Decomposition Reaction of Dioxetanone in Firefly Bioluminescence by Computer Experiment

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Abstract. Firefly luciferin (Ln) reacts with molecular oxygen in the presence of the enzyme luciferase $(E), the \, Mg^{+2} \, ion \, and \, ATP \, to \, form \, a \, four-membered \, cyclic \, peroxide, \, so-called \, dioxetanone, \, which \, has \, dioxetanone, \, dioxet$ not yet been observed by spectrophotometric techniques. Subsequently, dioxetanone decomposes into carbon dioxide (CO₂) and electronically excited oxyluciferin (Oxyln^{-*}), emitting yellow-green light. In order to clarify the characteristics of the elementary reaction path from dioxetanone to Oxyln-*, the potential energy curve of the singlet ground-state (S₀-PEC) along the reaction coordinate was obtained by the intrinsic reaction coordinate (IRC) calculations using the AM1 Hamiltonian. Furthermore, the potential energy curve of the singlet excited-state (S₁-PEC) was calculated, because dioxetanone decomposes to Oxyln-* along the reaction coordinate. The S₁-PEC relative to S₀-PEC was estimated at each point of the reaction coordinate using the INDO/S, where only the singly-excited configuration interactions (CI) constructed from 20 occupied and 20 unoccupied molecular orbitals (MOs) were considered. As a result of these calculations, it was concluded that (1) firefly dioxetanone might not be an intermediate but rather be in an unstable transition state; (2) the S₀-PEC has an activation barrier of 37.5 kcal/mol for dioxetanone formation and the reaction is exothermic along the S_0 -PEC; (3) the S₁-PEC approaches the S₀-PEC in a concave manner where dioxetanone decomposes to efficiently produce Oxyln-*; and (4) rupturing of an O-O bond in dioxetanone can trigger the coming and going of electrons in a "cradle" motion mediated by S_0 - and S_1 -PECs in the chemiexcitation step toward

Key words: dioxetanone, firefly bioluminescence, oxyluciferin, semiempirical molecular orbital method, transition state

1. Introduction

In *in vitro* firefly bioluminescence, the substrate luciferin (Ln) reacts with molecular oxygen in the presence of the enzyme luciferase (E), the Mg^{+2} ion and ATP to form luciferyl adenylate. Subsequently, it releases AMP to form a four-membered cyclic peroxide, namely dioxetanone, and decomposition of dioxetanone produces carbon dioxide (CO₂) and the electronically-excited oxyluciferin (Oxyln^{-*}), emitting yellow-green light (see Figure 1).

Figure 1. Model of the elementary reaction path in firefly bioluminescence; in the first step, firefly luciferin changes to luciferyl-AMP in the presence of luciferase (E), Mg⁺² and ATP. Subsequently, luciferyl-AMP produces oxyluciferin in an excited state (Oxyln^{-*}) via dioxetanone. Decomposition of dioxetanone yields yellow-green light accompanied by the electronic transition from Oxyln^{-*} to its ground state.

To date, numerous dioxetanes and dioxetanones have been synthesized in order to study experimentally and theoretically the chemiluminescent process accompanying the rupture of O–O bonds in these compounds [1]. Although the chemically initiated electron exchange luminescence (CIEEL) hypothesis has been important for guiding the design of synthetic products with a high quantum yield of chemiluminescence [2, 3], it has highlighted several problems in regard to the origin of enzyme-catalyzed bioluminescence with high quantum efficiency [4–6].

In general, a class of the synthesized dioxetanes is stabilized at room temperature to form the triplet- rather than the singlet-excited state through decomposition reaction by thermolysis. A quantum-chemical analysis of the chemiluminescence of 1,2-dioxetanones was performed by Tanaka and Tanaka [7] using *ab initio* molecular orbital (MO) calculations to determine the characteristics of the adiabatic potentials for the first triplet- and singlet-excited states. As distinct from the synthesized dioxetanes and dioxetanones, firefly dioxetanone is too unstable to detect directly by spectrophotometric techniques. Furthermore, ordinary flash photolysis with a time resolution of pico- to femtoseconds is useless, because the excitation light-pulse cannot trigger and control the primary oxygenation step of Ln, the rate-determining step, to form dioxetanone by the enzyme-specific reaction. It is therefore difficult to detect an enzyme-bound peroxide having a very short lifetime even by the transient-pulse techniques.

