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Efficient Firefly Chemi/Bioluminescence: Evidence for Chemiexcitation Resulting from the Decomposition of a Neutral Firefly Dioxetanone Molecule

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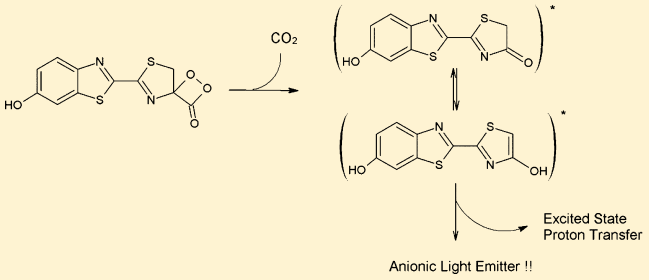
Supporting Information

ABSTRACT: Both experimental and theoretical methodologies were employed in order to study the possibility of excited state oxyluciferin being formed as the result of the decomposition of a neutral dioxetanone. Excitation measurements in water (at different pH values) and in methanol, along with computational calculations, demonstrated that the hydroxyl-benzothiazole group of firefly dioxetanone and six oxyluciferin analogues is only deprotonated in conditions not in line with the firefly bioluminescence reaction. Thus, a new mechanism involving a neutral firefly dioxetanone must be presented in order to explain the chemiexcitation of oxyluciferin. It was also studied for the first time the interaction between a molecule involved in the bioluminescence reaction (neutral firefly dioxetanone) and the real second conformation of firefly luciferase.



INTRODUCTION

Chemiluminescence is the emission of energy with limited emission of heat, as the result of a chemical reaction.[1] When this phenomenon occurs in a living organism, due to enzyme-catalyzed reaction, it is termed bioluminescence.[2−7] Both phenomena are gaining numeral practical applications in the fields of pharmaceutical, biomedical, and bioanalytical analysis, among others.[8−12]

The most important chemi/bioluminescence system is that of the fireflies, due to very high quantum yields (∼40−60%).[13,14] Firefly luciferase catalyzes a two-step reaction: first, D-luciferin (D-LH_2) reacts with adenosine-5′-triphosphate-Mg^{2+} (ATP-Mg^{2+}), leading to the generation of an adenylyl intermediate (D-LH_2-AMP); in the second step, D-LH_2-AMP is oxidized in the presence of molecular oxygen (O_2), which results in the formation of oxyluciferin ($OxyLH_2$), adenosine-5′-monophosphate (AMP), and carbon dioxide (CO_2).[2−7,15,16] $OxyLH_2$ is thought to be formed in its anionic keto-form, in a singlet excited state, decaying to the ground state with emission of visible light[17−19] (Scheme 1).

It was demonstrated that $OxyLH_2$ is produced in a singlet excited state due to the formation and subsequent decomposition of firefly dioxetanone (FiDiox)[1−7] (Scheme 1). The efficient chemiexcitation is then thought to occur due to crossing points between the singlet ground and excited potential energy surfaces on the reaction coordinate.[20,21] The efficiency of bioluminescence is then described in terms of quantum yield, which is controlled by the efficiency of the chemical reaction, the efficiency of crossing to the excited state, and the efficiency of the fluorescence of the excited state product.[1−7]

FiDiox is supposed to have a deprotonated benzothiazole hydroxyl group, both because $OxyLH_2$ is formed in an anionic form and due to energetic reasons. The deprotonation of this hydroxyl group is expected to lower the energy barrier of FiDiox decomposition to values more appropriated to a favorable reaction.[3,20−23] Also, this deprotonated electron-donating hydroxyl-benzothiazole moiety is expected to explain the efficient formation of singlet excited state products. Several authors have proposed mechanisms in which the deprotonation of the hydroxyl-benzothiazole moiety trigger charge transfer phenomena, which are responsible for the efficient singlet excitation.[3,20,23−25]

Very recently we have studied the binding of D-LH_2-AMP and two other adenylates to *Luciola cruciata* luciferase.[26] Our calculations have indicated that the hydroxyl-benzothiazole group of these adenylates is indeed protonated in the active site of this enzyme. Also, there is experimental data that indicates that, in the ground state, the hydroxyl-benzothiazole group of D-LH_2 and $OxyLH_2$ is only deprotonated in water (dielectric constant of 78) at basic pH (pH ≈ 7−10) and not in less polar solvents or at acidic pH.[27−31]

