A Theoretical Analysis of the Potential Role of π–π Stacking Interactions in the Photoprotolytic Cycle of Firefly Luciferin

ARTICLE *in* CHEMPHYSCHEM · AUGUST 2014

Impact Factor: 3.42 · DOI: 10.1002/cphc.201402558

2 AUTHORS:

Luís Pinto da Silva

University of Porto

55 PUBLICATIONS **448** CITATIONS

SEE PROFILE

Joaquim C G Esteves da Silva

University of Porto

302 PUBLICATIONS **2,936** CITATIONS

SEE PROFILE

ChemPubSoc Europe

DOI: 10.1002/cphc.201402558

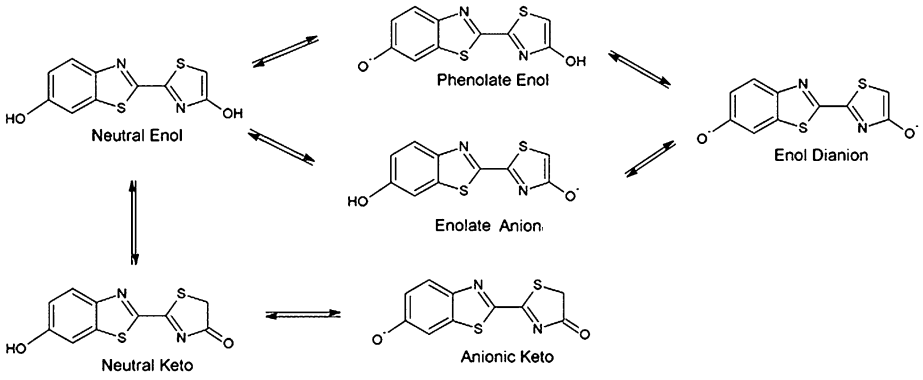
A Theoretical Analysis of the Potential Role of π–π Stacking Interactions in the Photoprotolytic Cycle of Firefly Luciferin

Luís Pinto da Silva and Joaquim C. G. Esteves da Silva*[a]

Firefly oxyluciferin is a photoacid that presents a pH-sensitive fluorescence, which results from pH-dependent changes on the conformation of self-aggregated π–π stacking complexes. Luciferin is a derivative of oxyluciferin with very similar fluorescence and photoacidic properties. This similarity indicates that luciferin is also expected to be able to form π–π stacking complexes, but no pH-sensitive fluorescence is found for this compound. Here, a theoretical approach is used to rationalize this finding. We have found that luciferin only forms π–π stacking complexes in the ground state at acidic pH. At basic pH and in the excited state, luciferin is present as a dianion. This species is not able to self-aggregate, owing to repulsive electrostatic interactions. Thus, this emissive species is not subject to π–π stacking interactions; this explains its pH-insensitive fluorescence.

1. Introduction

Proton transfer is a fundamental process with an important role in chemistry and biology.[1,2] Prime examples are keto–enol tautomerism, proton transport, and protein relay systems in membrane-spanning proteins and enzymes.[3,4] A special class of compounds involved in proton-transfer processes is represented by heterocyclic aromatic molecules.[5,6] These molecules are known to undergo significant changes in their acidity upon photoexcitation.[7] Molecules that increase their acidity are termed photoacids, whereas photobases increase their basicity. The excited-state proton transfer (ESPT) can be intramolecular if the acidic and basic moieties coexist in the same molecule, or intermolecular (e.g. ESPT to the solvent).[8]



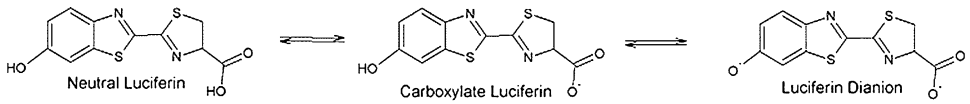
Scheme 1. Chemical equilibria of firefly oxyluciferin.

The firefly oxyluciferin (Scheme 1) family of fluorophores is a particular example of photoacids capable of ESPT to the solvent.[9] Up to now, the study of this family of photoacids has revealed many interesting, complex, and unexpected phenomena stemming from ESPT: efficient fluorescence quenching, photoacidity from an enol group, and photo-induced base-catalyzed keto–enol tautomerism.[9–12]

The most well-studied and well-known examples of this family are firefly oxyluciferin and luciferin (Scheme 1 and Scheme 2).[9,10] Both molecules are also key molecules in firefly bioluminescence. Bioluminescence can be characterized as light-emission occurring in a living organism, and is due to an enzyme-catalyzed chemical reaction.[13,14] In fireflies, this process is a two-step reaction catalyzed by the luciferase enzyme. In the first step, firefly luciferin reacts with adenosine-5′-triphosphate-Mg^{2+} (ATP-Mg^{2+}), which results in the formation of an adenylyl intermediate. The second step consists of the oxidation of this intermediate. The last step generates firefly oxyluciferin, which is formed directly in the singlet excited state.[15,16] Thus, this fluorophore is the bioluminescence emitter, decaying to the ground state with green to red emission (2.34 to 2.00 eV).

[a] Dr. L. Pinto da Silva, Prof. Dr. J. C. G. Esteves da Silva
Centro de Investigação em Química (CIQ-UP)
Departamento de Química e Bioquímica
Faculdade de Ciências, Universidade do Porto
R. Campo Alegre 697, 4169-007 Porto (Portugal)
E-mail: jcsilva@fc.up.pt

Supporting Information for this article is available on the WWW under http://dx.doi.org/10.1002/cphc.201402558.



Scheme 2. Chemical equilibria of firefly luciferin.

