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Systematic Theoretical Investigation on the Light Emitter of Firefly

Shu-Feng Chen,[†] Ya-Jun Liu,[*,†] Isabelle Navizet,[‡,§] Nicolas Ferré,[∥] Wei-Hai Fang,[*,†] and Roland Lindh[*,⊥]

[†]Key Laboratory of Theoretical and Computational Photochemistry, Ministry of Education, College of Chemistry, Beijing Normal University, Beijing 100875, China

[‡]Laboratoire Modélisation et Simulation Multi Echelle, Université Paris-Est, MSME UMR 8208 CNRS, 5 bd Descartes, 77454 Marne-la-Vallee, France

[§]Molecular Science Institute School of Chemistry, University of the Witwatersrand, PO Wits Johannesburg 2050 South Africa

[∥]Universités d'Aix-Marseille I, II, et III-CNRS UMR 6264: Laboratoire Chimie Provence, Equipe: Chimie Théorique, Faculté de St-Jérome, Case 521, 13397 Marseille Cedex 20, France

[⊥]Department of Quantum Chemistry, The Ångström Laboratory, Uppsala University, P.O. Box 518, S-75120 Uppsala, Sweden

Supporting Information

ABSTRACT: This is a systematic theoretical investigation on all the possible light emitters of firefly using a multireference method. Six chemical forms of oxyluciferin ($OxyLH_2$) molecules/anions were studied by a multistate complete active space second-order perturbation (MS-CASPT2) method in vacuum and dimethyl sulfoxide. The calculated results and subsequent analysis excluded enol-$OxyLH_2$, keto-$OxyLH_2$, and enolate-$OxyLH^-$ as possible light emitters. The remaining three candidates, phenolate-enol-$OxyLH^-$, phenolate-keto-$OxyLH^-$, and $OxyL^{2-}$, were further investigated in protein by a MS-CASPT2/molecular mechanics (MM) study to explain the natural bioluminescence of firefly. By comparison of the MS-CASPT2/MM calculated results of phenolate-enol-$OxyLH^-$, phenolate-keto-$OxyLH^-$, and $OxyL^{2-}$ with the experimental observation and detailed analysis, we concluded that the direct decomposition excited-state product of firefly dioxetanone in vivo and the only light emitter of firefly in natural bioluminescence is the first singlet excited state (S_1) of phenolate-keto-$OxyLH^-$.

INTRODUCTION

The firefly bioluminescence has for decades been studied experimentally. Theoretical calculation beyond semiempirical theory was first done by Orlova et al.[1] in 2003 using density functional theory (DFT). Its generally accepted mechanism is shown in Figure 1. The mechanism is now understood to the extent that intelligent modifications to improve the performance of luciferin, in industrial applications, based on this knowledge are reported. For example, recently, Reddy et al. demonstrated that an engineered modified luciferin molecule, a cyclic alkylaminoluciferin, exhibits more efficient light emission than the native form.[2] However, there are still three important concerns about the firefly bioluminescence, actually the most difficult three questions for understanding the firefly bioluminescence which need to be answered in detail. First, how does the ground-state (S_0) firefly dioxetanone decompose to the excited-state (S_1) oxyluciferin ($OxyLH_2$) molecules/anions? This has been theoretically investigated in gas phase,[3−6] although the explanations are not totally satisfactory. Second, how does the microenvironment of the light emitter(s) affect the emission wavelength? In protein (bioluminescence), the environmental effect mainly comes from the hydrogen bonds linking to the $OxyLH_2$, which has been verified by experimental[7−9] and theoretical[10,11] investigations. In solution (chemiluminescence), the light emission of excited $OxyLH_2$ is affected by the polarity and the pH value of the solvent, which were extensively explored experimentally.[12−18] The experiment[17] suggests that the light emitter is the S_1 state of phenolate-keto-$OxyLH^-$ (named $^1(OL^-)^*$ in ref 17). The authors concluded that the wavelength of the light emission from the S_1 state of phenolate-keto-$OxyLH^-$ is modulated by the polarity of the active-site environment of luciferase and the degree of covalent character of the O···H bond between phenolate oxygen of phenolate-keto-$OxyLH^-$ and a protonated basic moiety in the active site. The current investigation will focus on the third question. What is/are the firefly light emitter(s) in vivo? Six possible chemical forms of $OxyLH_2$ are listed in Figure 2. For consistency, we take the same names employed in ref 12. From 1971 to 2002, the general thought on light emitters of firefly was that both keto and enol/enolate forms of $OxyLH_2$ are responsible for the light emission.[7,13,15,19−21] However, from 2002 to 2009, the phenolate-keto-$OxyLH^-$ was considered as the only light emitter,[10,11,17,22−24] except for the mechanism of triple chemical equilibrium supposed by Ugarova.[25] Based on a series of fluorescence spectra and NMR data, Naumov et al.[12] in 2009 concluded that the enol form of $OxyLH_2$ can play a role in the emission, and the concentration ratio of the different chemical forms in solutions of $OxyLH_2$ was determined by several factors which affect the intricate triple chemical equilibrium, most notably the pH, solvent polarity, hydrogen bonding, presence of additional ions, and $\pi-\pi$ stacking. Moreover, in their very recent publication,[26] Naumov et al. investigated the spectral−structural effects of the keto−enol−enolate and phenol−phenolate equilibria of $OxyLH_2$ and concluded that the