Given that the active site of an enzyme (luciferase included) is characterized by a dielectric constant of 2.5−4, the bioluminescence reaction can occur in a pH range of ∼5−

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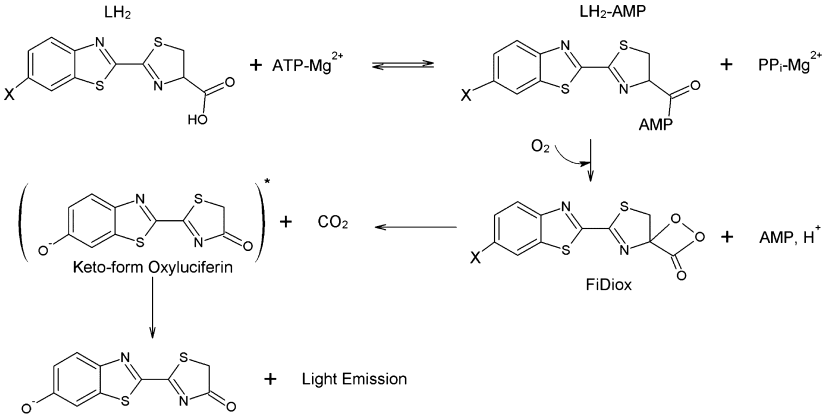
Scheme 1. Schematic Representation of the Firefly Bioluminescence Reaction; X Can Either Represent a Protonated or Deprotonated −OH Group



8,[2,15,32] and the hydroxyl-benzothiazole group of ground state D-LH_2, D-LH_2-AMP, and $OxyLH_2$ appear to be protonated in these conditions,[26−31] it is strange that the deprotonation of the same group in FiDiox can occur in the same conditions. It should be noted that the computational studies, in which was stated that the deprotonation of the hydroxyl-group was needed for an efficient bioluminescence, have only studied FiDiox outside of the enzymatic microenvironment.[20−23] Thus, they have ignored the geometric restraints imposed by the enzyme, and all types of intermolecular interactions that can occur between FiDiox and active site molecules. Moreover, we can not forget that an enzyme is a biological catalyst, so its job is to increase the rate of the chemical reactions that it catalyzes. So, any claim of the necessity of a deprotonated hydroxyl-group for an efficient firefly bioluminescence unsupported by studies inside the enzymatic environment is not a reliable one. Moreover, we cannot forget that it was computationally demonstrated that, during the decomposition of neutral FiDiox, there is a path for singlet excitation.[20]

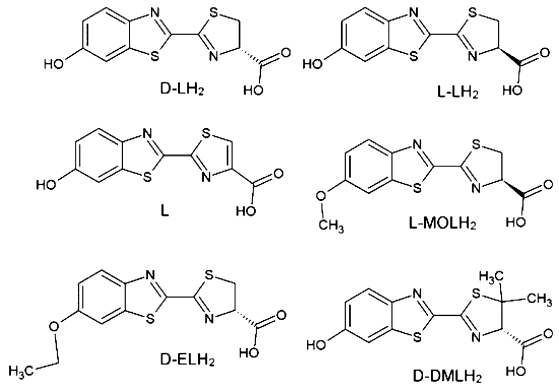
Even the evidence that excited state $OxyLH_2$ is produced in its anionic form[17−19] is not a determinant factor for the necessity of a deprotonated hydroxyl-group. More recently, it has being demonstrated that $OxyLH_2$ and analogues are very strong photoacids.[30,31,33−37] Thus, $OxyLH_2$ can be chemiexcited from a neutral FiDiox and then undergo an excited state proton transfer (ESPT) process with active site molecules, in order to lose a proton and form an excited state anionic species.

