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Study of coelenterazine luminescence: Electrostatic interactions as the controlling factor for efficient chemiexcitation

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ABSTRACT

Coelenterazine is a common substrate used by marine species in enzyme-catalyzed bioluminescent reactions, in which thermal energy is converted into excitation energy. Coelenterazine is also known to emit chemiluminescence, in the absence of enzymes. Moreover, the scaffold of this molecule is present in organisms of eight *phyla*, making it a prototypical system for marine chemi-/bioluminescence. The characterization of the chemiexcitation step responsible for light emission is essential for future applications in bioimaging, bioanalysis and biomedicine. We have found evidence to support the identification of a neutral dioxetanone intermediate as the responsible for efficient chemiexcitation. This is explained by attractive electrostatic interactions between the CO_2 and Coelenteramide moieties, which allow the reacting dioxetanone to spend time in a PES region of degeneracy between singlet ground and excited states. Contrary to expected, there is no relationship between electron (ET)/charge (CT) transfer, from an electron-rich moiety to the peroxide, and efficient chemiexcitation. Thus, neither Chemically Induced Electron-Exchange Luminescence (CIEEL) nor Charge Transfer-Initiated Luminescence (CTIL) can be used to explain imidazopyrazinone-based chemi-/bioluminescence. We have also found a concentration-dependent quenching effect, more prevalent at acidic pH.

1. Introduction

Bioluminescence that consists on the conversion of thermal energy into excitation energy, thereby leading to light emission [1–4], and has been attracting attention due to high quantum yields, relative non-toxicity of luciferins and high signal-to-noise ratio [5–8]. Also, in these reactions there is no autofluorescence arising from the background signal [9]. The lack of light excitation also eliminates problems regarding light-penetration into biologic tissues (except for emission) [10]. These properties make bioluminescent systems extremely helpful tools in the real-time and noninvasive imaging of target molecules in vivo [11–14], as well as potential excitation sources for self-illuminating systems in photodynamic therapy of cancer [10].

Bioluminescence organisms as different as the fireflies, fungi, fishes and bacteria [2,6,15–19]. Nevertheless, about 80% of all bioluminescent organisms can be found in the ocean [20,21]. Moreover, most of the marine organisms react with the same bioluminescent substrate: Coelenterazine (Scheme 1). The bioluminescent reaction involving this molecule proceeds as follows (Scheme 1): [22–25] the first step is the oxygenation of the imidazopyrazinone scaffold of Coelenterazine, to form a dioxetanone intermediate; the second step is the thermolysis of dioxetanone, which allows for a thermally-activated ground state (S_0) reaction to produce Coelenteramide in its first singlet excited state (S_1) [26–28].

Typical Coelenterazine-based reactions involve the presence of an enzyme named luciferase. As Coelenterazine, these luciferases can be found in a great variety of marine organisms, such the decapod shrimp *Oplophorus gracilirostris*, anthozoan *Renilla reniformis*, copepods *Gaussia princeps* and *Metridia longa*, jellyfish *Aequorea victoria* and the hydrozoan *Obelia longissimi* [3,4]. Currently, the *Gaussian* and *Renilla reniformis* luciferases are the ones with the highest number of practical applications [3,4].

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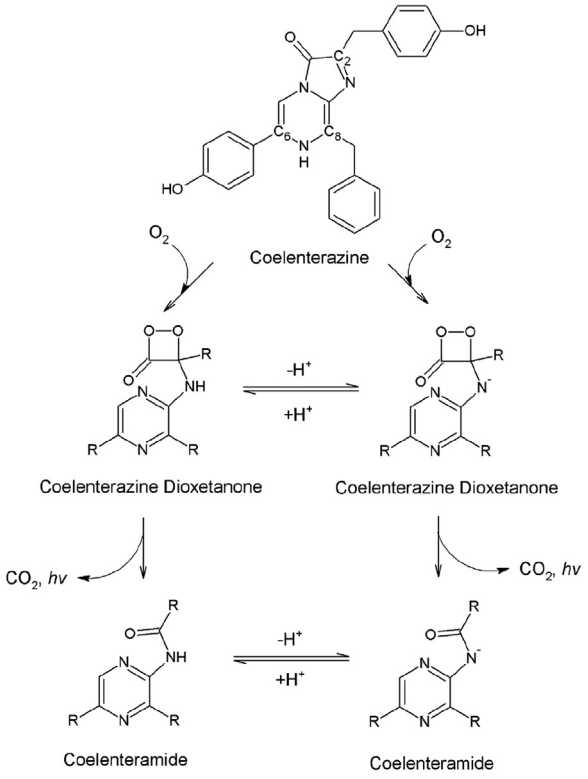

Scheme 1. Reaction mechanism of coelenterazine bioluminescence.

Coelenterazine also gains importance by being a prototypical system for most of marine bioluminescent systems, which encompass nearly 80% of all luminescent organisms [20,21]. This results from the fact that the imidazopyrazinone core of Coelenterazine is a common link among marine luminescent substrates, being found in *Cypridina* luciferin, dehydrocolenterazine and in *Watasenia* luciferin [3,25,29].