This system presents some useful characteristics, namely ATP-dependence and high quantum yield, which have triggered the development of numerous applications in the pharmaceutical, biomedical, and bioanalytical fields.[17–19] In order to optimize these applications of firefly bioluminescence and to develop new ones, it is necessary to fully understand this system. One of the research topics that attracts the most attention is the elucidation of the color tuning mechanism of firefly oxyluciferin.[20–24] This fluorophore is able to emit green to red light both in firefly bioluminescence and in solution.[20–24] Until now, no definitive consensus has been reached, but the available evidence indicates that the color is modulated by an electrostatic field generated by intermolecular interactions formed between oxyluciferin and nearby molecules.[25–32] Nevertheless, the complete elucidation of this topic is impaired by doubt regarding the identity of the light emitter. In solution and in the solid phase the enol species are predominant.[33,34] Some studies point out that in the bioluminescence reaction only the anionic keto species is needed to obtain the green to red light emission.[35,36] However, recent studies have provided evidence to support the formation of the oxyluciferin enolate anion, which results from ESPT from the neutral keto species to nearby adenosine-5′-monophosphate (AMP).[12,25] Thus, it appears that ESPT may play a key role in firefly bioluminescence.

One of the objectives of our group is to understand ESPT stemming from oxyluciferin by characterizing the photoprotolytic cycle of this molecule and its derivatives in aqueous solution.[9–11,25,37,38] Measurements in aqueous solution, under both acidic (pH≈4.5) and basic (pH≈9) conditions, revealed pH-sensitive fluorescence for oxyluciferin.[10] At acidic pH, the emission peaks at 2.25 eV, whereas at basic pH, the emission shifts to 2.30 eV.[10] Moreover, experimental measurements indicate that the fluorescence of oxyluciferin at acidic pH consists of both a fast decaying component and a slow one. In basic solution, the amplitude of the short-time component of the anionic species signal is less than 0.05, whereas in acidic solution its value is approximately 0.35.[10] The longtime-component lifetime is also different if the compound is excited at different pH.[10] The difference in lifetime and emission peaks indicates that the fluorescence at acidic and basic pH stems from two species. However, there is some evidence that indicates that the emission only stems from one species. Strong experimental findings demonstrate that only neutral and anionic enol species are present in aqueous solution.[33,34,39] More recently, Naumov and co-workers have proved that the light emitter at acidic pH is the enolate-anion species.[12] Thus, in order for two different fluorophores to exist in the photoprotolytic cycle of this photoacid, the phenolate or dianion enol would have to be the emitters at basic pH. However, in our work we only detected one pK_a value, whereas two would have been necessary to account for the two deprotonations needed to form the dianion enol.[10] The phenolate enol could have been a possible emitter at basic pH if the hydroxyl group was more acidic in the ground state than the enol group. However, our theoretical work has indicated that the enol group is more acidic than the hydroxyl group both in the ground and excited states.[40] Therefore, the pH-sensitive fluorescence should be explained considering only the enolate anion. In our opinion, this statement is supported by both experimental and theoretical results that indicate that the oxyluciferin fluorescence emitter is the enolate anion. Various studies have demonstrated that, at acidic pH, the fluorophore is the enolate anion due to photoexcitation of the neutral enol and subsequent ESPT.[10,12,33] At basic pH, the emitter should be formed after ground-state deprotonation of the neutral enol, which is followed by photoexcitation of the anion. Theoretical studies have indicated that this anion species is the enolate anion.[40]

More recently we have employed a computational approach in order to clarify the pH-sensitive fluorescence and photoprotolytic cycle of oxyluciferin in aqueous solution.[38] We have found that both the neutral enol and enolate anion forms of oxyluciferin are able to form π–π stacking complexes in the ground state. However, the complexes present different conformations due to the different charges of these two oxyluciferin species. Therefore, on photoexcitation, the resulting excited-state enolate-anion species will be formed in different π–π stacking arrangements, which depend on the pH. Even considering only one emitter, the enolate anion, the different conformations lead to a more red-shifted band at acidic/neutral pH than at basic pH. Therefore, we ascribed the pH-sensitive fluorescence to different geometries of π–π stacking complexes.[38]

Notably, this pH-sensitive fluorescence was not found for firefly luciferin.[10] As already mentioned, luciferin is a member of the oxyluciferin family of photoacids, with very similar photoacidic and fluorescence properties to those of oxyluciferin itself.[9,10,41] Nevertheless, the fluorescence of luciferin peaks at 2.34 eV, irrespective of the pH, and this corresponds to the luciferin dianion.[10,41,42] However, given its great structural similarity with oxyluciferin, luciferin can also be expected to be involved in the formation of π–π stacking complexes. So, this leads to the following question: is luciferin unable to form π–π stacking complexes, or are the π–π stacking interactions unable to modulate its fluorescence?

Thus, the objective of the present manuscript is to answer this question. We think that the elucidation of this topic is needed in order to understand the photoprotolytic cycle of these photoacids, and to gain a better grasp on the role of π–π stacking interactions in the fluorescence of aromatic molecules. To this end, we have used a computational approach to study firefly luciferin in aqueous solution. This approach is based on molecular dynamics (MD) simulations and quantum mechanical (QM) calculations. MD simulations allow us to

assess the possibility of the formation of π–π stacking complexes by taking into account the dynamics of the luciferin–water system. QM calculations can be used to obtain direct and detailed information (down to the atomic level) of the ground and excited-state luciferin species, which cannot be provided directly using experimental methodology.

