DOI 10.5012/bkcs.2010.31.04.895

Theoretical Study of the Relationships between Excited State Geometry Changes and Emission Energies of Oxyluciferin

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Received January 9, 2010, Accepted February 8, 2010

In order to find a relationship between firefly luciferases structure and bioluminescence spectra, we focus on excited substrate geometries which may be affected by rigid luciferases. Density functional theory (DFT) and time dependent DFT (TDDFT) were employed. Changes in only six bond lengths of the excited substrate are important in determining the emission spectra. Analysis of these bonds suggests the mechanism whereby luciferases restrict more or less the excited substrate geometries and to produce multicolor bioluminescence.

Key Words: Firefly, Oxyluciferin, Bioluminescence, Luciferases, TDDFT

Introduction

In firefly luminescence, the light emitter is the first excited state (S_1) of oxyluciferin which likely exists as an anionic keto form (named 'keto-1' in this article) (Figure 1).[1-4] Interestingly, while keto-1 exhibits red fluorescence in DMSO or in aqueous solution,[2,3,5-9] its bioluminescence spectra can be shifted to the green by interactions with the luciferases.[10-16] Several mechanisms have been proposed to explain this phenomenon.[1,2,4,6,11,12,14,17-25] To the present, the detailed mechanism of the bioluminescence and its shifts in color remain unclear due to the complexity of the microenvironment *in vivo*.

With improved spectrometry as well as computational methods, significant progresses have been reported recently both in experimental and theoretical studies.[4,20,26-27] In particularly, using a new X-ray diffractometer, Nakatsu *et al*. found that loose and tight luciferases structures, which may cause the substrate to have higher or lower energies in the excited state, can influence emission colors.[27] Accordingly, we propose a further theoretical explanation of the experiment (Figure 2).

As shown in Figure 2, following the changes in electronic structure from point E_i (initial excited state), the excited molecule usually relaxes to an energy minimum (point E_f (final excited state)) on the first excited state potential energy surface (PES) and emits a photon while return to the ground state PES at G_f (final ground state). In luciferases with tight active sites, the location and shape of the ground state and first excited PES would change by affect of tight luciferases, the location and the energy values of G_i, E_i, E_f and G_f were also changed to G_i', E_i', E_f' and G_f'. As a result, the light emitter (at the PES represented by dot line) decays to its ground state without (or with less) geometrical relaxation and the emission energy is larger than that of the red fluorescence $\Delta E(E_f - G_f)$ shown in Figure 2. Different luciferases may allow the excited substrate to vary its geometry to different extents (point E_f') which leads to multicolor bioluminescence. Theoretical calculation can be employed to examine proposal of the excited state transformations which is difficult to measure experimentally.

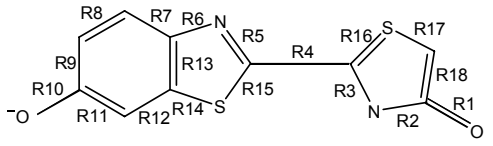

Figure 1. The anionic keto form of firefly oxyluciferin (keto-1), Bonds are labeled.

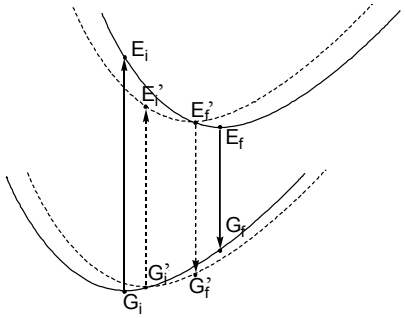

Figure 2. Schematic diagram of the relationships between luciferases structures and emission colors for red light bioluminescence in loose luciferase (solid lines) and green light bioluminescence in tight luciferase (dotted lines).

