hirtus*, *Lampyris noctiluca*, and *Lampyris turkestanicus*.[1,11]

Currently, one of the most intriguing and studied aspects of firefly light emission is the origin of the multicolor bioluminescence. Albeit the similarity of the substrate−product structures between all bioluminescent insects, their emission energies range from 2.14 to 2.34 to 2.00 eV.[12,13] The red shift can be induced by high temperatures, addition of divalent metal cations, and denaturation by addition of substances like urea.[14−16] The shifting from yellow-green (basic pH) to red emission is also achieved with a decrease in pH.[9] The understanding of this peculiar aspect could be of enormous importance in firefly research. Red-emitting Luc could be used in in vivo medical imaging, as red light is absorbed very poorly by mammalian tissues in comparison with the natural emitted light. Also, the control of the multicolor bioluminescence could be the basis for using Luc as a single dual reporter gene, a bioindicator of cellular stress, and a probe for intracellular changes of pH.[17]

Several hypotheses have been proposed over the years to try to explain this pH-dependent phenomenon:

(a) The first hypothesis was advanced by White et al. who suggested that the color variation was the result of the keto−enol tautomerism of the $OxyLH_2$ species (Scheme 2).[18] The keto form was supposed to emit red light, while the enol form was the yellow-green emitter. However, experiments with a keto-constrained $OxyLH_2$ analogue indicated that the light-emitting reaction only required a keto emitter.[19]

(b) McCapra et al.[20] proposed a mechanism based on theoretical calculations with the semiempirical functional AM1.[21] These calculations indicated that the color variation depended on the rotation around the C−C bond of the −N=C−C=N− moiety.

(c) The dependence of the bioluminescence color on the polarization of the internal microenvironment of Luc was advanced by several groups as a determining factor in the light-emitting mechanism. The higher the polarizability, the larger the red shift of bioluminescence.[4,22,23]

(d) Following its 2002 work,[17] Branchini et al. proposed that Luc modulates the color of the light emitted by controlling the resonance structure of the anionic keto form of

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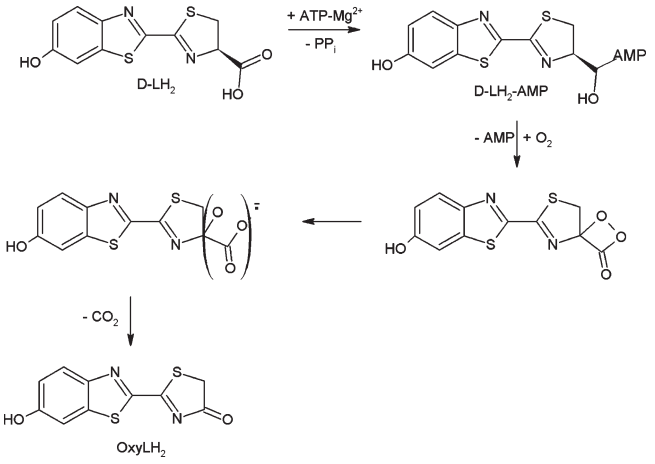
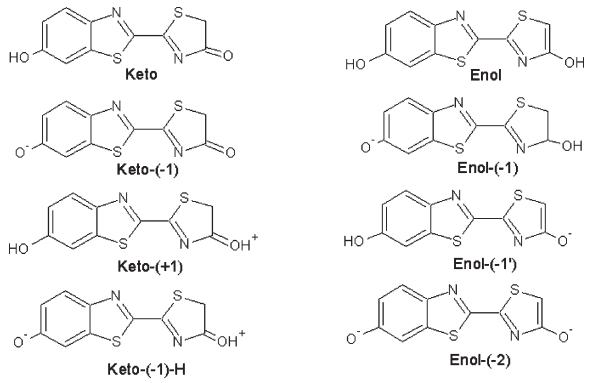
Scheme 1. Luc-Catalyzed Bioluminescence Reaction



Scheme 2. Tautomeric and Dissociation States of $OxyLH_2$



$OxyLH_2$.[24] A structure showing a −N=C−C=N− moiety would relax by emitting green light, while another one showing a =N−C=C−N= moiety would be the red emitter.