2. Results and Discussion

The luciferin dianion species is known to be the sole fluorophore in water over the entire pH range (Scheme 2).[9,10,41,42] Nevertheless, this species is formed from two different pathways that depend on the pH. At acidic pH, the dominant ground-state species is the carboxylate luciferin (Scheme 2). Upon photoexcitation, this molecule undergoes ESPT with the solvent, and this generates the luciferin dianion. At basic pH, the dianion is already formed in the ground state prior to photoexcitation.[9,10,41,42] This situation is very similar to that found for oxyluciferin, in which we found neutral enol and enolate-anion species instead of carboxylate and dianionic anions.

In order to try to model the photoprotolytic cycle, we created two complexes. One was composed of three carboxylate luciferin ground-state species in a water box up to 12 Å, and the other was composed of three luciferin ground-state dianions in a similar water box. The first complex was built in order to obtain the conformation of luciferin at acidic pH prior to photoexcitation, whereas the second complex was expected to model luciferin at basic pH. Both complexes were subjected to 30 000 steps of energy minimizations prior to MD simulations of 1 ns.

The three luciferin molecules in each complex are presented in Figure 1 as obtained in the final frame of the MD simulation.

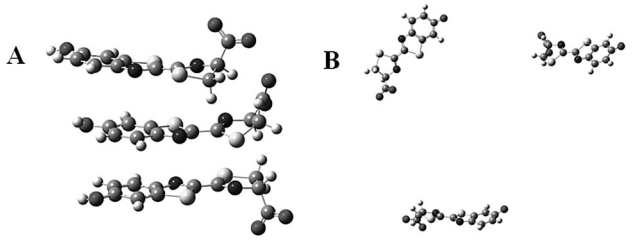


Figure 1. Ground-state A) carboxylate-anion and B) luciferin-dianion complexes at the final frame of MD simulations of 1 ns.

As shown in Figure 1, the carboxylate luciferin is capable of forming a sandwich-like π–π stacking complex, very similar to the one found for oxyluciferin (more specifically for the ground-state neutral enol).[38]

We then tried to assess the association energy of this π–π stacking complex by using Equation (1).

$$\Delta E_{\text{ass\_water}} = E_{\text{complex}} - (E_{\text{luciferin1}} + E_{\text{luciferin2}} + E_{\text{luciferin3}}) + \text{BSSE} + \Delta E_{\text{solv}} \quad (1)$$

where $\Delta E_{\text{ass water}}$ refers to the association energy of the complex in water, E_{complex} is the energy of the complex, and $E_{\text{luciferinx}}$ refers to the energy of each one of the luciferin molecules by itself. The basis set superposition error (BSSE) was obtained by the counterpoise method.[43,44] The BSSE arises from the overlap of the basis functions of the atoms of interacting molecules as they approach one another. The complex can thus be artificially stabilized as a monomer by utilizing extra basis functions from another monomer to describe its electron distribution. As the counterpoise method can only be used in vacuo, E_{complex} and $E_{\text{luciferinX}}$ were calculated in the gas phase. ΔE_{solv} is a term added to the equation in an attempt to add the solvent effects to $\Delta E_{\text{ass water}}$, and consists of Equation (2).

$$\Delta E_{\text{solv}} = \Delta E_{\text{complex}} - (\Delta E_{\text{luciferin1}} + \Delta E_{\text{luciferin2}} + \Delta E_{\text{luciferin3}}) \quad (2)$$

Each ΔE term consists of the difference between the energy of a structure in implicit water and that of the same structure in the gas phase. We also used Equation (3) to obtain this energy in the gas phase. The results are presented in Table 1.

$$\Delta E_{\text{ass\_vacuo}} = E_{\text{complex}} - (E_{\text{luciferin1}} + \Delta E_{\text{luciferin2}} + \Delta E_{\text{luciferin3}}) + \text{BSSE} \quad (3)$$

Table 1. Energy of association of the three oxyluciferin molecules in water and in the gas phase ($\Delta E_{\text{ass water}}$ and $\Delta E_{\text{ass vacuo}}$ in kcal mol^{-1}), the BSSE (in kcal mol^{-1}) and solvation energy (ΔE_{solv} in kcal mol^{-1}), calculated at the *w*B97XD/cc-pVDZ level of theory.

Water		Gas phase	
$\Delta E_{\text{ass water}}$	−26.4	$\Delta E_{\text{ass vacuo}}$	183.1
BSSE	90.0	BSSE	90.0
ΔE_{solv}	−209.5		

These results show that basis set superposition does indeed overly stabilize the π–π stacking complex by a significant 90 kcal mol^{-1}. It can also be seen that this complex is indeed stabilized in water by 26.4 kcal mol^{-1}, but is unstable in the gas phase. This large destabilization in vacuo is not unexpected. Each carboxylate luciferin is an anionic molecule, and this complex of three negative molecules should therefore generate significant repulsive electrostatic interactions. In water, stabilization is achieved due to solvent effects (ΔE_{solv} of −209.5 kcal mol^{-1}). This is in line with previous studies of π interactions, in which it was determined that solvents such as water maximize complementary electrostatics and minimize repulsive electrostatic interactions.[45]

Water also induces stabilization by further polarizing the luciferin molecules in the π–π stacking complex. In Table 2 the atomic Mulliken charges of each luciferin molecule (divided into the benzothiazole and carboxylic thiazole moieties) in the complex are presented, both in water and in the gas phase. It can be seen that in both media luciferin is a polar molecule with the negative charge density localized mainly on the carboxylic thiazole moiety. However, in the gas phase the benzo-

Table 2. Atomic Mulliken charges of the benzothiazole and carboxylic thiazole moieties of the ground-state luciferin complexes in water at the *w*B97XD/cc-pVDZ level of theory. The in vacuo values are given in parentheses.