It is necessary to find the quantitative relationships between excited substrate structures in different luciferases and the emission energies to gain an insight into multicolor bioluminescence. The changes in color tuning would be determined by various geometries of oxyluciferin has been suggested by others. For example, Liu *et al*. obtained different excited oxyluciferin structures using complete active space self-consistent field (CAS-SCF) method by choosing different active spaces.[28] Nakatani

et al. compared the two structures (gas phase and bond in luciferases) in the ground and the excited state using Hartree-Fock (HF) and configuration interaction with single excitation (CIS) methods.[4] The motivation for these was all a study of the resonance-based mechanism.[11] We systemically investigate the relationship with a different motive.

Computational Methods

Modeling the substrate in the luciferases active site is computationally very demanding. In currently, it is impossible to take into account the effect of whole enzyme structures to the ground and excited states geometry of substrate with quantum chemistry method, directly. So the effects, a variety of enzyme to the excited state substrate structure were simulated to find the important structural factors which affects multicolor bioluminescence. For our proposition, it is the changes in substrate geometry that is the focus.

In Figure 2, points G_i (initial ground state) and E_i have the same geometry and could be calculated by a method such as B3LYP (Becke's hybrid exchange functional and the correlation functional of Lee, Yang, and Parr)[29] which has been shown to successfully predict the ground and excited states properties of oxyluciferin.[30,31] This geometry is the initial form of excited substrate. The energy minimum geometry in the excited state (point E_f) can be found with time-dependent B3LYP (TD B3LYP). The two geometries obtained are the benchmarks in the whole discussion. And these are the points' limits of the transformed geometries (E_f' in Figure 2) of the excited substrates in loose or tight luciferases. After comparison of the two benchmarks, by TD B3LYP, we changed their bond lengths to study other probable excited state geometries (namely E_f') due to the luciferases. Based on these geometries, their emission energies (S_1-S_0) and total energies were also calculated. The 6-31+G* basis set was employed throughout.

The whole calculation was performed with TURBOMOLE.[32]

Results and Discussion

Geometry differences between points E_i and E_f. The oxyluciferin at G_i and E_i have the same geometry but different energies. While G_i is on the ground state and E_i is on the first excited state. Thus situation is similar to E_f and G_f. Comparing the geometries between E_i and E_f indicates the tendency for geometric relaxing action of the excited substrate without luciferases bounding. Oxyluciferin retains its shape and near planarity for E_i and E_f. The important dihedral angles indicate planarity and the bond angles change little as a result of changes in bond lengths. The differences in bond lengths are the keys to our observations.

Bond lengths of geometries of E_i and E_f are obtained in gas phase, benzene, methanol, dimethyl sulphoxide (DMSO) and water phase (Table 1-Table 5). The tendency of bond lengths changes in the five solvents are displayed in Figure 3.

It can be seen from Table 1-Table 5 and Figure 3 that the geometry changes from E_i to E_f have almost the same tendency although in the different polarity environment (so later studies in this article are performed only in gas phase). Some bonds shorten and some lengthen; some change significantly and other

Table 1. Bonds lengths, in angstrom unit, predicted for oxyluciferin (keto-1) in points E_i and E_f respectively with B3LYP/6-31+G* and TD B3LYP/6-31+G* in gas phase. Bond length differences are also listed

Bond[a]	1	2	3	4	5	6	7	8	9
E_i	1.227	1.375	1.318	1.413	1.329	1.342	1.428	1.368	1.472
E_f	1.234	1.374	1.319	1.440	1.317	1.397	1.413	1.388	1.449
$\Delta_{(E_f - E_i)}$	0.007	−0.001	0.001	0.027	−0.012	0.055	−0.015	0.020	−0.023
	10	11	12	13	14	15	16	17	18
E_i	1.257	1.453	1.376	1.447	1.762	1.791	1.805	1.822	1.553
E_f	1.266	1.463	1.409	1.412	1.743	1.779	1.791	1.828	1.553
$\Delta_{(E_f - E_i)}$	0.009	0.01	0.033	−0.035	−0.019	−0.012	−0.014	0.006	0.000

[a]Bond numbers see Figure 1.

Table 2. Bonds lengths, in angstrom unit, predicted for oxyluciferin (keto-1) in points E_i and E_f respectively with B3LYP/6-31+G* and TD B3LYP/6-31+G* in Benzene ($\varepsilon = 2.25$).