(e) Nakatsu et al. proposed another mechanism based on the recently solved three-dimensional structure of *Luciola cruciata* luciferase (LcLuc).[25] This structure and its mutant showed that this enzyme can assume two different conformations: an open conformation with a polar and loose active site and a closed conformation with a hydrophobic and rigid active site. Based on these findings, this group hypothesized that the geometrical rearrangement of the active site could lead to different relaxation of the light emitter. So, a nonrelaxed $OxyLH_2$ would emit yellow-green light, and a molecule that is permitted to relax would emit red light.

(f) More recently Hirano et al. proposed a mechanism derived from hypothesis (c).[26] They stated that the emission of light is modulated by the polarity of the active site internal environment and by the degree of covalent character of a bond between the anionic keto form and a protonated basic moiety present in the active site.

Thus, these proposed mechanisms are all based on changes in the properties of $OxyLH_2$, and/or changes in the internal microenvironment of the active site. However, there is still no consensus on which hypothesis best describes the mechanism behind the multicolor bioluminescence. One factor that impairs the experimental study of this question is the experimental instability of $OxyLH_2$ in a basic model solution. In fact, only rather recently, the crystal structure of this molecule was achieved for the first time.[27] Therefore, the majority of information was obtained from more stable analogues, as 5-methyl ($MOxyLH_2$), 5,5-dimethyl ($DMOxyLH_2$), and 6′-dehydroxy ($DHOxyLH_2$).[28−32] However, studies with these molecules only provide indirect information as they possess substitutions which impose significant steric hindrance for binding of the emitter to Luc, and $DMOxyLH_2$ is keto-constrained and cannot account for possible effects related to the keto−enol tautomerism. Moreover, these studes are performed outside the protein active center.

Thus, a computational approach to the firefly bioluminescence research is fundamental. Only computational studies appear to have the required level of accuracy and reliability for understanding this natural phenomenon. One of the main advantages of the use of theoretical calculations is the possibility of direct study of the properties of the various $OxyLH_2$ species in various solvents, without the need for more stable analogues. Also no experimental technique, unlike the available theoretical methods, allows us to perform direct and detailed studies, down to the atomistic level, of the interactions between the emitter molecule with molecules present in the active site of Luc and the effect of these interactions on the emission energies.

THEORETICAL STUDIES OF $OXYLH_2$ STRUCTURAL AND ELECTRONIC PROPERTIES

The light emitter $OxyLH_2$ is formed in the singlet excited state in the Luc-catalyzed reaction by attachment of O_2 to the thiazolone moiety of LH_2−AMP. This reaction releases AMP and forms a dioxetanone intermediate which decarboxylates into the emitter molecules.[1] Besides bioluminescence, $OxyLH_2$ also exhibits chemiluminescence. It was established that this molecule is in the keto form in dimethyl sulfoxide (DMSO) solution containing a small amount of potassium *tert*-butoxide, and it is transformed into the enol form in DMSO with a large amount of potassium *tert*-butoxide.[18,33,34] The enol form was thought to emit green light and the keto form red light.[18] It was this similarity between chemi- and bioluminescence which introduced the idea that the color tuning mechanism was the same in the two processes.[18,33] That is why that the first computational studies on bioluminescence focus on $OxyLH_2$ intrinsic properties to explain the multicolor bioluminescence. The first relevant study was performed by McCapra et al.[20] It was made in vacuo and by means of the semiempirical functional AM1 and proposed the twisted intramolecular charge-transfer (TICT) state mechanism. According to their calculations, the yellow-green emitter has a planar structure and is a saddle point on the potential energy surface. The twisting between the −N=C−C=N− stabilizes the molecule and leads to red emission. However, the method used has a tendency not to describe conjugated single bonds and underestimated the energy barrier between these two states.[35,36] This was later confirmed by another in vacuo study which used the more reliable ab initio excited-state method,[37] configuration interaction with single excitations (CIS).[38] These calculations predicted that the planar structure of both the enol and keto forms is a minimum on the S_1 potential energy surface and that the twisted structure is a saddle point.