Thus, and taking all these evidence into consideration, the objective of this work is to consider the possibility of the efficient firefly bioluminescence being a result of the decomposition of a neutral FiDiox. First, we will study the excitation spectra of six $OxyLH_2$ analogues, in various conditions of polarity and pH, in order to demonstrate that the hydroxyl-benzothiazole group cannot be deprotonated in the ground state, in conditions similar to that found in the bioluminescence reaction. Also, we will computationally verify if the deprotonation of the hydroxyl-benzothiazole group of FiDiox is similar to that experimentally verified for some of the six $OxyLH_2$ analogues studied here. Finally, we will be the first to study the interaction between neutral FiDiox and the active site of the newly found second catalytic conformation of *Photinus pyralis* luciferase.[41]

MATERIAL AND METHODS

Materials. The $OxyLH_2$ analogues studied in this article were D-LH_2, L-LH_2, dehydroLH_2 (L), L-6′methoxiLH_2 (L-$MOLH_2$), D-6′ethylLH_2 (D-ELH_2), and D-5,5-dimethylLH_2 (D-$DMLH_2$) (Chart 1). D-LH_2 and D-ELH_2 were purchased from

Chart 1. Schematic Representation of the Six $OxyLH_2$ Analogues



Sigma, as well as HEPES (4-(-2-hydroxyethyl)-1-piperazineethanesulfonic acid) and MES (2-(*N*-morpholino)-ethanesulfonic acid). HEPES buffer was used for basic pH, while MES was used for acid pH values. The pH values were adjusted by the addition of NaOH and HCl. In pH values below 3, the buffer effect was provided by the addition of the strong acid HCl. L, L-LH_2, L-$MOLH_2$, and D-$DMLH_2$ were synthesized and purified as described previously and were stocked in aqueous solutions.[38−42] Isocratic grade methanol was purchased from Merck. Sample used in absorption measurements that contained methanol, contained also 2−10% of water, due to the fact that the fluorophores were already dissolved in this solvent. The samples were prepared in deoxygenized solvents, in an inert atmosphere, in order to ensure that the fluorophores were stable.

Excitation Spectroscopy. Excitation spectra were recorded with a Horiba Jovin Yvon Fluoromax 4 TCSPC with an integration time of 0.1 s, using slit widths of 5 nm for excitation monochromators. The emission wavelengths used, in pH range 0.68−10.0 and water and methanol, were 530 nm for D-LH_2, L-LH_2, and D-$DMLH_2$, 550 nm for L, and 440 nm for L-$MOLH_2$ and D-ELH_2. Quartz cells, with a volume of 800 μL,

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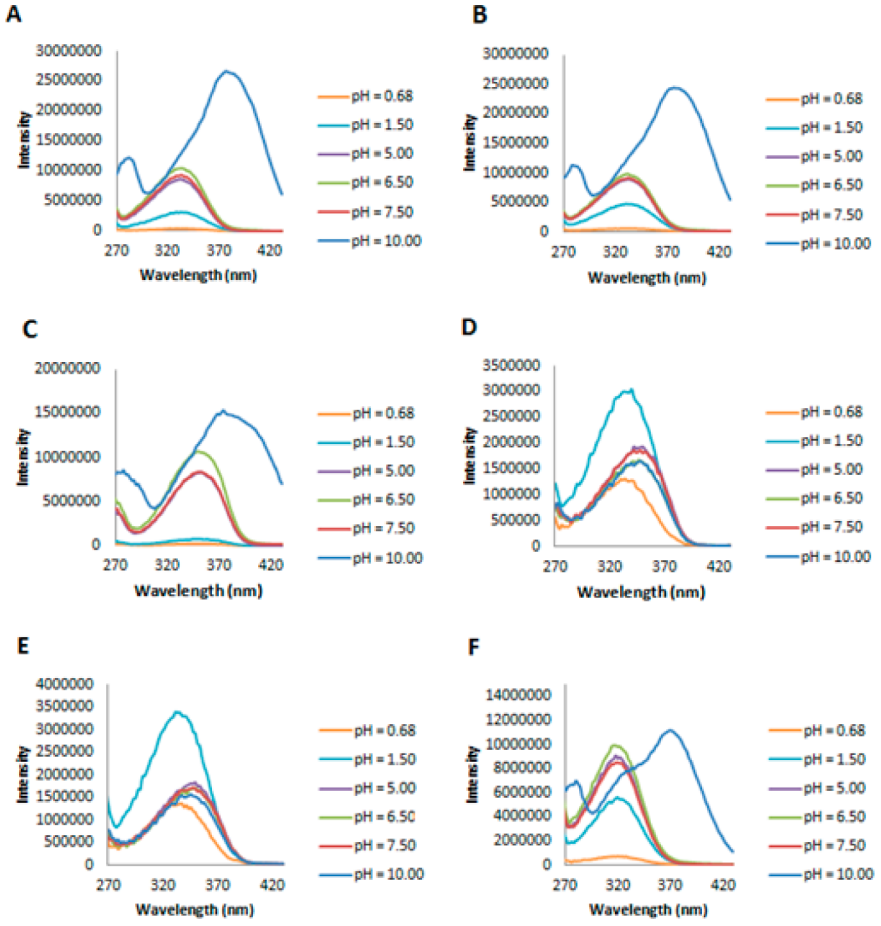