Bond[a]	1	2	3	4	5	6	7	8	9
E_i	1.229	1.375	1.320	1.414	1.328	1.344	1.428	1.368	1.468
E_f	1.234	1.374	1.318	1.440	1.317	1.397	1.413	1.388	1.449
$\Delta_{(E_f - E_i)}$	0.005	−0.001	−0.002	0.026	−0.011	0.053	−0.015	0.020	−0.019
	10	11	12	13	14	15	16	17	18
E_i	1.264	1.448	1.378	1.444	1.759	1.787	1.795	1.823	1.546
E_f	1.266	1.463	1.409	1.412	1.743	1.779	1.791	1.828	1.553
$\Delta_{(E_f - E_i)}$	0.002	0.015	0.031	−0.032	−0.016	−0.008	−0.004	0.005	0.007

Table 3. Bonds lengths, in angstrom unit, predicted for oxyluciferin (keto-1) in points E_i and E_f respectively with B3LYP/6-31+G* and TD B3LYP/6-31+G* in methanol (ε = 32.63).

Bond[a]	1	2	3	4	5	6	7	8	9
E_i	1.233	1.376	1.321	1.420	1.324	1.351	1.425	1.370	1.460
E_f	1.240	1.372	1.324	1.446	1.315	1.409	1.414	1.389	1.435
$\Delta_{(E_f - E_i)}$	0.007	−0.004	0.003	0.026	−0.009	0.058	−0.011	0.019	−0.025
	10	11	12	13	14	15	16	17	18
E_i	1.277	1.439	1.382	1.439	1.754	1.781	1.778	1.825	1.536
E_f	1.288	1.450	1.415	1.400	1.742	1.772	1.767	1.832	1.538
$\Delta_{(E_f - E_i)}$	0.011	0.011	0.033	−0.039	−0.012	−0.009	−0.011	0.007	0.002

Table 4. Bonds lengths, in angstrom unit, predicted for oxyluciferin (keto-1) in points E_i and E_f respectively with B3LYP/6-31+G* and TD B3LYP/6-31+G* in DMSO (ε = 46.7).

Bond[a]	1	2	3	4	5	6	7	8	9
E_i	1.233	1.376	1.322	1.419	1.324	1.351	1.426	1.370	1.460
E_f	1.240	1.372	1.324	1.446	1.315	1.410	1.414	1.388	1.435
$\Delta_{(E_f - E_i)}$	0.007	−0.004	0.002	0.027	−0.009	0.059	−0.012	0.018	−0.025
	10	11	12	13	14	15	16	17	18
E_i	1.277	1.439	1.383	1.438	1.753	1.780	1.777	1.825	1.535
E_f	1.288	1.450	1.415	1.400	1.741	1.771	1.766	1.832	1.537
$\Delta_{(E_f - E_i)}$	0.011	0.011	0.032	−0.038	−0.012	−0.009	−0.011	0.007	0.002

Table 5. Bonds lengths, in angstrom unit, predicted for oxyluciferin (keto-1) in points E_i and E_f respectively with B3LYP/6-31+G* and TD B3LYP/6-31+G* in water phase (ε = 78.39).

Bond[a]	1	2	3	4	5	6	7	8	9
E_i	1.233	1.376	1.322	1.420	1.324	1.351	1.425	1.370	1.459
E_f	1.241	1.372	1.324	1.446	1.315	1.410	1.414	1.388	1.435
$\Delta_{(E_f - E_i)}$	0.008	−0.004	0.002	0.026	−0.009	0.059	−0.011	0.018	−0.024
	10	11	12	13	14	15	16	17	18
E_i	1.278	1.439	1.383	1.438	1.753	1.780	1.777	1.825	1.535
E_f	1.289	1.450	1.415	1.400	1.742	1.771	1.766	1.832	1.537
$\Delta_{(E_f - E_i)}$	0.011	0.011	0.032	−0.038	−0.011	−0.009	−0.011	0.007	0.002

negligibly. Luciferases with tight active sites may have greater effects on the longer than the shorter bonds. So the longer bonds likely play an important role in the proposed mechanism. Four bonds, R4, R6, R8 and R12, which large elongate from E_i to E_f, are our focus. R4, which connects the benzothiazole moiety with the thiazoline side, of especial interest, it probably be influenced greatly by the tight environment which leads to changes in the emission color. In addition, whereas R9 and R13 shorten significantly that are also ought to be studied. Moreover, these key 6 bonds were changed much in CASSCF results, too.[25]