Carboxylate luciferin	Benzothiazole	Carboxylic thiazole
luciferin1	−0.003 (−0.136)	−1.029 (−0.992)
luciferin2	−0.011 (−0.104)	−0.947 (−0.951)
luciferin3	−0.003 (−0.097)	−1.029 (−1.022)
Luciferin dianion	**Benzothiazole**	**Carboxylic thiazole**
luciferin1	−0.938 (−0.944)	−1.061 (−1.057)
luciferin2	−0.886 (−0.897)	−1.112 (−1.103)
luciferin3	−0.931 (−0.966)	−1.068 (−1.031)

thiazole moiety still presents negative Mulliken charges of about −0.112. In water, the negative charge on this moiety is only about −0.006. Thus, even if the molecule is already polarized in both media, significant polarization still occurs on solvation. The interaction energy of π–π stacking complexes contains primarily four components: electrostatic, induction, dispersion, and exchange repulsion.[46,47] Induction and dispersion forces are attractive interactions that result from a permanent multipole or instantaneous dipole, respectively, and an induced dipole. Thus, if water increases the dipole moment of the carboxylate luciferin, it is expected that the attraction generated by induction and dispersion interactions will increase.

We also calculated the vertical excitation energy of the carboxylate luciferin π–π stacking complex in implicit water (Table 3). The computed value was 3.80 eV, which is well in line with the experimental value of 3.76 eV.[10,41] We also calculated the vertical excitation energy of each carboxylate species in order to evaluate the effect that π–π stacking has on the excited state properties of luciferin. Only one luciferin molecule presented a vertical excitation identical to that presented by the complex. The other two molecules presented significantly blue-shifted excitation energies (by 0.22 and 0.36 eV). This means that besides stabilizing the complex in the ground state, π–π stacking also stabilizes the Franck–Condon state. Although all the isolated luciferin molecules present excitation energies close to the experimental value, it is the complex that agrees best with the experiment.

Table 3. Vertical excitation energies (E_{ex}) of the ground state of the luciferin carboxylate and dianion species at the TD *w*B97XD/cc-pVDZ level of theory.

Carboxylate luciferin	E_{ex} [eV]
complex	3.80
luciferin1	3.98
luciferin2	3.80
luciferin3	4.12
Dianion luciferin	**E_{ex} [eV]**
complex	3.14
luciferin1	3.10
luciferin2	3.14
luciferin3	3.33

In Figure 1 the three ground-state luciferin dianions are presented in a water box. This is the complex corresponding to basic pH. Contrary to the case of the carboxylate luciferin and of oxyluciferin, the luciferin dianion is clearly not capable of generating π–π stacking complexes.[38] In fact, the dianion species are separated by more than 11 Å. This inability of forming π–π stacking complexes can be attributed to the double negative charge possessed by each molecule, which should generate repulsive electrostatic interactions that are impossible to overcome. Moreover, as seen in Table 2, the deprotonation of the hydroxyl group significantly reduces the polarization of the luciferin species (thereby reducing the possibility of potential dipole–dipole interactions).

We also calculated the vertical excitation of the three luciferin molecules together, and also of each one by itself (Table 3). As expected, the calculated vertical excitation energies are very similar to each other because the luciferin molecules should only interact with each other by long-range repulsive electrostatic interactions. Moreover, the vertical excitation energy of the three luciferin molecules (3.14 eV) is in line with the experimental value of 3.35 eV.[9,10,41]

The results presented so far indicate that at acidic pH, ground-state luciferin is present as a π–π stacking complex composed of the carboxylate luciferin species. At basic pH, the complex is disrupted by further deprotonation of luciferin, which generates the dianion species. The formation of the dianion increases the repulsive electrostatic interaction, and causes depolarization of this species, which makes the formation of π–π stacking complex unfavorable.

The next step in this study was to obtain stable conformations of the excited-state luciferin dianion in aqueous solution at basic and acidic pH. To this end, we used the geometries of the three luciferin species at basic and acidic pH (carboxylate and dianion species, Figure 1), as the initial structures for further MD simulations of 1 ns. The parameterized atomic charges were those of the dianion species at the Franck–Condon state. The Franck–Condon state was chosen in order to better compare these results with those obtained for the study of the pH-sensitive fluorescence of oxyluciferin, in which the same approach was used.[38] Moreover, the absorption spectrum often reflects the main excited-state features, with a difference that occurs due to geometry relaxation.[48,49] The strategy of using the Franck–Condon state to obtain information regarding the emissive state is routinely used with good results in a variety of topics.[50–60] The hydrogen atoms of the hydroxyl group of the three carboxylate luciferin anions were withdrawn in order to simulate the formation of the dianion species at acidic pH.[38]

The resulting conformations of the Franck–Condon luciferin dianion at acidic and basic pH are presented in Figure 2. Interestingly, at acidic pH the π–π stacking complex formed by the carboxylate luciferin species was disrupted by deprotonation of the luciferin dianion (Figure 2 A). In fact, one of the three luciferin molecules was about 24 Å away from the other two molecules in the final frame of the calculation. The other two

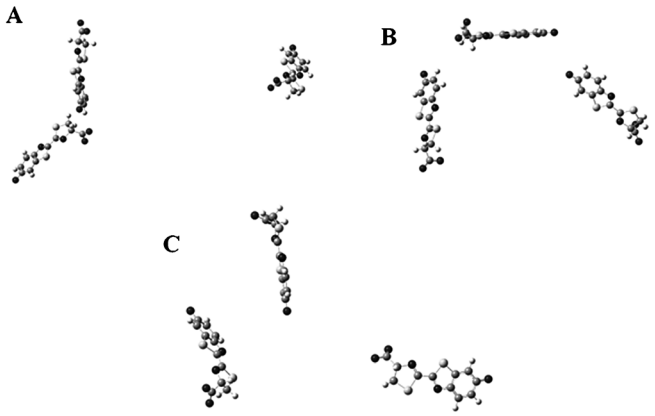


Figure 2. Excited-state luciferin-dianion complexes at acidic pH at the final frame of MD simulations of A) 1 ns and B) 1.5 ns, and C) at basic pH at the final frame of MD simulations of 1 ns.

