

# Theoretically obtained insight into the mechanism and dioxetanone species responsible for the singlet chemiexcitation of Coelenterazine



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## ARTICLE INFO

### Keywords:

Bioluminescence  
Chemiluminescence  
Coelenterazine  
Imidazopyrazinone  
Dioxetanone

## ABSTRACT

Coelenterazine is a widespread bioluminescent substrate for a diverse set of marine species. Moreover, its imidazopyrazinone core is present in eight *phyla* of bioluminescent organisms. Given their very attractive intrinsic properties, these bioluminescent systems have been used in bioimaging, photodynamic therapy of cancer, as gene reporter and in sensing applications, among others. While it is known that bioluminescence results from the thermolysis of high-energy dioxetanones, the mechanism and dioxetanone species responsible for the singlet chemiexcitation of Coelenterazine are not fully understood. The theoretical characterization of the reactions of model Coelenterazine dioxetanones showed that efficient chemiexcitation is caused by a neutral dioxetanone with limited electron and charge transfer, by accessing a region of the PES where ground and excited states are nearly-degenerated. This finding was supported by calculation of equilibrium constants, which showed that only neutral dioxetanone is present in conditions associated with bioluminescence. Moreover, while cationic amino-acids easily protonate amide dioxetanone, anionic ones cannot deprotonate the neutral species. These results indicate that, contrary to existent theories, efficient chemiexcitation can occur with significant electron and/or charge transfer. In fact, these processes can be prejudicial to chemiexcitation, as anionic dioxetanones showed a less efficient chemiexcitation despite the occurrence of significant electron and charge transfer.

## 1. Introduction

Bioluminescence and chemiluminescence assays, as gene reporters assays, bioimaging and ROS probing in biological environments, have attracted a great deal of attention given their low background noise, no problems regarding light-penetration into tissues (except of emission) and high sensitivity [1–3]. These processes consist on light emission from the oxidation of a substrate, whether in the presence of an enzyme (bioluminescence) or not (chemiluminescence).

While there are more than 700 *genera* known to produce bioluminescence, ranging from fireflies to fungi, [4] about 80% of luminescent organisms (as bacteria and fishes) reside in the ocean [5,6]. The majority of these organisms react with Coelenterazine, an ordinary substrate in luminescent organisms (Scheme 1) [3,4,7,8,9]. This molecule is able to emit light in both chemi- and bioluminescent reactions, by decomposing into Coelenteramide (the reaction product) and CO<sub>2</sub> [3,4,8,9]. Such reactions share similar mechanisms (Scheme 2): the

imidazopyrazinone core is oxidized by O<sub>2</sub> (or other ROS), which leads to the formation of Coelenteramide in an electronically singlet excited state. Excited state Coelenteramide latter decays to the ground state with emission of visible light [3,4,8,9].

Typical bioluminescent reactions of this molecule involve the presence of an enzyme (termed luciferase) that catalyzes the oxidation of Coelenterazine [4,8]. These luciferases have been found in a great variety of marine organisms, such as the decapod shrimp *Oplophorus gracilirostris*, the anthozoan *Renilla reniformis*, and of the copecods *Gaussia princeps* and *Metridia longa* [4,8]. Arguably, the *Gaussia* and *Renilla reniformis* luciferases are the ones that generated the highest number of practical applications [4,8]. It should be also noted that the imidazopyrazinone core of Coelenterazine is present in other marine luminescent substrates, as *Cypridina* and *Watasenia* luciferins, among others (Scheme 1) [9].

Light emission from Coelenterazine and related-imidazopyrazinones results from the formation of a cyclic peroxide intermediate

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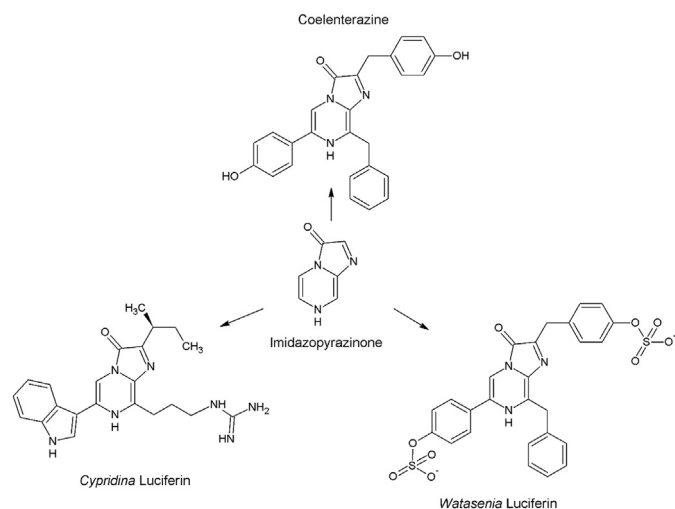
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<http://dx.doi.org/10.1016/j.jphotobiol.2017.07.012>

Received 31 March 2017; Received in revised form 1 July 2017; Accepted 14 July 2017