What are the effects of the four lengthening and two shortening of these bonds on emission energies and total energies? What happens if the bonds cannot change lengths on account of the luciferases with tight active site? Relevant studies are subsequently in next section.

Effects of the six bond lengths being constrained. As Figure 2 shows, beginning at the E_i geometry, the excited substrate is transformed into E_f and luciferases with tight active sites may impede this process especially for the six bonds which change evidently. Take R4 for example, on relaxation to a minimum on S_1, bond length is lengthen from 1.413 Å to 1.440 Å (Table 1). Accordingly fix R4 in 1.415 Å, 1.420 Å, 1.425 Å, 1.430 Å and 1.435 Å and respectively perform S_1 PES geometry optimization to simulate E_f'. This process simulates that R4 is constrained by luciferases during its lengthening. Similarly, R6, R8, R12, R9 and R13 are also dealt with the same methods (Be ware of that the changes of R9 and R13 are step down) (Table 6). Their emission energies and total energies are denoted $\lambda_{Ef'}$, $E_{Gf'}$ and $E_{Ef'}$.

Expectably, the more hindered for lengthening of the four bonds or the shortening of the two the greater the blue shift of the emission spectrum. With less hindered their total energy increase on the S_0 PES ($E_{Gf'}$) as expected since G_i' is a minimum on

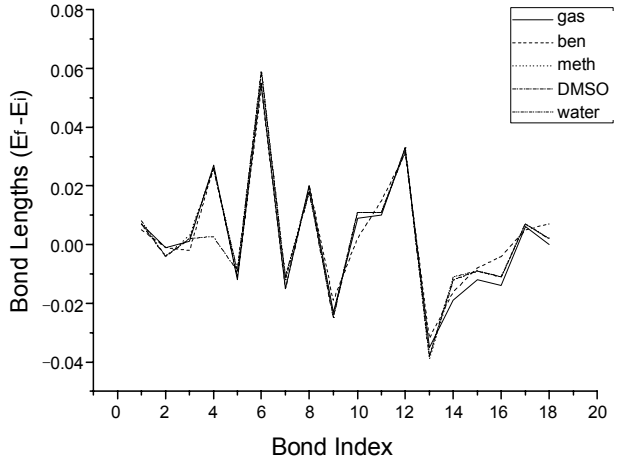

Figure 3. Oxyluciferin (keto-1) bond lengths changes in the excited state in different solvents.

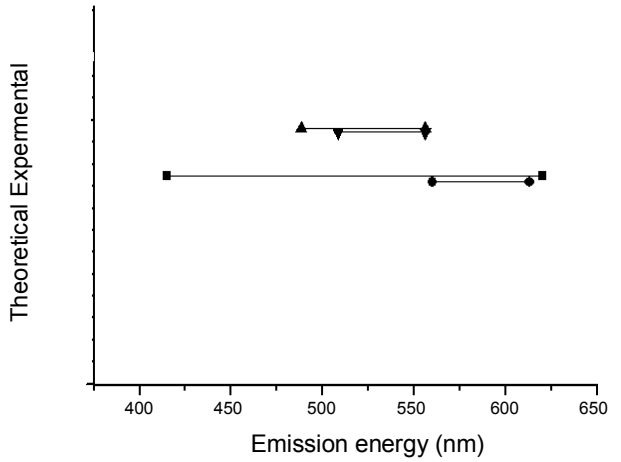

Figure 4. The proportion of blue shift (caused by restricted geometry theoretically and by luciferases effects experimentally) to the absorption-emission spectra difference.