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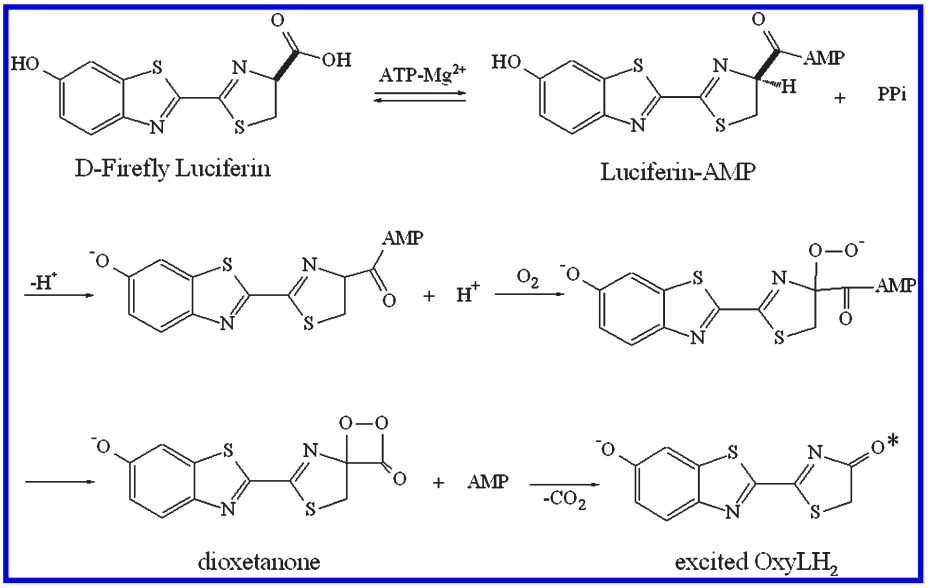

Figure 1. The generally accepted mechanism of firefly bioluminescence.

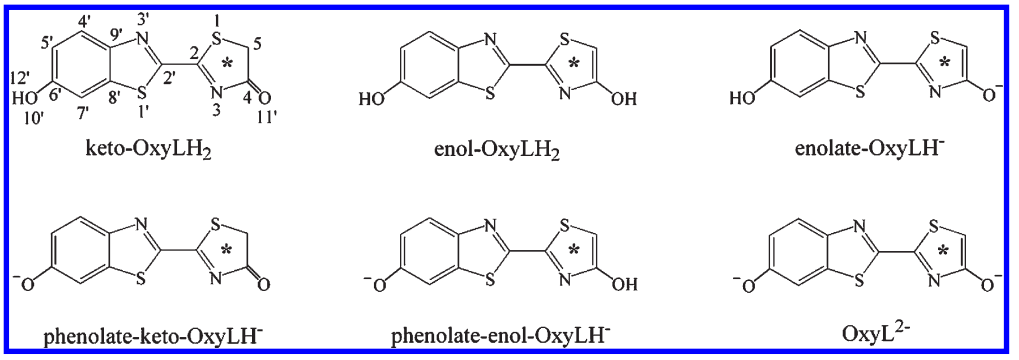

Figure 2. Molecular structures and atomic labels of all possible excited-state $OxyLH_2$ chemical forms.