Table 1. Absorption Energies (in eV) of the Various $OxyLH_2$, Calculated at Different Levels of Theory[a]

$OxyLH_2$	TD-B3LYP/6-31+G(d)	TD-B3LYP/6-31G(d)	TD-B3LYP/6-31+G(d,p)	CIS/6-31G(d)
keto-(3.35)[c,d]	3.32[b,37] 3.02[c,63] 3.32[b,64]	3.55[b,51] 3.42[c,51]	3.32[b,43]	5.15 [b,51] 5.07 [c,51]
enol	3.40 [b,37] 3.20 [c,63]	3.71 [b,51] 3.66 [c,51]		4.95 [b,51] 4.89 [c,51]
keto-(−1) (2.99) [c,e]	2.54 [b,37] 2.45 [c,63] 2.54 [b,64] 2.55 [c,64]	2.72 [b,51] 2.75 [c,51]	2.54 [b,43]	3.49 [b,51] 3.55 [b,51]
enol-(−1)	2.48 [b,37] 2.54 [c,63]	2.65 [b,51] 2.77 [c,51]		3.58 [b,51] 3.81 [c,51]
enol-(−1′)	2.06 [b,37]	2.01 [b,51] 2.41 [c,51]		3.74 [b,51] 4.16 [c,51]
enol-(−2)	2.36 [b,37] 2.49 [c,63]	2.58 [b,51] 2.67 [c,51]		4.22 [b,51] 4.22 [b,51]
keto-(+1)	2.40 [c,63] 2.27 [b,64] 2.47 [c,64]			
keto-(−1)-H	2.39 [b,64] 2.41 [c,64]			

[a] Experimental values are in parentheses.[50] [b] Gas phase. [c] Water. [d] pH 1.0. [e] pH 10.0.

Table 2. Emission Energies (in eV) of the Various $OxyLH_2$, Calculated at Different Levels of Theory[a]

$OxyLH_2$	TD-B3LYP/6-31+G(d)	TD-B3LYP/6-31G(d)	SAC−CI	CIS/6-31G(d)
keto	2.71 [c,63] 2.99 [b,64]	3.17 [b,51] 3.12 [c,51]	2.95 [d,57]	4.13 [b,51] 4.03 [c,51]
enol (2.77)[d]	2.73 [c,63]	3.07 [b,51] 3.03 [c,51]	2.83 [d,57]	3.77 [b,51] 3.72 [c,51]
keto-(−1) (2.01)[d]	2.20 [c,63] 2.23 [b,64] 2.30 [c,64]	2.61 [b,51] 2.63 [c,51]	2.08 [d,57]	3.34 [b,51] 3.40 [c,51]
enol-(−1) (2.22−2.24,[c] 2.16) [d]	2.44 [c,63]	2.59 [b,51] 2.65 [c,51]	2.25 [d,57]	3.22 [b,51] 3.33 [c,51]
enol-(−1′)		1.97 [b,51] 2.19 [c,51]	2.14 [d,57]	2.89 [b,51] 3.22 [c,51]
enol-(−2) (2.30,[c] 2.10) [d]	2.17 [c,63]	2.19 [b,51] 2.27 [b,51]	2.07 [d,57]	3.14 [b,51] 3.17 [c,51]
keto-(+1)	2.05 [c,63] 1.70 [b,64] 2.10 [b,64]			
keto-(−1)-H	1.79 [b,64] 1.99 [c,64]			

[a] Experimental values are in parentheses.[27] [b] Gas phase. [c] Water. [d] DMSO.