Based on the results obtained by the computational analysis of the Ln chemiluminescence in DMSO (dimethyl sulfoxide) induced by adding potassium tertiary-butoxide (t-BuOK) in order to clarify the optical properties of Ln and Oxyln^{-*} [8–10], we here examined the elementary reaction process of the decomposition of dioxetanone to Oxyln^{-*} along the reaction coordinate by means of the semiempirical MO method. First, dioxetanone was assumed to be in a transition

state, and second, the potential energy curve of the singlet ground-state (S_0 -PEC) along the reaction coordinate was obtained by calculating the intrinsic reaction coordinate (IRC). Third, the potential energy curve of the singlet excited-state (S_1 -PEC) relative to the S_0 -PEC was calculated at each point of the reaction coordinate. Following the results obtained by these calculations, the possibility of the non-adiabatic transition from the S_0 -PEC to the S_1 -PEC to produce Oxyln^{-*} accompanied by the decomposition of dioxetanone is discussed together with the characteristic changes of atomic orbital (AO) coefficients and electron changes in the highest occupied MO (HOMO) and the lowest unoccupied one (LUMO) along the reaction coordinate.

2. Method of Calculations

In order to study the decomposition reaction path of dioxetanone toward the formation of $Oxyln^{-*}$ by the semiempirical MO method, we use the Austin Model 1 (AM1) Hamiltonian to perform geometric optimization of dioxetanone in the ground-state. The S_0 -PEC is obtained by the IRC calculation along the reaction coordinate where the interaction of Ln with E is not taken into account, but if it were considered, the activation energy necessary to form dioxetanone would be lower due to its catalytic effect.

The MO methods used in these calculations are included in the program package of WinMOPAC Ver 3.5 (Fujitsu, Japan). The details of the procedures used for the calculations are as follows. First, the "SADDLE" calculation is performed to search for an initial geometry of dioxetanone according to the geometries of the reactant and the product obtained by AM1. Second, using the "TS" method the initial geometry of dioxetanone in the transition state is optimized. Third, by applying the keyword "FORCE" in AM1 which functions as calculating the normal modes of vibration in the molecule, it is examined whether the obtained structure of dioxetanone is in a true transition state. If it has only one imaginary frequency, it is regarded as a true transition state; if not, another candidate dioxetanone structure must be searched for using same iteration procedure until a suitable geometry can be found. Based on the optimized dioxetanone, the IRC calculation is applied to obtain the reaction path of the decomposition of dioxetanone from the reactant to the product. Finally, the relative excitation energy at each point of the reaction coordinate is calculated by the intermediate neglect of differential overlap for spectroscopy (INDO/S), where in the configuration interaction (CI) only the singly-excited 400 configurations are included as constructed from 20 occupied and 20 unoccupied MOs, to obtain the S₁-PEC along the reaction coordinate of dioxetanone decomposition.

3. Results and Discussion

Figure 2 shows the IR spectrum of the geometry-optimized dioxetanone obtained by vibrational analysis of the normal mode using the keyword "FORCE" in AM1,

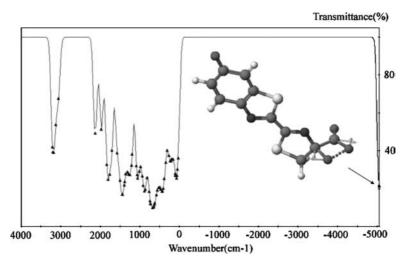


Figure 2. IR spectrum of dioxetanone obtained by "FORCE" calculation in AM1.

where the dissociation of the O–O bond in the four-membered cyclic peroxide has only one mode with imaginary frequency.

This structure thus corresponds to the true transition state which breaks down to carbon dioxide and $Oxyln^{-*}$. Next, based on the optimized geometry of dioxetanone, the IRC calculation was executed to obtain the S_0 -PEC along the reaction coordinate for the decompositions of dioxetanone. From Figure 3, it is found that the S_0 -PEC has an energy barrier of 37.5 kcal/mol and a heat of formation difference

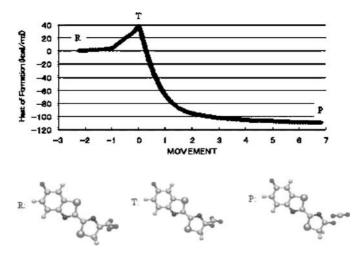


Figure 3. The singlet ground-state potential energy curve along the reaction coordinate for decomposition of dioxetanone; from the reactant (R) to the product (P; $Oxyln^{-*} + CO_2$) via the transition state (T) of dioxetanone.

between the reactant and the product of 109 kcal/mol, and consequently the decomposition reaction through the ground state is exothermic. Figure 3 also shows geometries of the reactant (a quasi-linear hydroperoxide anion), dioxetanone and the product ($Oxyln^-$ and CO_2).