phenol−enolate form of $OxyLH_2$ (see enolate-$OxyLH^-$ in Figure 2) is a yellow-emitting species. However, these in vitro experiments deal with the chemiluminescence of $OxyLH_2$, not the bioluminescence. So, which is/are the light emitters of firefly bioluminescence? Where does/do the emitter(s) come from in vivo? Is it/are they the direct product(s) of the firefly dioxetanone decomposition or a tautomerization product from another excited state of $OxyLH_2$? To answer these questions, Min et al.[27] did the following theoretical investigation: They selected Arg220, His247, AMP, Water324, Phe249, Gly343, and Ser349 in the X-ray structure of luciferase (2D1R)[28] to simulate the luciferase environment. The respective and cooperative effects of those residues and water molecule on the electronic absorption and emission spectra of $OxyLH_2$ were investigated by DFT using the B3LYP, B3PW91, and PBE1KCIS functionals. Min et al. concluded that phenolate-keto-$OxyLH^-$ not the enol forms produced yellow-green luminescence under the composite effects of the simulated environment. However, this investigation is far from systematic. Milne et al. performed a more systematic study based on the ground-state optimization of the six isomers of oxyluciferin using fragment molecular orbital-1:restricted Hartree-Fock time-dependent DFT (FMO-1:RHF-TDDFT), including the side chains of residues closer than 7.5 Å to oxyluciferin.[29] From the absorption spectra, they concluded that phenolate-keto-$OxyLH^-$ was likely both the yellow-green and red light-emitting species. It is still impossible for experiments to detect which is/are the direct excited-state product(s) of the firefly dioxetanone decomposition and which is/are the actual light emitter(s) of firefly bioluminescence in nature. We will here give a clear answer to these questions by reliable theoretical investigation. The report is supplemented by Supporting Information providing additional information and details.

COMPUTATIONAL METHODS

The complete active space SCF (CASSCF)[30] method was used to optimize the S_0 and S_1 geometries of six $OxyLH_2$ molecules/anions (see Figure 2). Their vertical excitation energies (T_v), transition energies (T_e) of emission ($S_1 \rightarrow S_0$) and oscillator strengths (f) were calculated using the multistate complete active space second-order perturbation (MS-CASPT2) method.[31] These calculations were performed in both the gas phase and dimethyl sulfoxide (DMSO) solution. The selected active space '18 electrons in 15 orbitals' (18-in-15) of all kinds of conformers are listed in Figure S1, Supporting Information. The double-ζ ANO-RCC[32] basis sets (ANO-RCC-VDZP) were used for all the calculations. All these calculations were performed using the MOLCAS 6.4 quantum chemistry software.[33] The three candidates, phenolate-enol-$OxyLH^-$, phenolate-keto-$OxyLH^-$,

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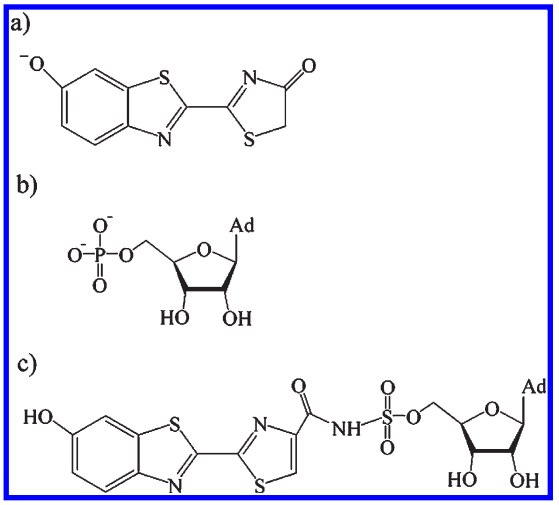

Figure 3. Structures of: (a) phenolate-keto-OxyLH$^-$, (b) AMP, and (c) DLSA.