Figure 1. Excitation spectra of D-LH_2 (A), L-LH_2 (B), L (C), L-$MOLH_2$ (D), D-ELH_2 (E), and D-$DMLH_2$ (F), in various pH values.

were used. The fluorophores were studied at the concentration of 10 μM.

Computational Methods. Ground state geometry optimizations and frequency calculations were performed at the mPWKCIS/6-31G(d,p) level of theory, while the energies were recalculated at the mPWKCIS/6-31+G(d,p) level of theory.[44] The geometry optimizations were performed in vacuo. Some energies were recalculated with implicit solvent effects, by using the conductor-like polarized continuum model (CPCM) and the UAKS radii.[45]

We have used the mPWKCIS functional in geometry optimizations calculations due to good results in the optimizations of other dioxetanones.[46−48] The 6-31G(d,p) basis set was used in geometry optimizations due to its previous use in the geometry optimization of anionic and neutral FiDiox.[20]

The PDB structure 4G37 (*Photinus pyralis* luciferase trapped in the second catalytic conformation) was used as a starting structure in the study of the interaction of neutral FiDiox with luciferase.[43] Hydrogen atoms were added to the 4G37 structure by the MolProbity web server.[49] From the resulting structures were withdrawn the ligand DLSA and active site molecules Ala348, Arg218, Asp422, Gln448, Gly/341/339/316/315, His245, Lys443, Phe247, Ser 314/347, Thr343, Tyr340, and Wat772. These molecules were chosen due to their proximity to FiDiox and to their presence on previous studies of firefly bioluminescence.[19,26,43,50−57] The ligand DLSA was transformed in neutral FiDiox and AMP with the aid of the Avogadro software.[58] An initial guess for the geometry of this complex was obtained with the geometry optimization function of Avogadro.[58] Further geometry calculations were obtained with our own N-layered integrated molecular orbital and molecular mechanics (ONIOM) method.[59] Neutral FiDiox was included in the high layer, in which was used the mPWKCIS/6-31G(d,p) level of theory, while AMP and the remaining active site molecules were included in the low layer (in which the Dreiding force field was used).[60]

All the theoretical calculations were performed with the Gaussian03 software package.[61]

RESULTS AND DISCUSSION

Effect of the pH on the Excitation Measurements. Figure 1 and Table S1, Supporting Information, refer to the absorption of OxyLH_2 analogues at various values of pH.

Analyses of the excitation spectra (and Table S1, Supporting Information) demonstrate that L, L-$MOLH_2$, and D-ELH_2 present the longer wavelength maxima, while D-$DMLH_2$ presents the lower (pH 0.68−7.50). However, at pH 10.0, the excitation maxima of L, D-$DMLH_2$, D-LH_2, and L-LH_2 suffer from an accentuated red-shift. This phenomenon is expected to be caused by deprotonation of the benzothiazole oxygen, which is supported by the fact that the two molecules that do not present this red-shift cannot undergo this deprotonation (L-$MOLH_2$ and D-ELH_2, Chart 1). Naumov and co-worker have already demonstrated the importance of deprotonated benzothiazole oxygen for a more red-shift emission and excitation.[28] These results are in line with previous experimental studies of D-LH_2 and L.[29−31,33−36] These data