S_0. And the total energy decreases on the S_1 PES ($E_{Ef'}$) indicates a tendency to relax to a minimum. Thus a redshift spectrum is predicted.

The changes of emission and total energies were linear with changes in the six bond lengths. Thus different rigid luciferases active sites have possibilities to produce multicolor bioluminescence by influencing the excited substrate geometries. So far, each of the six bonds was studied individually. More, the six could be impeded synchronously and to have greater influence on the light emission. We selectively draw returned some of the six bonds back to their lengths at E_i while the others are performed geometry optimization on the S_1 PES to simulate the hindered transformations to E_f. Twenty-four new geometries, as well as their emission and total energies, are obtained (Table 7). Emphasizing the blue shifts caused by these restricted bond lengths, the emission energies are arranged in increasing order. The two reference points, E_i and E_f also are given.

As table 7 shows, during the process from E_i and E_f, if no more than R9 is hindered that emission energy causes approximately 4 nm blue shift. Gradually while the six are all hindered that approximately 48 nm blue shift is caused. Among the six bond lengths, R6 alone has as much as a 26 nm influence on the emission energy. R12 and R13 causes emission changes of over 12 nm. R4, R8 and R9 cause shifted about 4nm. This sum of the shifts 47.81 nm is more than two-part of the 67.81 nm which shift from λ_{Ei} (488.30 nm) to λ_{Ef} (556.11 nm). Numerically, $\lambda^{Theo}{}_{\%} = 47.81\ nm/67.81\ nm = 0.71$. $\lambda^{Theo}{}_{\%}$ denotes: 1). Theoreti-

Table 6. Emission energies, $\lambda_{Ef'}$, in nm, and total energies, $E_{Gf'}$ and $E_{Ef'}$, in hrtree, pedicted for oxyluciferin at E_f' with TD B3LYP/6-31+G*; one bond length is fixed at a time while the others are optimized in the first excited state

Fixed Bond Lengths	$\lambda_{Ef'}$	$E_{Gf'}$	$E_{Ef'}$
R4			
1.415	551.47	−1440.0229	−1439.9403
1.420	552.40	−1440.0229	−1439.9404
1.425	553.31	−1440.0228	−1439.9405
1.430	554.33	−1440.0227	−1439.9405
1.435	555.25	−1440.0226	−1439.9406
R6			
1.350	533.67	−1440.0247	−1439.9393
1.360	538.15	−1440.0245	−1439.9398
1.370	542.90	−1440.0241	−1439.9402
1.380	547.78	−1440.0236	−1439.9404
1.390	552.71	−1440.0230	−1439.9406
R8			
1.370	551.70	−1440.0229	−1439.9403
1.375	552.90	−1440.0229	−1439.9404
1.380	554.15	−1440.0228	−1439.9405
1.385	555.17	−1440.0226	−1439.9406
R12			
1.380	545.06	−1440.0236	−1439.9400
1.385	546.88	−1440.0235	−1439.9402
1.390	548.76	−1440.0234	−1439.9404
1.395	550.58	−1440.0232	−1439.9405
1.400	552.58	−1440.0230	−1439.9405
1.405	554.46	−1440.0227	−1439.9406
R9			
1.470	552.39	−1440.0228	−1439.9403
1.465	553.25	−1440.0228	−1439.9404
1.460	554.13	−1440.0227	−1439.9405
1.455	555.03	−1440.0227	−1439.9406
1.450	555.95	−1440.0225	−1439.9406
R13			
1.440	546.78	−1440.0234	−1439.9401
1.435	548.31	−1440.0233	−1439.9402
1.430	549.95	−1440.0232	−1439.9404
1.425	551.61	−1440.0231	−1439.9405
1.420	553.30	−1440.0229	−1439.9405
1.415	554.94	−1440.0227	−1439.9406

Table 7. Emission energies, $\lambda_{Ef'}$, in nm, and total energies, $E_{Gf'}$ and $E_{Ef'}$, in hartree, predicted for oxyluciferin at E_f' with TD B3LYP/6-31+G*; one bond length is fixed at its value at E_i at a time while the others are optimized in the first excited state