The dependence of the S_0 - and the S_1 -PECs on the $R_{C_4-C_6}$ and R_{O-O} are depicted in Figure 4, where the concave approach of the S_1 - to the S_0 -PEC hereafter called the "concave approach" region, is seen along the reaction coordinate. As the reactant reaches the transition state, the O–O bond stretches slowly, while the C_4 - C_6 bond between C_4 in Oxyln⁻ and C_6 in CO_2 remains nearly constant. After formation of dioxetanone in the transition state, the C_4 - C_6 bond elongates gradually. At $R_{C_4-C_6} \sim 1.77$ Å, where the vertical $S_0 \rightarrow S_1$ excitation energy ΔE has a minimum value of 4 kcal/mol, the C_4 - C_6 bond further increases with a slightly steeper curve. Successively, the O–O and the C_4 - C_6 bonds continue to increase, and at \sim 2.1 Å the latter becomes longer than the former, indicating that Oxyln^{-*} and CO_2 are produced. It should be noted here that the excitation energy of Oxyln^{-*}, estimated as \sim 53 kcal/mol (\sim 540 nm), is high enough to emit yellow-green light.

Figure 5 is an enlargement of the concave approach region that shows the concave approach of the S_1 - to the S_0 -PEC proceeding with the unimolecular decomposition of dioxetanone, which accompanies a finite probability of the non-adiabatic transition to produce Oxyln^{-*}.

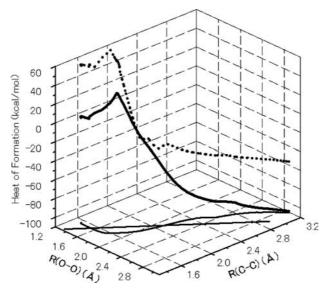


Figure 4. Potential energy curves of the singlet ground- (solid lines) and excited-states (dotted line) for the decomposition of dioxetanone vs. the bond distances of R_{O-O} and $R_{C_4-C_6}$ (units in Å) along the reaction coordinate.

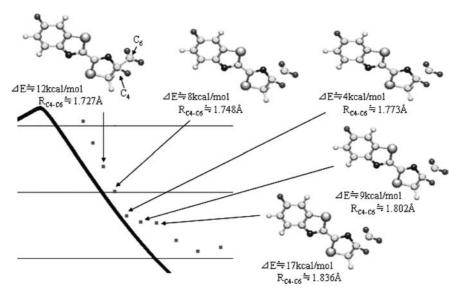


Figure 5. Enlargement of the concave approach region, where ΔE (= E_1 - E_0) is the energy difference between the excited (E_1) and the ground (E_0) state energies at each point, and $R_{C_4-C_6}$ is the bond distance between C_4 in Oxyln⁻ and C_6 in CO_2 . The E_0 and E_1 are represented by the solid and dotted lines, respectively.

Since the vertical $S_0 \rightarrow S_1$ transition can be approximately assigned to one electron excitation from HOMO to LUMO, in order to examine the electronic properties of the decomposition process, the change in the atomic orbital (AO) coefficients of HOMO and LUMO is shown in Figure 6 along the reaction coordinate. At three points on the reaction coordinate, R, S and U, the AO coefficients of HOMO and LUMO are spatially distributed. The AO coefficient distribution of HOMO hardly changes at all from R to U, while that of LUMO changes drastically for U, where the AO coefficients at the benzothiazolyl- and thiazolinone-rings seem to be almost zero but with finite amplitudes localized at the four-membered cyclic peroxide ring. Around the concave approach region from D_1 to D_3 , the spatial distribution of the AO coefficients of HOMO at D₁ indicates the emergence of non-zero values at the four-membered peroxide ring. Concerning LUMO, on the other hand, the distribution of the AO coefficients is almost zero except in the region of the four-membered peroxide ring, and the finite AO coefficients located around the benzothiazolyl- and the thiazolinone-rings gradually recover as the decomposition reaction proceeds from D₁ to D₃. Furthermore, the AO coefficients of LUMO again have finite values as the decomposition reaction of dioxetanone is completed from N to the product Oxyln^{-*} at P along the reaction coordinate. After the formation of Oxyln⁻ and CO₂, the AO coefficients of HOMO show different spatial distributions from those of R and the other six structures.