The paper of Orlova et al. was the first work to give a theoretical insight on the stability of the different $OxyLH_2$ species and their vertical excitation energies.[37] Theoretical calculations with the B3LYP functional[39] and 6-31+G(d) split-valence basis set augmented with *d*-type polarization and diffuse functions[40] predicted that the anionic keto-trans conformer is more stable than keto-cis by 5.3 kcal/mol. This was an important breakthrough as the earlier modeling studies of the active site assumed a cis-conformation for LH_2 and $OxyLH_2$.[41,42] This study was complemented by calculations (B3LYP/6-31+G(d,p) of Liu et al., which predicted that all of the trans-conformers are more stable than the cis species by a range of 4.9−6.2 kcal/mol.[43] Orlova et al. also predicted that enol and enol-(−1) are less stable by 8.5 and 19.9 kcal/mol than their corresponding keto forms.[37] This was another pivotal discovery because combined with Branchini et al. conclusions, directed the research of firefly bioluminescence for the study of the keto forms of $OxyLH_2$.[19] Another important contribution from this work was the first study of the in vacuo vertical excitation energies of the $OxyLH_2$, which are summarized in Table 1. For this, excited-state predictions were made by means of time-dependent (TD) hybrid time density functional theory (DFT) method (TD-B3LYP)[44,45] and, for additional calibration, the Zerner's intermediate neglect of differential overlap (ZINDO) semiempirical method.[46,47] Both methods were already proven reliable in some excited-state calculations.[48,49] The results obtained with TD-B3LYP gave excellent agreement with experiment at pH = 1.[50] This opened the way for the use of TD-DFT in the study of $OxyLH_2$ electronic properties.

The conclusions regarding bioluminescence that could be derived from this study are however limited, as aqueous solvents always play an important role in vivo and can affect significantly excited-states energies. Ren and Goddard were the first to include solvent effects in their vertical excitation energies calculations (Table 1).[51] Moreover, they were the first to perform the systematic excited-state optimizations of $OxyLH_2$ and the calculation of the corresponding emission energies (Table 2). The excited-state calculations were made with the TD-B3LYP and CIS methods with the 6-31G(d) basis set. It should be noted that the basis set used lacked diffuse functions, which are necessary for the accurate prediction of excited-state energies.[37] The predictions of solvent effects were made with the self-consistent isodensity polarized continuum model (SCI PCM) with parameters set for water.[52] The obtained results showed the importance of solvent effects on the prediction of the excitation and emission energies of the anionic species, as they underwent large shifts to the blue when in comparison with in vacuo results. The neutral species were affected to a lesser extent and suffer only small shifts to the red. The CIS method correctly predicted the changes in the excited-state geometries but needed a scaling of the wavelengths to be in reasonable agreement with the TD-B3LYP results and with experiment. This indicated that calculations with the CIS method should be limited to geometries optimization and that the prediction of $OxyLH_2$ electronic properties should be made with more accurate computational methods.

Goddard's group continued the theoretical study of the S_1 state of $OxyLH_2$ by performing multireference calculations.[53] They used the complete active space self-consistent-field (CASSCF) method[54] and the multiconfigurational complete active space second-order perturbation theory (CASPT2) in the in vacuo study of the structural and electronic properties of keto-(−1) and enol-(−1).[55,56] The CASSCF method can be used to predict accurate ground- and excited-states structures, and CASPT2 is used to include dynamic electron correlation corrections, which can be useful for obtaining reliable excitation and emission energies. These methods are considered more accurate and

reliable than the CIS and TD-DFT methods. The group confirmed Orlova et al. results by showing that planar keto-(−1) and enol-(−1) are minima on both the S_0 and the S_1 potential energy surfaces and that the twisted forms are transition states.[37] They also predicted emission energies in the range of 2.35−2.53 eV for keto-(−1) and 2.42−2.74 eV for enol-(−1). The strong oscillator strengths predicted are consistent with a strong S_1-S_0 vertical emission.

Nakatani et al.[57] used the symmetry adapted cluster[58]/symmetry adapted cluster−configuration interaction (SAC/SAC−CI)[59] method for the study of $OxyLH_2$.[60,61] This method can be used for balanced description of the electron correlation effects in both ground and excited states. One of the objectives delineated by this group was the study of the chemiluminescence of $OxyLH_2$ in DMSO, as described in refs 18,33, and 34. To simulate the DMSO environment, a polarized continuum model (PCM) was used, with the dielectric constant of 46.7.[62] Their calculation of the emission energies (Table 2) characterized the neutral species as blue emitter, excluding them from the candidates for the emitters. The emission energy of keto-(−1) agreed well with red emission, while the anionic enol forms all emit in the yellow-green region. However, enol-(−2) was predicted to be more stable by 6.6−8.3 kcal/mol than the other species, characterizing it as the yellow-green emitter. It should be noted that the SAC−CI method was used in single point calculations, while the optimizations were performed with the CIS method. This emphasized the reliability of this method in geometry optimizations calculations.