and OxyL^{2-}, were investigated in protein by MS-CASPT2/MM study. According to the experimental conclusion,[28] the open-form luciferase with AMP and OxyLH$_2$ (PDB code: 2D1R.pdb) and the closed-form luciferase with 5′-*O*-[*N*-(dehydroluciferyl-)-sulfamoyl]adenosine (DLSA) (PDB code: 2D1S.pdb) were used as the initial guesses for the S_0 and S_1 states of the three candidates, respectively, and denoted as model-open and -closed, respectively. For phenolate-keto-OxyLH$^-$, the DLSA was replaced by phenolate-keto-OxyLH$^-$ + AMP (see Figure 3) in model-closed. The quantum mechanical (QM) and molecular mechanical (MM) calculations were performed on the basis of a modification of MOLCAS[33] and TINKER[34,35] programs, respectively. For more computational details, please see the Supporting Information.

RESULTS AND DISCUSSION

Structural Variations and Emission Spectra of the Six Possible Emitters. The CASSCF-optimized main geometrical parameters of the S_0 and S_1 states of the chemical forms of OxyLH$_2$ in vacuum are listed in Table S1, Supporting Information. The predominant variations in geometries from ground to excited state are the −N=C−C=N− unit for keto-OxyLH$_2$, enol-OxyLH$_2$, enolate-OxyLH$^-$, and OxyL^{2-}. Upon excitation to the S_1 state, $N_{3'}-C_{2'}$ and C_2-N_3 bonds stretch while the $C_{2'}-C_2$ bond shortens. For phenolate-keto-OxyLH$^-$, $O_{10'}-C_{6'}$, $O_{11'}-C_4$, N_3-C_2, and $C_{2'}-C_2$ bond distances in the S_1 state are longer than in the S_0 state, but $N_{3'}-C_{2'}$ is shorter. For phenolate-enol-OxyLH$^-$, $O_{10'}-C_{6'}$, $O_{11'}-C_4$, and $N_{3'}-C_{2'}$ bond lengths are longer and C_2-N_3, $C_{2'}-C_2$ bonds shorter in the S_1 state compared to the S_0 state. The MS-CASPT2 calculated T_e and f values of the S_1 states of the six chemical forms of OxyLH$_2$ in vacuum, DMSO, and protein are listed in Table 1. Their MS-CASPT2 calculated energies, T_v, T_e, and other detailed information are listed in Table 2 and Tables S2, S3, and S4, Supporting Information, respectively.

Exclusion of the Neutral Forms from the Possible Light Emitters. We first discriminate candidates due to the computed emission energies and oscillator strengths. We note that the neutral forms should be excluded from the possible light emitters for the following reasons. First, their MS-CASPT2 calculated T_e values in vacuum and DMSO are much outside the visible light

Table 1. The MS-CASPT2 Calculated Transition Energies T_e (in eV) and f Values of the S_1 State of the Chemical Forms of Oxyluciferin

	T_e (f)		
	in vacuum	in DMSO	in protein
keto-OxyLH$_2$	3.53 (0.32)	3.50 (0.31)	−
enol-OxyLH$_2$	3.52 (0.43)	3.37 (0.45)	−
enolate-OxyLH$^-$	1.76 (0.27)	2.18 (0.19)	−
phenolate-keto-OxyLH$^-$	2.10 (0.71)	2.31 (0.58)	2.15 (0.62)
phenolate-enol-OxyLH$^-$	2.25 (0.62)	2.57 (0.45)	2.18 (0.59)
OxyL^{2-}	2.04 (0.34)	2.20 (0.52)	2.18 (0.19)

Table 2. The MS-CASPT2 Calculated Relative Energies of the Chemical Forms of OxyLH$_2$ (in kcal/mol) from the Reference −1440.65317045 au

	in vacuum		in DMSO		in protein	
	S_0	S_1	S_0	S_1	S_0	S_1
keto-OxyLH$_2$	0.00	80.16	−12.54	69.42	−	−
enol-OxyLH$_2$	0.39	83.96	−15.23	64.83	−	−
enolate-OxyLH$^-$	345.45	387.32	285.69	337.39	−	−
phenolate-keto-OxyLH$^-$	327.00	376.83	279.13	333.36	−498.57	−504.48
phenolate-enol-OxyLH$^-$	341.43	393.46	284.83	344.26	−499.96	−507.83
OxyL^{2-}	741.32	791.10	585.14	640.12	−148.51	−154.72