In order to study the dependence of the electron charge distribution of HOMO on the reaction coordinate, the electron charge on each atom of HOMO was

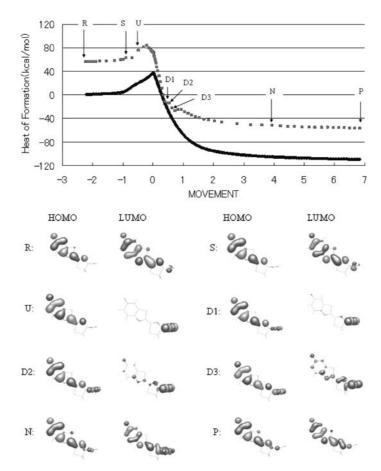


Figure 6. Change in the atomic orbital (AO) coefficients of highest occupied molecular orbitals (HOMOs) and lowest unoccupied molecular orbitals (LUMOs) along the reaction coordinate from the reactant R to the product P through the concave approach region. The solid and dotted lines mean the energy curves of the single ground- and the first excited-states, respectively.

conveniently arranged into the two parts, namely that on the benzothiazolyl-ring side, and the remaining charge, which we refer to simply as "the other portion". In Figure 7a, before arriving at the concave approach region along the S₀-PEC, the fractional electron charge on the benzothiazolyl-ring side and that on the other portion are almost constant. While passing through the concave approach region, the electron-charge polarization occurs transitorily from the former to the latter. This characteristic change of electron charge distribution of HOMO near the concave approach region can be ascribed to both the steep decrease in the electron charge on the benzothiazolyl-ring side and the increase in that charge on the two oxygens in the four-membered peroxide ring in dioxetanone. The dependence of the electron charge distribution of LUMO on the reaction coordinate is shown in Figure 7b,

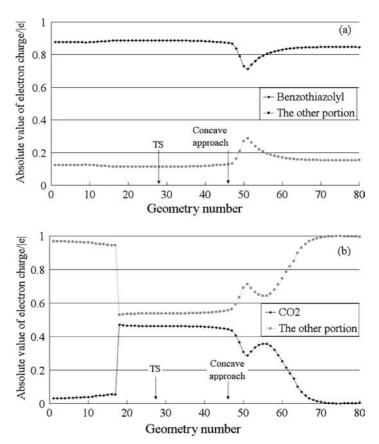


Figure 7. The dependence of the electron charge distribution (a) in the benzothiazolyl fragment and the other portion of HOMO, and (b) in CO_2 and the other portion of LUMO on the reaction coordinate.

where the electron charge on each atom of LUMO was conveniently arranged into the two parts, namely that on the CO₂ side and the "other portion".

In the case of reactant R, there is nearly a zero electron-charge on the CO_2 side. At the structure U (see Figure 6), the electron charge on the CO_2 side steeply increases due to the electron-charge localization on the four-membered peroxide side. Moreover, passing through the concave approach region after the electron charge on the CO_2 side increases, that charge transfers back to the other portion corresponding to the formation of $Oxyln^{-*}$. Two Os in four-membered cyclic peroxide, C_4 , C_6 and the phenolate anion- O^- , contribute mainly to the characteristic change of the electron-charge distribution of LUMO.

Based on the results of the calculations, the conclusions can be summarized as follows: (1) firefly dioxetanone might not be an intermediate, but rather an unstable molecule in a transition state; (2) the S_0 -PEC obtained by IRC along the reaction coordinate has an activation barrier of 37.5 kcal/mol for dioxetanone

formation and the reaction is exothermic along the S_0 -PEC; (3) the S_1 -PEC approaches the S_0 -PEC in a concave manner and the decomposition of dioxetanone proceeds to efficiently produce $Oxyln^{-*}$; and (4) concerning the change in electron charge on each atom of HOMO and LUMO along the reaction coordinate, it is found that (i) before the decomposition reaction from the reactant to the transition state, there is, around the concave approach region, a movement in the electron charge of HOMO on the benzothiazolyl-ring side to the thiazolinone-ring side, and that (ii) from the transition state to the product, the electron charge of LUMO, which is localized completely at the four-membered cyclic peroxide bond on transition from the S_0 - to the S_1 -PEC, returns to the conjugated ring side of $Oxyln^{-*}$.