were close together in a T-displaced conformation, not in a π–π stacking dimer. This behavior is different from that seen for oxyluciferin at acidic pH, at which oxyluciferin was found to be present in a disordered π–π stacking complex.[38] We attribute this inability of the luciferin dianion to form π–π stacking complexes to the significant repulsive electrostatic interaction between the molecules (given the double negative charge of each dianion) and the depolarization resulting from the carboxylate→dianion conversion (Table 2). Also, these molecules are known to become less polarized upon excitation.[60,61]

Despite not forming a π–π stacking complex, the two luciferin molecules present in the T-displaced conformation are close enough to exert a significant effect on each other's excited-state properties. In order to see if this conformation was maintained or if was only a transitory state resulting from the fact that the initial conformation was a π–π stacking complex, we subjected to this luciferin–water complex to another MD simulation of 500 ps. The resulting conformation of the three luciferin dianions is presented in Figure 2B. It can be seen that with a longer simulation time, the T-displaced conformation ceased to exist and all of the luciferin molecules were distant from each other.

The conformation of the Franck–Condon luciferin dianion at basic pH is presented in Figure 2C. As in the ground state (Figure 2B), the luciferin molecules were not involved in a π–π stacking complex. In fact, they were not close to each other. This is not in line with the results found for oxyluciferin, which was present as a π–π stacking complex at basic pH in the ground and excited states.[38]

The results presented so far have demonstrated that luciferin is only able to form ground-state π–π stacking complexes at acidic pH, under which conditions it takes the form of the carboxylate luciferin anion. The complex is able to form due to solvent effects, which are thought to maximize complementary electrostatics and minimize repulsive electrostatic interactions.[45] At basic pH in the ground state, and in the excited state, under which conditions luciferin is found as the dianion species, this molecule is not able to form π–π stacking complexes. This can be attributed to the deprotonation of the hydroxyl group, which increases the negative charge of each luciferin molecule to two. This is expected to increase the repulsive electrostatic interactions between the luciferin molecules to a point at which they cannot be overcome by solvent effects. Also, the deprotonation of this group reduces the polarization of luciferin, as this results in the molecule having a negative charge at each extreme of its scaffold. This depolarization is also expected to affect the dipole–dipole interactions between the luciferin molecules present in solution.

The calculated excitation energies of the luciferin dianion at acidic (both at 1 and 1.5 ns of MD simulation) and basic pH are presented in Table 4. The vertical excitation of each luciferin molecule by itself is also presented in the same table.

Table 4. Vertical excitation energies (E_{ex}) of the Franck–Condon states of the luciferin dianion species at the final frame of the MD simulation of 1 ns (presented in Figure 2A,C), at the TD *w*B97XD/cc-pVDZ level of theory.

Luciferin dianion (acidic pH)	E_{ex}[a] [eV]
complex	3.05 (3.16)
luciferin1	3.22 (3.02)
luciferin2	3.25 (3.24)
luciferin3	3.05 (3.17)
Luciferin dianion (basic pH)	**E_{ex}[a] [eV]**
complex	3.12
luciferin1	3.00
luciferin2	3.21
luciferin3	3.13

[a] The values in parentheses are the excitation energies of the luciferin dianion species at the final frame of the MD simulation of 1.5 ns (presented in Figure 2B).

The excitation energies of the three complexes are all similar to each other, with differences of only 0.04 to 0.12 eV. For oxyluciferin differences of 0.25 eV were found at acidic and basic pH.[38] Also, the difference between the vertical excitation energies of the luciferin complexes and the average of the energies of each luciferin molecule is not very significant (between 0.01 and 0.13 eV). This finding indicates that the interactions between the luciferin molecules (expected to be long-range electrostatic) do not significantly affect the vertical energy of the complexes.

All of these findings support the pH-insensitivity of luciferin in aqueous solution.[10] Even if photoexcited at acidic pH from a π–π stacking complex, luciferin emits light as a dianion that only interacts with other luciferin molecules by long-range electrostatic interactions. This is in stark contrast with oxyluciferin. The oxyluciferin fluorescence emitter, the enolate anion, emits light in π–π stacking complexes at both acidic and basic pH, but the conformation of the complexes is dependent on the pH. These different structures result in different π–π stacking interactions between the oxyluciferin, and lead to different emission wavelengths that depend on the pH.[38] In conclusion, our results indicate that luciferin only forms π–π stacking complexes in the ground state at acidic pH. Thus, this type of com-

plex and the resulting π–π stacking interactions do not have a significant effect on the photophysical properties and photoprotolytic cycle of luciferin. Moreover, these findings further support that the oxyluciferin π–π stacking complexes are the basis for the pH-sensitive fluorescence of this fluorophore.[10,38]

3. Conclusions

Time-resolved measurements have revealed that the fluorescence of firefly oxyluciferin in aqueous solution is pH sensitive. At acidic pH the emission peaks at 2.25 eV, whereas at basic pH it shifts to 2.30 eV. Our recent theoretical study explained this feature by demonstrating that oxyluciferin is able to form self-aggregated π–π stacking complexes in both the ground and the excited state, and at acidic and basic pH. However, the conformations of these complexes are different at basic and acidic pH, and this leads to different types of π–π stacking interactions between the oxyluciferin molecules. The pH-dependent changes in these intermolecular interactions are then able to modulate the emission, thus generating pH-sensitive fluorescence.