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clearly indicate that, even in a polar environment as water, the hydroxyl-benzothiazole group of this type of molecule is not easily deprotonated in the ground state. In fact, only at basic pH (pH of 10) it is seen a red-shift associated with that deprotonation.[29−31,33−36] This was also seen for oxyluciferin.[30] As we know that the bioluminescence reaction also occurs at acidic pH (pH ≈ 5−6),[2,15] it appears to be very difficult to believe that an anionic FiDiox molecule is involved in the chemiexcitation of $OxyLH_2$. As for the higher excitation intensity of the deprotonated form, when in comparison with the protonated form, Naumov and co-worker have shown that, for an oxyluciferin derivative, the absorbance is higher with higher pH and wavelength.[28] Thus, a higher absorbance of the deprotonated form, could explain the higher excitation intensities.

Considering previous and present results,[29−31,33−36] these LH_2 molecules should be an anionic species in the ground state during nearly all the pH range (the carboxylic group has a pK_a ≈ 3). In the ground state, in the pH range of ∼8−10, the hydroxyl-benzothiazole group is also deprotonated. Thus, these results indicate that the ground state deprotonation of the hydroxyl-benzothiazole group of FiDiox should only occur at basic pH. However, the bioluminescence reaction is known to occur at both acidic and basic pH.[2,15,30,31,33−37] Therefore, it appears that the deprotonation of the hydroxyl-benzothiazole group of FiDiox does not occur at the same conditions of the firefly bioluminescence reaction.

Effect of the Polarity on the Excitation Measurements. Figure 2 and Table S2, Supporting Information, refer

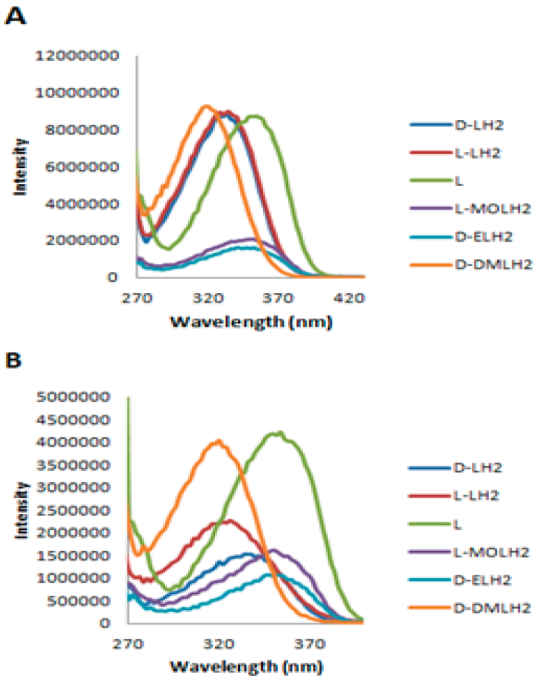

Figure 2. Excitation spectra in water (A) and methanol (B).

to the excitation of $OxyLH_2$ analogues in water and methanol. Despite the important information provided by the measurements in aqueous solutions at various pH values, the active site of firefly luciferase is expected to be much less polar than an aqueous solution.[32,50,52] Therefore, measurements in a less polar environment were needed.

Analyses of the excitation spectra (and Table S2, Supporting Information) demonstrate that L, L-$MOLH_2$, and D-ELH_2 present the longer wavelength maxima. Also, the wavelength maxima (Table S2, Supporting Information) indicate that the hydroxyl-benzothiazole group is protonated in two different solvents. Moreover, it can also be seen that the polarity of the solvent does not have a relevant effect in the excitation spectra of these analogues, as the wavelength maxima are basically the same in the two solvents.