(1.78−3.10 eV), as shown in Table 1. Actually, we previously calculated the T_e value of keto-OxyLH$_2$ in the wild-type protein by QM/MM method.[10] The predicted T_e value, 3.11 eV ($f \approx 0.1$), is not within the visible light spectrum. Second, the f value of keto-OxyLH$_2$ is substantially lower than that of the phenolate-keto-OxyLH$^-$ in vacuum, DMSO, or protein. Finally, in the intramolecular charge-transfer induced luminescence (ICTIL) mechanism of bioluminescence, the excited light emitter is formed by a charge transfer from an anionic remote π orbital to the σ^* of the O−O bond of the dioxetanone.[3−5] The negative charge on oxygen 10′ is essential for effective excited-state charge transfer and lowers the emission energy. This has been demonstrated both theoretically[3−5] and experimentally.[26] All together, clear facts in line with the experimental observations[12,17] imply that the neutral forms of OxyLH$_2$ are not the light-emitting species.

Exclusion of Enolate-OxyLH$^-$ from the Possible Light Emitters. Although the T_e of enolate-OxyLH$^-$ in DMSO is in the range of visible light, the f value of enolate-OxyLH$^-$ is substantially lower than those of the phenolate-keto-OxyLH$^-$ and phenolate-enol-OxyLH$^-$ (see Table 1). Moreover, according to Tables S3 and S4 and Figure S1, Supporting Information, the S_1 state of enolate-OxyLH$^-$ is formed by a charge transfer from the thiazole ring to the benzothiazole ring, whereas the S_1 states of phenolate-keto-OxyLH$^-$ and phenolate-enol-OxyLH$^-$ were formed in a contrary way. From the viewpoint of the origin of the excited OxyLH$_2$, the bioluminescence mechanism requires a negative charge on oxygen 10′.[3−5,26] So all the structures whose phenol group are protonated are excluded as light emitters of firefly bioluminescence; that is, in addition to the two species

eliminated already by arguments presented in the previous section, we also exclude the enolate-OxyLH$^-$ structure.

Three Phenolate Forms of Possible Light Emitters in Protein. Based on the above analysis and the calculated T_e and f values in vacuum and DMSO (see Table 1), the S_1 states of phenolate-keto-OxyLH$^-$, phenolate-enol-OxyLH$^-$, and OxyL^{2-} are possible light emitters of firefly. To simulate the actual firefly bioluminescence in nature, we further calculated the T_e and f values of the S_1 states of phenolate-keto-OxyLH$^-$, phenolate-enol-OxyLH$^-$, and OxyL^{2-} in protein (employing the 2D1S and 2D1R pdb X-ray structures in ref 28) by the QM/MM method. The calculated results are listed in Table 1. Their T_e values in protein showed that the S_1 states of phenolate-keto-OxyLH$^-$, phenolate-enol-OxyLH$^-$, and OxyL^{2-} can emit yellow-green light, which is the natural emission light of firefly. This is in line with the experimental conclusion drawn in 2005 by Ugarova et al.[25] This paper concluded that the observed bioluminescence spectrum could be explained as a superposition of these three forms of electronically excited OxyLH$_2$ (see Figure 1 of ref 25 notice the different nominations). However, the studies up until now are not conclusive with respect to which is/are the most important light emitter(s) involved in the firefly bioluminescence. To be more precise, the previous analysis on this subject[1,11,12,17,23,24,27] was based on the assumption that the excited-state OxyLH$_2$ forms studied were the direct products of the firefly dioxetanone decomposition, and conclusions were drawn on the basis of the emitted light in relation to the three candidate emitters. However, the direct and dominant product(s) of the dioxetanone decomposition in vivo is/are still not determined. The previous literatures[3−6] tacitly assumed that the direct product of the decomposition of the firefly dioxetanone anion is the phenolate-keto-OxyLH$^-$ S_1 state in vacuum. Several studies suggest the same state as the primary product of the chemiexcitation process from the anionic dioxetanone intermediate in vacuum.[18,36−38] We will below discuss arguments which support the presence of a single decomposition product.