Up to this time, the chemi-excitation process of Ln has been interpreted by the CIEEL hypothesis proposed independently by Schuster [2] and McCapra [3]. According to Schuster, Oxyln^{-*} is only formed once, when CO_2 is released where dioxetanone decomposes through intramolecular electron-transfer upon breaking of the O–O bond. On the other hand, McCapra deduced that dioxetanone decomposes by the intermolecular electron transfer from the hydroxyl group of Ln to CO_2^+ , and subsequently the back electron transfer from CO_2^+ to the Oxyln radical (Oxyln*) makes Oxyln**.

According to our conclusion (4), $Oxyln^{-*}$ can be formed by a one-step process in which a non-adiabatic transition from S_0 - to S_1 -PEC occurs at the concave approach region rather than by a two-step process in which the back transfer happens from CO_2^- to $Oxyln^{\bullet}$ only after the two clearly distinguishable radicals are generated. The lifetime of $Oxyln^{-*}$ formation caused by the going and returning of electrons in a "cradle" motion mediated through S_0 - and S_1 -PECs must be shorter than not only that of fluorescence but also that of the vibrational relaxation in the excited state. Thus the decomposition reaction might be expected to proceed under the time scale of ps. Although one of the relaxation channels from $Oxyln^{-*}$ in the singlet excited-state is opened to the intersystem crossing, the probability of spin inversion must be very low because the electron spins in the singlet excited-state remain correlated due to the extremely fast decomposition of dioxetanone.

One of the primary factors for the high quantum yield of firefly bioluminescence is the specific interaction of Ln with E. Based on our conclusion (1), the decomposition reaction of dioxetanone is not initiated by its specific interaction with E, but proceeds unimoleculary and spontaneously along the reaction coordinate by thermal excitation. Therefore, the specific interaction of dioxetanone with E probably plays a role in increasing the rate of the decomposition reaction by lowering the energy-barrier height of S_0 -PEC.

Although application of the semiempirical MO method to the study of the primary process of firefly bioluminescence proved to be effective, further studies will be needed to examine whether the obtained results also hold by first-principles calculation.

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References

- Matsumoto, M.: Advanced Chemistry of Dioxetane-Based Chemiluminescent Substrates Originating from Bioluminescence, *J. Photochemi. Photobiol. C* 5 (2004), 27–53.
- Koo, J.-Y., Schmidt, S.P. and Schuster, G.B.: Bioluminescence of the Firefly: Key Steps in the Formation of the Electronically Excited State for Model Systems, *Proc. Natl. Acad. Sci. USA* 75 (1978), 30–33.
- 3. McCapra, F.: Alternative Mechanism for Dioxetane Decomposition, *J.C.S. Chem. Comm.* (1977), 946–948.
- 4. Wilson, T.: Comments on the Mechanisms of Chemi- and Biolumi nescence, *Photochem. Photobiol.* **62** (1995), 601–606.
- McCapra, F.: Chemiluminescence and Bioluminescence, J. Photochem. Photobiol. A Chem. 51 (1990), 21–28.
- Mager, H.I.X. and Tu, S.-C.: Chemical Aspects of Bioluminescence, *Photochem. Photobiol.* 62 (1995), 607–614.
- Tanaka, C. and Tanaka, J.: Ab Initio Molecular Orbital Studies on the Chemiluminescence of 1,2-Dioxetanes, J. Phys. Chem. A 104 (2000), 2078–2090.
- 8. Wada, N. and Shibata, R.: Absorption Spectral Changes of Firefly Luciferin in Deoxygenated Dimethyl Sulfoxide, *J. Phys. Soc. JPN.* **66** (1997), 3312–3313.
- Wada, N. and Sameshima, K.: Ab Initio Calculation for D-(-)-Luciferin and its Intermediates in Dimethyl Sulfoxide, in J.F. Case, P.J. Herring, B.H. Robinson, S.H.D. Haddock, L.J. Kricka and P.E. Stanley (eds.), *Bioluminescence and Chemiluminescence*. World Scientific, Singapore, 2000, pp. 251–254.
- Wada, N. and Sameshima, K.: Optical Properties of Firefly Oxy-Luciferin, the Light Emitter in Firefly Chemi- and Bioluminescence, in P.E. Stanley and L.J. Kricka (eds.), *Bioluminescence* and Chemiluminescence: Progress and Current Applications. World Scientific, Singapore, 2002, pp. 67–70.