Fixed Bond Lengths	$\lambda_{Ef'}$	$E_{Gf'}$	$E_{Ef'}$
E_f	556.11	−1440.0225	−1439.9406
R9	552.02	−1440.0228	−1439.9403
R4	551.30	−1440.0228	−1439.9402
R8	551.25	−1440.0229	−1439.9403
R8R9	548.30	−1440.0231	−1439.9400
R4R9	546.76	−1440.0232	−1439.9399
R4R8	546.03	−1440.0233	−1439.9399
R13	544.57	−1440.0235	−1439.9398
R12	543.66	−1440.0237	−1439.9399
R4R13	540.87	−1440.0237	−1439.9395
R9R13	539.99	−1440.0239	−1439.9395
R9R12	539.33	−1440.0240	−1439.9395
R4R12	538.97	−1440.0240	−1439.9395
R8R12	538.85	−1440.0241	−1439.9395
R4R8R12	531.00	−1440.0245	−1439.9387
R6	529.97	−1440.0248	−1439.9388
R4R6	527.85	−1440.0249	−1439.9386
R6R9	525.82	−1440.0252	−1439.9385
R6R8	525.46	−1440.0252	−1439.9385
R4R6R8	522.99	−1440.0254	−1439.9383
R6R12	521.50	−1440.0256	−1439.9383
R4R6R12	519.55	−1440.0257	−1439.9380
R6R8R12	517.19	−1440.0260	−1439.9379
R4R6R8R12	514.98	−1440.0262	−1439.9377
R4R6R8R9R12R13	508.3	−1440.0268	−1439.9372
E_i	488.30	−1440.0287	−1439.9354

cally, six bond lengths control 71 percent of the emission energy difference between E_f and E_i; 2). With six bonds, the influences on the emission energy is limited to 0.71. With different rigid active sites in the luciferases, the six can be probably partially restricted and then the changes in emission energies vary for the "0.71 proportion".

Notice that λ_{Ei} and λ_{Ef} is equal the theoretical absorption and emission spectra of the substrate. Experimentally, the two spectra in aqueous solution are at 415 and 620 nm respectively[7] and the bioluminescence observed by Nakatsu and his colleagues that ranges from 560 nm to 613 nm.[27] Here, $\lambda^{Exp}{}_{\%}$ = (613 nm - 560 nm) / (620 nm - 415 nm) = 0.26. Even though bioluminescence spectra, due to the use of luciferases mutants, range more widely (approximately 100 nm), $\lambda^{Theo}{}_{\%}$ is enough to cover $\lambda^{Exp}{}_{\%}$ (Figure 4). Namely, blue shift caused by restricted geometry is enough to produce equal multicolor light which caused by bioluminescence.

The tendency of total energies on the S_0 ($E_{Gf'}$) and S_1 ($E_{Ef'}$) PES in table 7 can be described by Figure 2. In a word, the energy minimum of the substrate on the S_1 PES is moved away from the original state (E_f) by restricted geometry. As a result, the color of the emission is determined by the new position of the excited energy minimum (E_f').

Conclusions

A model is proposed and examined to try to explain how tight active sites in firefly luciferases produce shorter wavelengths bioluminescence. The focus of this work is not on the interaction between luciferases and substrate. It based on an observation, which was reported by Nakatsu *et al*.,[27] that the emission energy of substrate is affected by the rigidity of luciferases. Accordingly, we propose the more immediate cause is geometries differences of excited substrate.

During geometric transformations in the excited state, the light emitter is restricted by the more rigid microenvironment. TDDFT calculations predicted that four bond lengths lengthen and two shorten markedly and would be affected most by the tightness of the active sites. The more the bond lengths changes are impeded the more the emission spectra shift to the blue. The variation in the spectrum ranges over that of observed bioluminescence changes. Further the simulating that how luciferases restrict oxyluciferin geometry transformation is under studying.

Acknowledgments. This work was supported by the National Natural Science Foundation of China (No.20673045, 20973078) and by the Natural Sciences and Engineering Research Council of Canada.

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