Direct Product of Decomposition of Firefly Dioxetanone Anion In Vivo. The X-ray coordinates of the light emitter OxyLH$_2$ after emission, i.e., in the ground state along with AMP inside the protein were detected experimentally (see the ligand in 2D1R PDB file of ref 28). To analyze and assign the chemical nature of this reaction product, we optimized the S_0 states of our candidate emitters, phenolate-keto-OxyLH$^-$, phenolate-enol-OxyLH$^-$, and OxyL^{2-}, in a protein model based on the 2D1R X-ray structure. The detailed geometrical parameters are listed in Table S4, Supporting Information. Although the low resolution of the X-ray experimental data, the X-ray detected bonds C_4-C_5 (1.50 Å) and $O_{11'}-C_4$ (1.23 Å) are only compatible with a keto species. The three QM/MM optimized structures, whose principal geometrical parameters are listed in Table S5, Supporting Information, confirm the keto nature of the ligand in the X-ray structure. Furthermore, the QM/MM calculated phenolate-enol-OxyLH$^-$ structure agrees with the experiment crystal structure of enol-OxyLH$_2$ of ref 12. This leads to the conclusion that the product of the deexcitation of the light emitter is the phenolate-keto-OxyLH$^-$ S_0 state, implying the nature of the light emitter to be the phenolate-keto-OxyLH$^-$ S_1 state.

However, is the phenolate-keto-OxyLH$^-$ S_1 state the direct product of the decomposition of firefly dioxetanone or not? The phenolate-keto-OxyLH$^-$ S_1 state could come from the tautomerism of the phenolate-enol-OxyLH$^-$ S_1 state, if the phenolate-enol-OxyLH$^-$ S_1 state is the direct decomposition product. Alternatively, the phenolate-keto-OxyLH$^-$ S_1 state could be derived from an initial OxyL^{2-} S_1 state. A theoretical study on the decomposition process of the firefly dioxetanone in protein can answer these questions. Unfortunately, the reaction is complicated, contains characteristics like charge transfer, nearly degenerated excited states, and probably proton transfer to/from the solvent in protein. Theoretical research cannot presently give a reliable description for this decomposition inside the protein, even in gas phase. Indeed, for the calculation of the firefly dioxetanone decomposition, a big active space beyond regular CASSCF calculation is needed.[39] However, we can give a reliable analysis with respect to the stabilities of the possible products of this decomposition, i.e., the stabilities of the phenolate-keto-OxyLH$^-$, phenolate-enol-OxyLH$^-$ and OxyL^{2-} S_1 states. To investigate this we calculated the energies of these species in vacuum and DMSO by the MS-CASPT2/CASSCF method and in protein by the QM(MS-CASPT2/CASSCF)/MM method. The calculated energies are listed in Table 2. Here we find that the phenolate-keto-OxyLH$^-$ S_1 state is 16.63 and 10.91 kcal/mol more stable than the phenolate-enol-OxyLH$^-$ S_1 state in vacuum and DMSO, respectively. However, in protein, the phenolate-enol-OxyLH$^-$ S_1 state is 3.35 kcal/mol more stable than phenolate-keto-OxyLH$^-$ S_1 state. We observed the same inversion for the S_0 state. We checked the optimized structures in protein and found that the phenolate-enol-OxyLH$^-$ in the S_0 and S_1 states form H-bonds with the nearby phosphate anion, while phenolate-keto-OxyLH$^-$ does not (see Figure S2, Supporting Information). This is in accordance with the observation of Naumov et al.[12] They obtained a crystal structure of isolated enol-OxyLH$_2$ which assembled as head-to-tail H-bonded dimers. Furthermore, the enol form that was in this study theoretically verified to be stabilized in the dimer as a result of an hydrogen bonding. We can also explain the change of relative stability of the species inside the protein compared to the in gas calculation by the below presented small model calculations. We optimized the geometries (see Figure S3, Supporting Information) and calculated the energies of the phenolate-keto-OxyLH$^-$ and phenolate-enol-OxyLH$^-$ S_1 states in vacuum, with one H_2O and $H_2PO_4^-$ molecule nearby $O_{11'}$ by the TD CAM-B3LYP method. As Table S6, Supporting Information, showed, the energy difference between the phenolate-keto-OxyLH$^-$ and phenolate-enol-OxyLH$^-$ S_1 states decreased to almost zero by the H-bonding network created by the presence of $H_2PO_4^-$. The surrounding residues and AMP presence in the enzyme cavity leads to a stabilization of the phenolate-enol-OxyLH$^-$ S_1 state as compared to the phenolate-keto-OxyLH$^-$. Regardless of that the tautomerization of enol to keto forms would have to overcome the activation barrier, the thermal stability of the enol form over the keto form, in both the excited and ground state, suggests that this reaction path is closed to the formation of a phenolate-keto-OxyLH$^-$ species, as observed in the X-ray experiment. Hence, a hypothesis of the phenolate-enol-OxyLH$^-$ S_1 state being the direct product of the dioxetanone decomposition has to be rejected. In the hypothesis of OxyL^{2-} being the direct product of the dioxetanone decomposition, one should note that the OxyL^{2-} S_1 state would not directly react to the phenolate-keto-OxyLH$^-$ S_1 state but rather first form the phenolate-enol-OxyLH$^-$ S_1 state by a proton transfer from the solvent (proton transfer with the solvent is less than 10 ns, the lifetime of excited state is 1 ns). Furthermore, the f value of OxyL^{2-} S_1 state is much smaller than the f values of the phenolate-keto-OxyLH$^-$ and phenolate-enol-OxyLH$^-$ S_1 states. This is another