The recent use of more accurate ab initio methods led to the abandonment of TD-DFT methods in $OxyLH_2$ research.[43,53,57] These methods were being criticized for its predictions on $OxyLH_2$ due to possible charge-transfer (CT) states, for which TD-DFT shows large errors.[53,61] However, Li et al. showed that CT was large for the twisted forms of $OxyLH_2$ but small for the planar ones, devaluing this flaw of TD-DFT methods in firefly bioluminescence research.[63] This group also tried to study the pH-dependent fluorescence spectra of $OxyLH_2$ in aqueous solution, by means of TD-B3LYP/6-31+G(d). Solvent effects were treated with the PCM model, with parameters set for water. Their results (Table 2) attributed the blue fluorescence peak (450 nm, 8 > pH > 3) to the neutral keto and enolic species and the yellow-green (560 nm, pH > 9) to enol-(−1). To explain the red peak (620 nm, pH < 3), a new species keto-(+1) was considered.

MICROSOLVATION STUDIES OF MULTICOLOR BIOLUMINESCENCE

The studies presented so far provided valuable information regarding $OxyLH_2$ structural and electronic properties and gave us some insights about its chemiluminescence. However, these studies did not take into account the microenvironment of Luc active site, and some of these studies do not take into account solvent effects of any kind. Due to the complexity of a protein active site and the various interactions that its molecules can make with the protein substrate, it is not likely that the effect of Luc on the emission can be disregarded or minimized. Therefore, these studies are limited to chemiluminescence and some indirect information of the firefly light-emission phenomenon.

Some authors have begun to address this problem by creating more complex simulations, which are focused on some aspects of Luc microenvironment. However, as the size of Luc−$OxyLH_2$ is incompatible with the most accurate quantum mechanics methods, more simplified models are still used and can provide important information. Liu et al. studied the effect of the polarization of the microenvironment on the emission energies by connecting a H_2O or a CH_2Cl_2 molecule to keto to simulate solvents of different polarity, at the B3LYP/6-31+G(d,p) level.[43] The in vacuo TD-B3LYP/6-31+G(d,p) calculated excitation energies (Table 1) decreased on the order of keto, keto-CH_2Cl_2, keto-H_2O, and keto-(−1), which in their model above corresponded to an increase in the polarization of the microenvironment. This is consistent with hypothesis (c) described above. However, it should be noted that an accurate study of the effect of the polarization of the microenvironment should be made including the crucial solvent effects, which are disregarded in this study. According to Ren and Goddard results, the inclusion of solvent effects could provoke a large blue shift of keto-(−1), when comparing with the in vacuo results, and a small red shift of keto and keto-X complexes.[51] These possible shifts could suffice for altering the excitation energies ordering presented by Liu et al..[43] Moreover in our opinion, the addition of these molecules to keto simulates the interactions between this species with molecules present in the Luc active site rather than simulating different solvents. This indicates that the addiction of CH_2Cl_2 or H_2O to keto-(−1) could provoke different effects on its emission energies than caused in the case of keto and so change this descending order. This group also demonstrated that keto-(−1), which had excitation energies (2.54 eV) closer to experiment, has a flat potential energy surface which allows an easy shifting of the minimum between different resonance structures by means of CASSCF geometry optimizations and multistate CASPT2 single point calculations. They also showed that the relaxation of keto-(−1) on S_1 can change significantly the emission energies. In conclusion, they stated that the hypotheses (c−e) are all plausible.