The results here presented indicate that not even in environments of lower polarity it can be observed the deprotonation of the hydroxyl-benzothiazole group. This further indicates that it is more likely that the hydroxyl-benzothiazole group of FiDiox is protonated inside of the active site of firefly luciferase. This is in line with the protonated state of D-LH_2-AMP and two other adenylates inside of *Luciola cruciata* luciferase.[26] Moreover, it is also in line with the experimental data regarding LH_2 and $OxyLH_2$.[27−31] Finally, this is in line with the possibility advanced by Naumov and co-workers, which is that the light emitter of *Cypridina* luminescence is chemiexcited from a neutral dioxetanone.[62]

Computational Study of the Ground State Deprotonation of Neutral FiDiox. In order to see if the deprotonation of the hydroxyl-benzothiazole group of neutral FiDiox can be comparable to the deprotonation of the same group in the six $OxyLH_2$ derivatives referred above, we have optimized the geometry of neutral FiDiox, D-LH_2, L, and D-$DMLH_2$ at the mPWKCIS/6-31G(d,p) level of theory. A relaxed coordinate scan was made in order to see the energetics of the O−H bond elongation of the hydroxyl group of these four molecules. The energies were re-evaluated at the mPWKCIS/6-31+G(d,p) level of theory. These single-point calculations were performed in implicit water, methanol, and with a dielectric constant of 4 (Figure 3) in order to simulate the hydrophobic environment of the luciferase active site.

It can be seen that the behavior of the O−H bond elongation of neutral FiDiox is very similar to that presented by the other three molecules, in the three different implicit environments. Moreover, we can see that, with decreasing polarity, the higher the energy needed for O−H bond elongations. Thus, these calculations indicate that the hydroxyl-benzothiazole group of neutral FiDiox behaves similarly to the ones present in the $OxyLH_2$ derivatives, which indicates that the probability of being an anionic FiDiox molecule to chemiexcited $OxyLH_2$ is apparently very low.

Interaction of Neutral FiDiox with *Photinus pyralis* Luciferase. Rather recently it was demonstrated by crystallography experiments, that firefly luciferase can adopt two very different conformations for the adenylation and oxidative steps, due to ∼140 °C terminal domain rotation.[43] This discovery clashes with previous assumptions that the conformation of the active site, during the two steps of the bioluminescence reaction, was very similar.[19,26,50−57] Therefore, despite numerous computational studies, apparently there is no study performed in the real conformation of the luciferase active site, at the oxidative step. In order to fill this gap in the existent literature, we have studied the interaction of FiDiox with active site molecules of this conformation and see the effect exerted by them in the geometric and charge parameters of FiDiox when compared with its in vacuo structure (Figure 4).

In Chart 2 are identified the different moieties analyzed in this subsection. In Table 1 are presented the atomic Mulliken charges of the $OxyLH_2$, CO_2, and Diox moieties, at the

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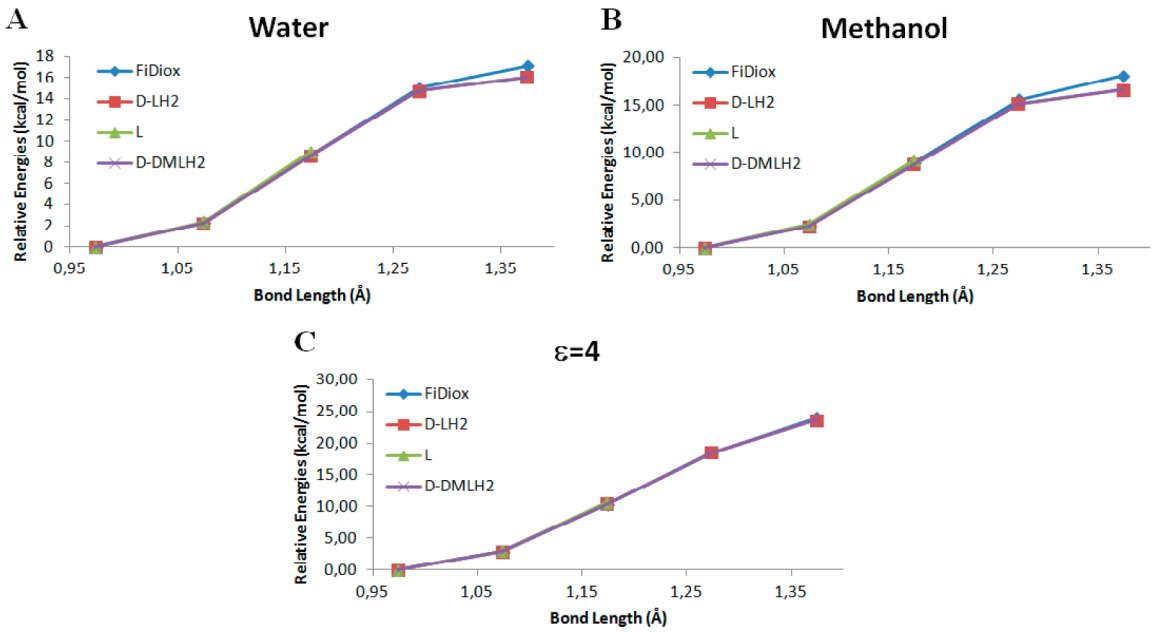