Firefly luciferin is a derivative of oxyluciferin with very similar fluorescence properties. This similarity indicates that luciferin should also be able to form π–π stacking complexes. However, no pH-sensitive fluorescence has been observed for this molecule. Thus, a question arises: is luciferin not able to form π–π stacking complexes, or are the π–π stacking interactions not able to modulate its fluorescence? The objective of this manuscript was to answer this question.

We employed a theoretical approach based on MD simulations and QM calculations to study the photodynamics of the photoprotolytic cycle of luciferin. We have found that this molecule is only able to form π–π stacking complexes in the ground state at acidic pH, under which conditions it is present as a carboxylate anion. At basic pH and in the excited state this molecule is present as a dianion. This further deprotonation of luciferin is expected to increase the repulsive electrostatic interactions to a level at which the formation of π–π stacking complexes is not possible. Thus, in the excited state luciferin is not subjected to π–π stacking interactions, but only to long-range repulsive electrostatic interactions, and so presents a very similar fluorescence peak over the entire pH range.

Theoretical Section

The ground-state geometries of luciferin species were obtained with the PBE0 functional and the cc-pVDZ basis set with no solvent effects.[62] This functional was used due to its good results in the calculations of geometries of organic molecules.[63–65]

Stable luciferin–water complexes were obtained by means of molecular-mechanics-based methods. TIP3P water molecules were added up to 12 Å by the LEAP module of the AMBER suite of programs.[66] We used this water-box size due to good results in previous theoretical studies of the interaction of firefly luciferin, dehydroluciferin, and oxyluciferin with firefly luciferase and/or with water molecules.[29,32,63] Six Na^+ cations were added to the complexes at basic pH, thereby neutralizing the charge of the complex. This was carried out in order to simulate the frequent addition of NaOH to aqueous solution in order to increase the pH of the solution. This approach was also used in our recent study of the photoprotolytic cycle of oxyluciferin in water.[38] One phase of energy minimizations (30000 steps) was performed by using the Not (just) Another Molecular Dynamics (program) (NAMD) MD code with AMBER potential functions, parameters, and file formats.[67] In this process, the particle mesh Ewalds method was used to include the long-range interactions.[68] Nonbonded interactions were considered with 14 Å cutoffs. The integration step size was 2 fs, and all bonds involving hydrogen and heavy atoms were constrained. All steps were performed in a NVT ensemble with a temperature of 298.15 K.

The ground-state geometries of the studied molecules were used in their parameterization with the ANTECHAMBER module of AMBER and the general AMBER force field.[69] The charges of the ground-state molecules were derived by using the restrained electrostatic potential (RESP) method at the *w*B97XD/aug-cc-pVDZ level of theory.[70] The Franck–Condon charges of luciferin were parameterized with the RESP method by performing single-point time-dependent (TD) *w*B97XD/aug-cc-pVDZ calculations on the PBE0/cc-pVDZ geometries.[71]

The ground state and vertical excitation energies of luciferin, after MD simulations, were calculated at the *w*B97XD/cc-pVDZ level of theory (TD *w*B97XD/cc-pVDZ for the Franck–Condon states). The conductor-like polarized continuum model (CPCM) was used to simulate implicit water.[72] We used this density functional due to good results in local $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$, charge transfer and Rydberg excited states.[73] Moreover, *w*B97XD was designed with an empirical dispersion correction term, which is needed for a correct modelling of π interactions.[70]

All QM calculations were performed with the Gaussian 09 program package.[74]

Acknowledgements

Financial support from Fundação para Ciência e Tecnologia (FCT, Lisbon) (Programa Operacional Temático Factores de Competitividade ;COMPETE) e comparticipado pelo Fundo Comunitário Europeu (FEDER; Project PTDC/QUI/71366/2006) is acknowledged. A Ph.D. grant to L.P.d.S. (SFRH/76612/2011), attributed by FCT, is also acknowledged.

Keywords: fluorescence · oxyluciferin · photoacidity · photoprotolytic cycle · pi interactions

[1] M. A. Lill, V. Helms, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 2778–2781.
[2] G. Mathias, D. Marx, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 6980–6985.
[3] S. J. Formosinho, L. G. Arnaut, *J. Photochem. Photobiol. A* **1993**, *75*, 21–48.
[4] A. H. Zewail, *Pure Appl. Chem.* **2000**, *72*, 2219–2231.
[5] J. Waluk, *Acc. Chem. Res.* **2003**, *36*, 832–838.
[6] S. Mente, M. Maroncelli, *J. Phys. Chem. A* **1998**, *102*, 3860–3876.
[7] N. Agmon, *J. Phys. Chem. A* **2005**, *109*, 13–35.
[8] T. Förster, *Pure Appl. Chem.* **1970**, *24*, 443–450.
[9] L. Pinto da Silva, R. Simkovitch, D. Huppert, J. C. G. Esteves da Silva, *ChemPhysChem* **2013**, *14*, 3441–3446.
[10] Y. Erez, I. Presiado, R. Gepshtein, L. Pinto da Silva, J. C. G. Esteves da Silva, D. Huppert, *J. Phys. Chem. A* **2012**, *116*, 7452–7461.
[11] L. Pinto da Silva, R. Sinkovitch, D. Huppert, J. C. G. Esteves da Silva, *J. Photochem. Photobiol. A* **2013**, *266*, 47–54.