Available online 20 July 2017

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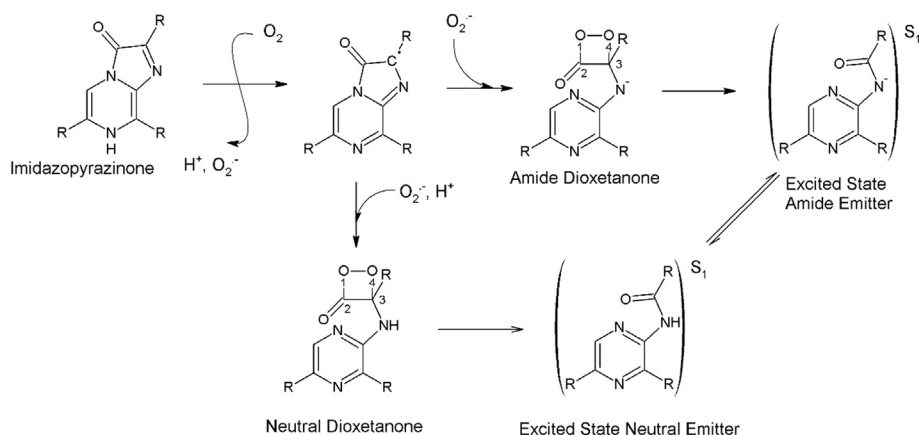


**Scheme 1.** Chemical structure of imidazopyrazinone and related chemi- and bioluminescent substrates.

(dioxetanone), which decomposition provides a thermally-activated ground state reaction to produce singlet excited states (Scheme 2) [10,11]. The resulting chemiexcitation arises from crossing points between the ground state and excited state potential energy surfaces (PES) on the reaction coordinate. Other examples of chemiluminescent cyclic peroxides include dioxetanes and dioxetanediones, and other dioxetanone molecules [10–17].

The first explanation provided for the efficient generation of singlet excited states by these peroxides was provided by the Chemically Induced Electron-Exchange Luminescence (CIEEL) mechanism [18,19]. According to CIEEL, there is an electron transfer (ET) from an oxidizable electron-rich moiety to the peroxide with formation of a radical ion pair. Back ET (BET) from the carbonyl radical anion to the radical cation leads to the formation of singlet excited states with high efficiency, due to charge annihilation. However, the CIEEL mechanism has been put into question due to the re-examination of the chemiluminescent quantum yields of prime CIEEL examples: diphenoyl peroxide and dimethyl-dioxetanone [20,21]. These new studies revealed that these systems present rather low quantum yields for supposedly efficient CIEEL decays. In fact, the measured quantum yields were significantly lower than the ones initially measured by Koo and Schuster, which allowed them to propose the CIEEL theory in the first place [18–21]. Thus, it does appear that the proposed CIEEL mechanism lacks a solid basis. These conclusions were also supported by the study of polyacene endoperoxide and two different dioxetanones [21].

Given the failure of CIEEL, some authors have tried to explain singlet chemiexcitation with mechanisms in which neither full ET/BET nor the formation of a radical ion pair are involved [22–27]. Instead,



**Scheme 2.** Schematic representation of the chemi- and bioluminescent reaction of imidazopyrazinone-based compounds.

the generation of singlet excited states is explained by more subtle charge transfer (CT) and back CT (BCT) between an ionized electron-rich moiety and the cyclic peroxide [22–27]. So, in these mechanisms, the presence of an ionized group is important as it triggers the CT/BCT steps that lead to efficient chemiexcitation, and finely tune the activation energy of the thermal reaction [22–27].

In the case of Coelenterazine and related imidazopyrazinone species, this has led to the identification of the amide dioxetanone species as the responsible for efficient chemiexcitation (Schemes 3 and S1) [22,24,27]. However, the identification of the amide dioxetanone and the requirement for an ionized group do not correlate so well with the available experimental and theoretical data.

Two different works have provided indirect evidence that in firefly and *Cypridina* luminescence, singlet chemiexcitation results from a neutral dioxetanone and not from an ionized one [28,29]. Other experimental studies regarding the chemiluminescence of imidazopyrazinone-based compounds, have attributed a more efficient singlet chemiexcitation to neutral dioxetanones than to ionized ones [30,31]. These results are supported by theoretical calculations, as they have found a more efficient pathway for singlet chemiexcitation for neutral dioxetanones [22,23,32]. This results from neutral dioxetanones having access to large and flat region of the PES where the ground and singlet excited states are degenerate/near-degenerate. This region of the PES appears to be only accessed with limited CT/ET, and not by ionized dioxetanones [32]. Thus, it is possible that while CT/ET steps might be important for determining the activation barrier of the thermal reaction, they might not be fundamental for the chemiexcitation itself [32–35].

Thus, the data discussed above indicate that efficient singlet chemiexcitation results from the thermolysis of a neutral dioxetanone, in a process with limited CT/ET. If correct, this would mean that mechanisms based on CT/BCT steps and ionized species are unable to explain efficient singlet chemiexcitation for Coelenterazine and related imidazopyrazinones, and so, alternative explanations must be sought out.

It should be noted that the proposal of the amide species as responsible for efficient chemiexcitation has been based mainly on energetic reasons [22,25]. That is, these authors have theoretically compared the activation energies for the thermolysis of the neutral and amide species and, given that both provided pathways for chemiexcitation, identified the one with the lowest activation energy as the responsible for chemiexcitation [22,25]. However, this approach is insufficient due to different reasons. One of them is that in some cases, as of a *Cypridina* dioxetanone model, the difference between the activation energy of the neutral and amide species was only of 2.9 kcal mol<sup>-1</sup> [22]. This is too small a difference, considering that the calculations were made outside of the enzyme (a biological catalyst), to allow the determination (based only on energetic criteria) of the responsible for efficient chemiexcitation. Moreover, the activation energies calculated for the neutral species are in line with the activation parameters

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