argument which disqualifies the dianion as the light emitter, as firefly has large bioluminescence efficiency. Thus the suggestion that the direct products are a phenolate-enol-OxyLH$^-$ or OxyL^{2-} S_1 state has to be excluded. Hence, we conclude that the direct product of firefly dioxetanone decomposition in vivo is the phenolate-keto-OxyLH$^-$ S_1 state. The T_e value of the phenolate-keto-OxyLH$^-$ S_1 state in protein is predicted by the QM/MM calculation to be 2.15 eV (see Table 1), which exactly corresponds to the firefly naturally produced yellow-green light, and the multicolor bioluminescence phenomenon is mainly tuned by the polarization of the microenvironment, which has been discussed theoretically and experimentally.[10,12,17,40−42]

CONCLUSION

This is the first systematic theoretical investigation on all the possible light emitters of firefly using a multireference method. Six chemical forms of oxyluciferin (OxyLH$_2$) molecules/anions were studied by the MS-CASPT2 method in vacuum and DMSO. Based on the T_e, f values, and ICTIL mechanism of bioluminescence, enol-OxyLH$_2$, keto-OxyLH$_2$ and enolate-OxyLH$^-$ were excluded as possible light emitters. The three remaining candidates, phenolate-keto-OxyLH$^-$, phenolate-enol-OxyLH$^-$ and OxyL^{2-}, were further investigated in protein by the MS-CASPT2/MM calculations to explain the natural bioluminescence of firefly. They are all possible light emitters of firefly if just based on the MS-CASPT2/MM calculated T_e values of 2.15, 2.18, and 2.18 eV, respectively. By comparison of the MS-CASPT2/MM calculated geometries and stabilities of phenolate-enol-OxyLH$^-$, phenolate-keto-OxyLH$^-$, and OxyL^{2-} with the experimental observed crystal data and the detailed analysis on the possibility of the tautomerization reactions among them in protein, we concluded that the direct decomposition product of firefly dioxetanone and the only light emitter of firefly bioluminescence is the phenolate-keto-OxyLH$^-$ S_1 state. The MS-CASPT2/MM results are proved to be the reference on which all the other calculations related to bioluminescence should be compared with.

ASSOCIATED CONTENT

Supporting Information. Selected active CASSCF orbitals of six OxyLH$_2$ molecules/anions, H-bonding network by the CASSCF/MM optimization, and TD CAM-B3LYP optimized structures were presented in Figures S1, S2 and S3, respectively. The CASSCF optimized geometries and T_v, T_e and charge densities, the CASSCF/MM optimized geometries in protein, and the TD-DFT calculated energy differences between the S_1 states of phenolate-enol-OxyLH$^-$ and phenolate-keto-OxyLH$^-$ were summarized in Tables S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: roland.lindh@kvac.uu.se (R. L.), yajun.liu@bnu.edu.cn (Y.-J. L.), fangwh@bnu.edu.cn (W.-H. F.).

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