Min et al. tried to study the role of the resonance structure of keto-(−1) on the multicolor bioluminescence by performing a TD-DFT investigation on the origin of the red chemiluminescence.[64] They connected a sodium or an ammonium cation to the benzothiazole or the thiazolone oxygen of keto-(−1) and studied their absorption and emission energies with the B3LYP, B3PW91, and PBE1KCIS functionals and the 6-31+G(d) basis set.[39,65,66] The PCM model and the conductor-like screening model (COSMOS) were used to simulate an aqueous environment.[67] They demonstrated that the interactions of keto-(−1) with the two cations caused similar blue shifts, while connected to the benzothiazole oxygen, and similar red shifts when interacting with the thiazolone moiety. These opposite effects caused by different interactions between the same molecules emphasizes the importance of the various interactions that can be formed in the complex active site microenvironment between the light emitter and the molecules present in Luc active sites. In this work it was considered a novel emitter, keto-(−1)-H. This new species could be of some importance in the bioluminescence phenomenon due to its emission wavelength (624.1−650.7 nm), which is close to the experimental value of 620 nm at acid pH.[1]

In order to assess the role of the rigidity of Luc active site on light emission, as described by Nakatsu et al.,[25] Li et al. focused on the study of the excited-state geometry of keto-(−1).[68] According to this group, the emitter, following changes in the electronic structure from a initial excited state on the potential energy surface, usually relaxes to a energy minimum and emits a photon while returning to the ground state. The different

conformations of Luc could affect the energy values of these points in the potential energy surface, modulating the color of bioluminescence. Their B3LYP/6-31+G(d) and TD-B3LYP/6-31+G(d) calculations demonstrated that changes in six bond lengths of keto-(−1) excited-state geometry can determine the emission spectra. The more the bond lengths changes are impeded, the more the emission energies increase. It should be noted, however, that this study was performed without imposing any constraints on these geometry changes. Due to the high complexity of interactions between the molecules present in protein active site and to the steric constraints imposed by the active site to the substrate caused by the proximity to active site molecules, it is expected that Luc has a great influence on bond lengths changes of $OxyLH_2$ and is unlikely that the emitter has the necessary flexibility to suffer so many significant changes in its geometry.

Cai et al. calculated the excitation energies of keto-(1) in response to different electrostatic fields computed using TD-DFT functionals.[84] They found the existence of a correlation between the wavelength shift with the projection of the electrostatic field on the molecular plane and the intensity of fluorescence can be affected by field modulation. However, neither the study of the effect of polarity in these electrostatic fields nor the integration of this simple model in the in vitro pH-dependent color variation were performed. Also, in this paper the use of the local density approximation (LDA) was validated, a much less computational demanding functional than B3LYP which is used in numerous optical calculations.[85,86]

INTEGRATION OF LUC ENVIRONMENT IN BIOLUMINESCENCE RESEARCH

Apart from these simplified models, the latest tendency in the theoretical bioluminescence research is the incorporation of extended portions of the active site in the calculations. Despite the valuable information that can be derived from more simplistic simulations, these studies ignore the steric and electrostatic contributions from Luc active site. Thus, some groups have recently begun to study the active site contributions to the light-emitting reaction. The first study in this field was made by Nakatani et al.[57] They performed quantum (QM) and molecular mechanical (MM) calculations, based on the X-ray structure of Luc and some working models derived from experimental studies, to determine the structure of the excited-state Luc−$OxyLH_2$ complex.[41,42,69] This group stated that the Luc environment shifted the emission energy of keto-(−1) to the green region (2.08−2.33 eV) and that Arg218, His245 (Luc numbering), and the phosphate group of AMP gave dominant contributions to this effect. It was also stated that the anionic enol species have emission energies close to experiment, but the keto−enol tautomerism is energetically unfavorable in Luc environment. The resonance-based mechanism was dismissed by these authors, as they did not find significant changes in the resonance structure in the excited state. However, some caution is needed in the analysis of this conclusion. The authors reached to this dismissal by comparing the bond lengths of $OxyLH_2$ in the gas phase and in their model. However, by Nakatsu et al. findings, Luc can adopt either a tight and hydrophobic active site or a loose and polar one.[25] Thus, these differences in Luc internal microenvironment can suffice for changes in the resonance structure of $OxyLH_2$. This group continued their studies in bioluminescence by performing in silico mutagenesis SAC−CI experiments.[70] By analyzing the contributions of several amino acid residues to the emission color tuning, they demonstrated the blue-shift effect of Arg223, Glu344, and Asp422. The replacement of these amino acid residues by an alanine caused a red-shift, thus predicting potential targets for future mutagenesis studies.