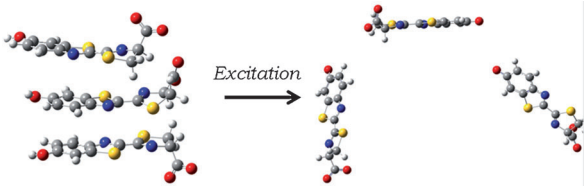
[12] K. M. Solntsev, S. P. Laptenok, P. Naumov, *J. Am. Chem. Soc.* **2012**, *134*, 16452 – 16455.
[13] T. Wilson, J. W. Hastings, *Annu. Rev. Cell Dev. Biol.* **1998**, *14*, 197 – 230.
[14] V. R. Viviani, *Cell. Mol. Life Sci.* **2002**, *59*, 1833 – 1850.
[15] H. Fraga, *Photochem. Photobiol. Sci.* **2008**, *7*, 146 – 158.
[16] S. M. Marques, J. C. G. Esteves da Silva, *IUBMB Life* **2009**, *61*, 6 – 17.
[17] A. Roda, M. Guardigli, *Anal. Bioanal. Chem.* **2012**, *402*, 69 – 76.
[18] A. Roda, M. Guardigli, E. Michelini, M. Mirasoli, *Anal. Bioanal. Chem.* **2009**, *393*, 109 – 123.
[19] K. Hochgräfe, E. M. Mandelkow, *Mol. Neurobiol.* **2013**, *47*, 868 – 882.
[20] J. Vieira, L. Pinto da Silva, J. C. G. Esteves da Silva, *J. Photochem. Photobiol. B* **2012**, *117*, 33 – 39.
[21] S. Hosseinkhani, *Cell. Mol. Life Sci.* **2011**, *68*, 1167 – 1182.
[22] L. Pinto da Silva, J. C. G. Esteves da Silva, *J. Chem. Theory Comput.* **2011**, *7*, 809 – 817.
[23] J. Y. Hasegawa, K. J. Fujimoto, H. Nakatsuji, *ChemPhysChem* **2011**, *12*, 3106 – 3115.
[24] V. R. Viviani, F. G. C. Arnoldi, A. J. S. Neto, T. L. Oehlmeyer, E. J. H. Bechara, Y. Ohmiya, *Photochem. Photobiol. Sci.* **2008**, *7*, 159 – 169.
[25] L. Pinto da Silva, J. C. G. Esteves da Silva, *J. Phys. Chem. B* **2014**, DOI: 10.1021/jp5036458.
[26] D. Cai, M. A. Marques, F. Nogueira, *J. Phys. Chem. B* **2013**, *117*, 13725 – 13730.
[27] N. Nakatani, J. Y. Hasegawa, H. Nakatsuji, *J. Am. Chem. Soc.* **2007**, *129*, 8756 – 8765.
[28] I. Navizet, Y. J. Liu, N. Ferré, H. Y. Xiao, W. H. Fang, R. Lindh, *J. Am. Chem. Soc.* **2010**, *132*, 706 – 712.
[29] L. Pinto da Silva, J. C. G. Esteves da Silva, *ChemPhysChem* **2011**, *12*, 3002 – 3008.
[30] C. G. Min, A. M. Ren, J. F. Guo, L. Y. Zou, J. D. Goddard, C. C. Sun, *ChemPhysChem* **2010**, *11*, 2199 – 2204.
[31] Y. Wang, Y. Hayamizu, H. Akiyama, *J. Phys. Chem. B* **2014**, *118*, 2070 – 2076.
[32] L. Pinto da Silva, J. C. G. Esteves da Silva, *J. Phys. Chem. B* **2012**, *116*, 2008 – 2013.
[33] J. C. G. Esteves da Silva, J. M. C. S. Magalhães, R. Fontes, *Tetrahedron Lett.* **2001**, *42*, 8173 – 8176.
[34] P. Naumov, Y. Ozawa, K. Ohkubo, S. Fukuzumi, *J. Am. Chem. Soc.* **2009**, *131*, 11590 – 11605.
[35] V. R. Viviani, D. R. Neves, D. T. Amaral, R. A. Prado, T. Matsuhashi, T. Hirano, *Biochemistry* **2014**, DOI: 10.1021/bi500160m.
[36] B. R. Branchini, M. H. Murtiashaw, R. A. Magyar, N. C. Portier, M. C. Ruggiero, J. G. Stroh, *J. Am. Chem. Soc.* **2002**, *124*, 2112 – 2113.
[37] I. Presiado, Y. Erez, R. Simkovitch, S. Shomer, R. Gepshtein, L. Pinto da Silva, J. C. G. Esteve da Silva, D. Huppert, *J. Phys. Chem. A* **2012**, *116*, 10770 – 10779.
[38] L. Pinto da Silva, R. Simkovitch, D. Huppert, J. C. G. Esteves da Silva, *ChemPhysChem* **2013**, *14*, 2711 – 2716.
[39] O. V. Maltsev, L. Yue, M. Rebarz, L. Hintermann, M. Sliwa, C. Ruckebusch, L. Pejov, Y. J. Liu, P. Naumov, *Chem. Eur. J.* **2014**, DOI. 10.1002/chem.201400210.
[40] L. Pinto da Silva, J. C. G. Esteves da Silva, *ChemPhysChem* **2011**, *12*, 951 – 960.
[41] Y. Erez, D. Huppert, *J. Phys. Chem. A* **2010**, *114*, 8075 – 8082.
[42] Y. Erez, I. Presiado, R. Gepshtein, D. Huppert, *J. Phys. Chem. A* **2011**, *115*, 1617 – 1626.
[43] S. F. Boys, F. Bernardi, *Mol. Phys.* **1970**, *19*, 553 – 566.
[44] S. Simon, M. Duran, J. J. Dannenberg, *J. Chem. Phys.* **1996**, *105*, 11024 – 11031.
[45] C. R. Martinez, B. L. Iverson, *Chem. Sci.* **2012**, *3*, 2191 – 2201.
[46] R. Podeszwa, R. Bukowski, K. Szalewicz, *J. Phys. Chem. A* **2006**, *110*, 10345 – 10354.
[47] M. O. Sinnokrot, C. D. Sherrill, *J. Am. Chem. Soc.* **2004**, *126*, 7690 – 7697.