Figure 3. Relative energies of FiDiox, D-LH_2, L, and D-$DMLH_2$ at different hydroxyl O−H bond lengths (in Å) in implicit water (A), methanol (B), and with a dielectric constant (ε) of 4.

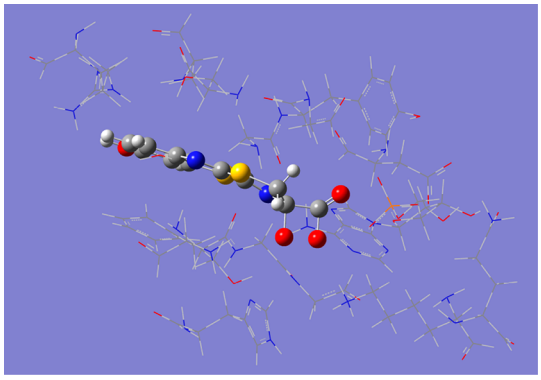

Figure 4. Neutral FiDiox interacting with active site molecules of the second conformation of *Photinus pyralis* luciferase.

Chart 2. Schematic Representation of Neutral FiDiox, and the Dioxetanone (Diox), $OxyLH_2$, and CO_2 Moieties

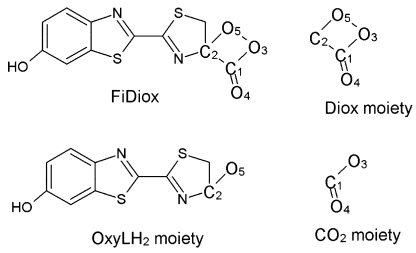

Table 1. Atomic Mulliken Charges of Diox, $OxyLH_2$, and CO_2 Moieties of in Vacuo FiDiox and Neutral FiDiox Optimized with Active Site Molecules (FiDiox-Luc)

	in vacuo FiDiox	FiDiox-Luc
Diox	−0.17	−0.27
$OxyLH_2$	0.05	0.04
CO_2	−0.05	−0.04

mPWKCIS/6-31+G(d,p) level of theory. These in vacuo single-point calculations only included the FiDiox molecules and not the active site molecules. It can be seen that the two FiDiox molecules present different charge distribution. The in vacuo $OxyLH_2$ moiety is positive by 0.05, while the CO_2 moiety is negative by −0.05. In the case of the FiDiox molecule, which resulted from the optimization calculation with active site molecules, the $OxyLH_2$ moiety is positive by 0.04, while the CO_2 moiety is negative by −0.04. In the case of the Diox moiety, it can be seen that it is negative in both cases, but more negative (by 0.10) in the case of the FiDiox that resulted from the geometry optimization with active site molecules.

In Table 2 are presented the atomic Mulliken charges of the atoms that constitute the Diox moiety. It can be seen that both

Table 2. Atomic Mulliken Charges of O_5, C_2, C_1, O_3, and O_4 of in Vacuo FiDiox and Neutral FiDiox Optimized with Active Site Molecules (FiDiox-Luc)

in vacuo FiDiox		FiDiox-Luc	
O_5	−0.13	O_5	−0.12
C_2	0.01	C_2	−0.11
C_1	0.40	C_1	0.41
O_3	−0.10	O_3	−0.10
O_4	−0.35	O_4	−0.35

O_5 and O_3 are negative atoms, with very similar charges, in both cases. The negative charge of O_4 is the same in both cases. In the case of C_1, there is a very small increase in its positive charge in the FiDiox that resulted from the geometry optimization with active site molecules, when compared with the in vacuo FiDiox. In the case of C_2, there is a large increase of negative charge when we compare the FiDiox that resulted from the geometry optimization with active site molecules with the in vacuo FiDiox.