Tagami et al. performed a similar study on LcLuc bioluminescence.[71] They employed the multilayer fragment molecular orbital method in combination with CIS(D) calculations[72,73] in the study of the emission energies resulting from the interaction between $OxyLH_2$ and the wild-type and mutant crystal structures determined by Nakatsu et al.[25] The experimental results were not well reproduced but were improved by the use of the whole structure of the enzymes in the calculations. These results further emphasize the importance of Luc contribution for the color tuning mechanism.

Another QM/MM investigation was conducted by Navizet et al., which was based on the open and closed conformation of LcLuc.[74] In this work, the authors create several models of the open and closed conformations of LcLuc structure. The main differences between the models were the number of the water molecules present in the active site and the performance of extensive molecular dynamics simulation on one of the models. This work has special importance on the multicolor bioluminescence research, as it is the only one to take into to account the different conformations of Luc active site in more complex calculations. Their CASPT2/CASSCF calculations on keto-(−1) demonstrated that the polarization of the microenvironment of the benzothiazole moiety have a crucial effect on the light emission. Moreover, the results obtained were in disagreement with the experimental conclusion that the rigidity of the active site controls the bioluminescence color and reduces the role of Luc different conformations on light emission to the modulation of the polarity of the microenvironment. It should be noted that these pivotal conclusions were achieved with a noteworthy reproduction of the spectral parameters of light production.

Three of the latest computational studies on firefly bioluminescence also focus on the contribution of the active site molecules to the multicolor variation. Milne et al. use the fragment molecular orbital method to study the effect of some amino acid residues, water molecules, and AMP on the excitation energies of some $OxyLH_2$ species.[75] This study assumes some importance as it is the only one to perform a systematic analyses of the effect of an extended portion of the active site on more $OxyLH_2$ species than keto-(−1). Moreover, it introduces a sense of pH variation on the simulation by considering different protonation states for AMP. Based on their calculations, the group proposed that keto-(−1) is the yellow-green and red emitter. However, this conclusion was reached by a somewhat confusing comparison between their calculated excitation energies and the experimental emission energies. Furthermore, the calculations were based on the assumption that AMP has a pK_a value of 6.23, which due to the variable internal environments of enzymatic active site may not be true in the case of Luc.[76]

Min et al. continued their TD-DFT-based studies on firefly bioluminescence, by constructing a complex between keto-(−1), AMP, and some other important active site molecules in order to simulate Luc contribution to the yellow-green bioluminescence.[77] This study gains importance relative to others here described, as is the only to include implicit solvent effects by means of the COSMO model with parameters set for water, in their TD-(B3LYP, B3PW91, PBE1KCIS)/6-31+G(d) calculations. The results obtained indicate that keto-(−1) is the yellow-green

emitter, which is consistent with Nakatani et al. and Milne et al.[57,75] However, some doubt is shed on these results by the use of water to simulate the environment of yellow-green emission. It is unlikely that the polar environment of an aqueous solution could be used in a reliable simulation of a hydrophobic active site, as predicted by Nakatsu et al.[25] Moreover, in the literature is described that for active site simulations a dielectric constant of 4 gives good agreement with experiment, which has large differences relative to the dielectric constant of water (∼78).[78−81] Therefore, it is reasonable to speculate if the emission energies of keto-(−1)-Luc would suffer an undesirable shift, when used with a proper dielectric constant, or if the polarity of the different conformations of Luc do not have any significant impact on bioluminescence.

Mao studied the sequence-induced color variation of Luc by performing dynamics simulations.[82] An elastic network model was used with a coarse-grained representation of protein and a harmonic potential describing the interactions of its components.[83] This work permitted the identification of several hotspot residues that are coupled to the active site and identified the B subdomain as being mainly responsible for the multicolor variation. However, this study was based on certain assumptions that clash with previous knowledge regarding firefly bioluminescence. First, the Luc structures considered for this work were unbound Luc as the open conformation and LcLuc bound to AMP and $OxyLH_2$ as the closed conformation, while most studies considered LcLuc bound to DLSA as the closed conformation and LcLuc bound to AMP and $OxyLH_2$ as the open conformations.[25,69] Moreover, the hotspots were identified by measuring changes in the mean-square fluctuations of the amino acid residues, a measure that in the opinion of the author may indicate the impact of the residues in the function of the protein. However, as the closed conformation here employed is very similar to the structure of LcLuc bound to ATP in the beginning of the bioluminescence reaction and no excitation and/or emission energies calculations were performed, it is not evident that the function modulated by these residues is the emission of light or the adenylation step that initiates this reaction.[25]