[48] B. F. Milne, M. A. Marques, F. Nogueira, *Phys. Chem. Chem. Phys.* **2010**, *12*, 14285 – 14293.
[49] D. Cai, M. A. L. Marques, B. F. Milnes, F. Nogueira, *J. Phys. Chem. Lett.* **2010**, *1*, 2781 – 2787.
[50] D. Cai, M. A. Marques, F. Nogueira, *J. Phys. Chem. B* **2011**, *115*, 329 – 332.
[51] L. Pinto da Silva, P. J. O. Ferreia, D. J. R. Duarte, M. S. Miranda, J. C. G. Esteves da Silva, *J. Phys. Chem. A* **2014**, *118*, 1511 – 1518.
[52] M. S. Miranda, L. Pinto da Silva, J. C. G. Esteves da Silva, *J. Phys. Org. Chem.* **2014**, *27*, 47 – 56.
[53] K. Støchkel, C. N. Hansen, J. Houmøller, L. M. Nielsen, M. Linares, P. Norman, F. Nogueira, O. V. Maltsev, L. Hintermann, S. B. Nielsen, P. Naumov, B. F. Milne, *J. Am. Chem. Soc.* **2013**, *135*, 6485 – 6493.
[54] M. Rosenberg, T. I. Solling, *Chem. Phys. Lett.* **2010**, *484*, 113 – 118.
[55] N. Singla, P. Chowdhury, *Chem. Phys. Lett.* **2012**, *548*, 71 – 79.
[56] B. K. Paul, S. Mahanta, R. B. Singh, N. Guchhait, *J. Phys. Chem. A* **2010**, *114*, 2618 – 2627.
[57] S. Mahanta, B. K. Paul, R. B. Singh, N. Guchhait, *J. Comput. Chem.* **2011**, *32*, 1 – 14.
[58] R. B. Singh, S. Mahanta, N. Guchhait, *J. Photochem. Photobiol. A* **2008**, *200*, 325 – 333.
[59] R. de Vivie-Riedle, V. De Waele, L. Kurtz, E. Riedle, *J. Phys. Chem. A* **2003**, *107*, 10591 – 10599.
[60] L. Pinto da Silva, J. C. G. Esteves da Silva, *Photochem. Photobiol. Sci.* **2013**, *12*, 2028 – 2035.
[61] L. Pinto da Silva, J. C. G. Esteves da Silva, *Chem. Phys. Lett.* **2014**, *608*, 45 – 49.
[62] C. Adamo, V. Barone, *J. Chem. Phys.* **1999**, *110*, 6158 – 6169.
[63] L. Pinto da Silva, J. C. G. Esteves da Silva, *Int. J. Quantum Chem.* **2013**, *113*, 45 – 51.
[64] D. Jacquemin, E. A. Perpète, I. Ciofini, C. Adamo, *Theor. Chem. Acc.* **2011**, *128*, 127 – 136.
[65] D. Jacquemin, V. Wathelet, E. A. Perpète, C. Adamo, *J. Chem. Theory Comput.* **2009**, *5*, 2420 – 2435.
[66] D. A. Case, T. E. Cheatham, T. Darden, H. Gohlke, R. Luo, K. M. Merz, A. Onufriev, C. Simmelring, B. Wang, R. Woods, *J. Comput. Chem.* **2005**, *26*, 1668 – 1688.
[67] J. C. Phillips, R. Braun, W. Wang, J. Gumbart, W. Tajkhorshid, E. Villa, C. Chipot, R. D. Skell, L. Kale, K. Schulten, *J. Comput. Chem.* **2005**, *26*, 1781 – 1802.
[68] U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee, L. G. Pedersen, *J. Chem. Phys.* **1995**, *103*, 8577 – 8593.
[69] J. M. Wang, R. M. Wolf, J. W. Caldwell, P. A. Kollman, D. A. Case, *J. Comput. Chem.* **2004**, *25*, 1157 – 1174.
[70] J. D. Chai, M. Head-Gordon, *Phys. Chem. Chem. Phys.* **2008**, *10*, 6615 – 6620.
[71] G. Scalmani, M. J. Frisch, B. Mennucci, J. Tomasi, R. Cammi, V. Barone, *J. Chem. Phys.* **2006**, *124*, 094107 .
[72] M. Cossi, N. Rega, G. Scalmani, V. Barone, *J. Comput. Chem.* **2003**, *24*, 669 – 681.
[73] C. Adamo, D. Jacquemin, *Chem. Soc. Rev.* **2013**, *42*, 845 – 856.
[74] Gaussian 09 (Revision A.02), M. J. Frisch et al., Gaussian Inc., Wallingford, CT, **2009**.

Received: August 1, 2014
Published online on ■■ ■■, 2014

*L. Pinto da Silva, J. C. G. Esteves da Silva**

■■ – ■■

A Theoretical Analysis of the Potential Role of π–π Stacking Interactions in the Photoprotolytic Cycle of Firefly Luciferin



Insensitive fluorescence: The potential role of π interactions in the fluorescence of luciferin is studied theoretically. This species is found to form π–π stacking complexes only in the ground state at acidic pH. Upon excitation, these complexes are disrupted, owing to repulsive electrostatic interactions. Thus, π interactions do not play any role in the fluorescence of luciferin, which is contrary to the case of oxyluciferin.