In Table 3 are presented the geometric parameters of the Diox moiety. It can be seen that only negligible differences between the geometries of the Diox moieties of FiDiox. In

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Table 3. Bond Lengths (in Å) and Angles and Dihedral (in deg) of the Diox Moiety of in Vacuo FiDiox and Neutral FiDiox Optimized with Active Site Molecules (FiDiox-Luc)

	in vacuo FiDiox	FiDiox-Luc
O_5-O_3	1.53	1.53
C_2-C_1	1.54	1.54
C_2-O_5	1.48	1.48
C_1-O_3	1.38	1.38
$O_5-C_2-C_1$	86.38	86.26
$O_3-C_1-C_2$	93.23	93.25
$O_5-C_2-C_1-O_3$	−0.38	−0.53

Figure 5, there is a visual comparison between the in vacuo FiDiox and the FiDiox that resulted from the geometry optimization with active site molecules, in which we can see that the geometry $OxyLH_2$ moiety was affected by interaction with active site molecules.

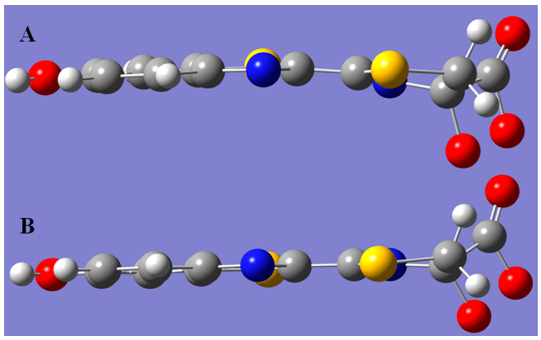

Figure 5. Structures of neutral FiDiox when optimized with active site molecules (A) and in vacuo FiDiox.

CONCLUSIONS

The possibility of $OxyLH_2$ being chemiexcited from the decomposition of a neutral FiDiox molecule was studied by means of both experimental and theoretical means.

Excitations measurements of six $OxyLH_2$ derivatives, in aqueous solutions of different pH values and in methanol, demonstrated that the hydroxyl-benzothiazole group is only deprotonated in water at basic pH. Theoretical calculations demonstrated that the hydroxyl-benzothiazole group of FiDiox has the same behavior as that of the same group in the above referred six $OxyLH_2$ analogues. The findings that this functional group is only deprotonated in polar environments ($\varepsilon \approx 78$) and at basic pH, while the bioluminescence reaction occurs in hydrophobic environments ($\varepsilon \approx 2.5-4$) and in a wide range of pH (~5−8), indicates that there is no possibility of existing an anionic FiDiox molecule in active site of firefly luciferase. Thus, our results indicate that chemiexcitation must occur from the decomposition of a neutral FiDiox molecule, as it is thought to occur in the case of *Cypridina* luminescence.[62] Anionic $OxyLH_2$ can then be formed by an ESPT process with an active site molecule, which can be expected due to numerous reports of the strong photoacidity of this type of molecules. These findings uncovered a brand new chemiexcitation mechanism for anionic $OxyLH_2$ formation.

In this work, it was also computationally studied for the first time the interaction between a molecule involved in the bioluminescence reaction, with the active site of the real conformation of luciferase for the oxidative step. Neutral FiDiox geometry was optimized with active site molecules of this conformation, and the effect exerted by these active site molecules on its geometric and charge density parameters was evaluated (by comparing the results with those obtained for in vacuo FiDiox).

Efforts are being made in order to study the decomposition reaction of neutral FiDiox in the active site of the real second conformation of firefly luciferase and to find a proton acceptor for the ESPT process between excited state $OxyLH_2$ and an active site molecule.

ASSOCIATED CONTENT

Supporting Information

Excitation wavelength maxima of the six $OxyLH_2$ derivatives and Cartesian coordinates of important molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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