FUTURE PERSPECTIVES ON COMPUTATIONAL STUDIES OF BIOLUMINESCENCE

Computational calculations have been a powerful tool in the research of the $OxyLH_2$ molecule. The studies performed to date have given us valuable insight on the species structural and electronic properties and its role on chemiluminescence in solvents like water and DMSO, without the need for more stable analogues.

The use of a computational approach in the study of firefly bioluminescence has also been recurrent but with less conclusive results. The use of computational techniques emphasized the importance of Luc in the light-emitting phenomenon and has provided qualitative analyses for the contribution of some key amino acid residues, which could be useful in mutagenesis studies. However, there is still some controversy and lack of substantiated information on other aspects of the bioluminescence phenomenon.

For example, the latest theoretical studies are considering keto-(−1) as the yellow-green emitter. However most authors, based on Branchini et al. results, already assume that keto-(−1) is the most probable emitter and do not study the enolic species in their simulations.[19] It should be noted that experiment made with the keto analogue only demonstrated that the keto species could produce yellow-green bioluminescence, and no experimental evidence excluded the enolic species from bioluminescence. However, some clarification to this topic may be provided by two very recent papers. Navizet et al. performed an analysis on six $OxyLH_2$ chemical forms (keto, enol, and the respective anions) using a multireference method.[87] Their MS-CASPT2 calculations in vacuo and in DMSO excluded keto, enol, and enol-(−1′) as possible light emitters, while MS-CASPT2/MM calculations on the remaining species indicated that keto-(−1) is the direct excited-state product of firefly dioxetanone and the sole light emitter. Also, our own group studied the effect of pH on the chemical equilibrium of $OxyLH_2$ (keto-(−1)-H, keto, enol, and respective anions) in the open and closed conformations of the Luc active site.[88] To this end, we simulated solvent effects by using the CPCM model with two different dielectric constants (4 and 78). Our TD-PBE0[89] calculations indicated that keto-(−1) is the sole species present in both conformations at the pH range of interest.

Furthermore, the majority of the studies do not take into account changes in the polarity of the active site, as described by Nakatsu et al.,[25] when they are studying the red-shift of the color of emitted light. Moreover, most of the studies described here do not take into account solvent effects of any kind, focusing instead on in vacuo calculations. This could lead to erroneous results as Ren and Goddard and Min et al. described the significant differences in the emission energies that can arise from when we compare results obtained in the presence or absence of solvent.[51,64] This leads to the questions: if solvent effects are considered, then will keto-(−1) emission energies still agree well with experiment? If not, then which is the real yellow-green emitter? If yes, then does the solvent polarity affect the bioluminescence color? To what extent does the polarity of the microenvironment affect firefly bioluminescence?

There is also some lack of knowledge regarding the active site of Luc that can prevent obtaining reliable results from the more complex models. For example, there has been some discrepancy in the protonation state of a histidine residue located near the thiazolone moiety of the emitter. The variation of the protonation state of AMP was considered pivotal in the red shift, but no study was made in order to validate this variation. Moreover no study considered, at least explicitly, a proton acceptor for the C_4 proton of LH_2.[1] This could be of great importance, as it could generate an unexpected protonation state and cause a rearrangement of the internal interaction of the active site.

Computational studies have been undeniably fundamental in building our knowledge of the firefly multicolor bioluminescence. Thus, it could be of pivotal importance in future lines of research, more specifically, on the study of contributions of all $OxyLH_2$ species to the color tuning mechanism, the study on Luc microenvironment contribution with solvent effects, the correct characterization of Luc active site, and the study of $OxyLH_2$ formation.

AUTHOR INFORMATION

Corresponding Author
*E-mail: jcsilva@fc.up.pt.

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