Coelenterazine and related imidazopyrazinone-based substrates are also able to emit chemiluminescence in solution (such as water, DMSO and diglyme) without an enzyme, in an identical mechanism to that of the bioluminescent reaction [22–25,30–32]. The most significant difference between chemiluminescence and bioluminescence is the higher quantum yield of the latter process [22–25,30–32]. Due to these similarities, chemiluminescence has been used as a model for the study of the bioluminescent reaction. Chemiluminescence has also been gaining importance as a probe for superoxide anion [33–35], which has a role as signaling molecules in processes such as cellular communication and cell division, as well as playing a role in several chronic diseases.

The chemiexcitation step is allowed by the thermolysis of dioxetanone intermediates not only in imidazopyrazinone-based systems, but also for fireflies, earthworms and *Latia* [1,2]. Similar cyclic peroxides (as dioxetanes) are also present in chemiluminescent systems, such as acridinium esters and AMPPD [36,37]. The reaction of luminol also involves a cyclic peroxide, but its reaction mechanism is different from other known chemiluminescent systems [37]. The efficient generation of excited states was initially explained by the Chemically Induced Electron-Exchange Luminescence (CIEEL) [38,39]. It postulates that there is an electron transfer (ET) from an oxidazable electron-rich moiety to the peroxide with formation of a radical ion pair. The radical ion pair undergoes back electron transfer (BET) from the newly formed carbonyl radical anion to the radical cation, leading to excited state formation with high efficiency due to charge annihilation. However, the CIEEL theory has been put into question after the chemiluminescence of diphenoyl peroxide and dimethyldioxetanone, prime examples of CIEEL, was re-examined [40,41]. These studies showed that these peroxides presented rather low quantum yields for a supposedly efficient CIEEL decay. Given these inconsistencies, different authors have tried to improve the CIEEL mechanism, which lead to the development of the Charge Transfer-Initiated Luminescence (CTIL) mechanism [36,42–45]. Now, there is neither full ET and BET processes, nor radical ion pairs. Instead, the generation of S_1 states is explained by gradual charge transfer (CT) and back CT (BCT) between an ionized electron-rich moiety and the cyclic peroxide.

It should be noted that dioxetanone derivatives decompose within two kinds of forms: neutral and anionic (Scheme 1) [1,2,40–50]. However, only the thermolysis of the anion has been found to follow the CTIL mechanism, with CT and BCT occurring in concert with O_1–O_4 and C_2–C_3 bond breaking, respectively [40–49]. Protonation leads to minimal CT and ET processes, with thermolysis found to proceed via homolytic cleavage of O_1–O_4 [40–49]. In the case of imidazopyrazinone-based systems, this has led to the identification of anionic dioxetanone as the responsible for efficient chemiexcitation [42,44]. However, this identification fails to address numerous experimental and theoretical findings.

Theoretical calculations at the density functional theory (DFT) level have attributed a more efficient S_1 chemiexcitation pathway to neutral dioxetanones than to anionic ones [42,44,46–48,50]. These results from neutral dioxetanones having access to a large and flat region of the potential energy surface (PES) where S_0 and S_1 are degenerate/near-degenerate. The presence of this degeneracy region in the thermolysis of neutral dioxetanones, and its absence for anionic ones, has been confirmed by higher-level multireference calculations [43,51,52]. Other authors have also attributed the efficient chemiexcitation to neutral dioxetanones instead of anionic ones, based on experimental characterization of chemiluminescent imidazopyrazinones [22–24].

It should also be said that the identification of anionic dioxetanone as the responsible for efficient chemiexcitation, has been based on energetic reasons [42–45]. However, this reasoning alone is insufficient to explain this complicated phenomenon. For one, no experimental activation energies are available for the thermolysis of imidazopyrazinone-based dioxetanones to serve as reference. Also, the calculations performed so far were made outside of luciferase, meaning that the obtained energies are not necessarily the same as the ones observed during bioluminescence. Finally, the activation energies calculated for the neutral species [42,44,46–48,50] are in line with experimental activation energies obtained for other dioxetanes and dioxetanones (~20 kcal mol^{-1}) [53–55].

Our previous experimental and theoretical chemiluminescent investigation into the *Cypridina* system showed that it has a pH-dependent behavior: a higher reaction rate is found at basic pH, while higher light intensity is found at acidic pH, despite the chemiluminophore being the same (neutral oxyluciferin) [50]. Calculations at the DFT and TD-DFT level correlated the higher reaction rate at basic pH with the lower activation energy of anionic dioxetanone, while the higher light output at acidic pH is associated with the thermolysis of neutral dioxetanone due to its ability to access a long region of S_0-S_1 degeneracy [50]. So, our results regarding the *Cypridina* system suggest that neutral dioxetanone is the species responsible for efficient chemiexcitation in the bioluminescent reaction. Also of note was the finding that there is no clear relationship between ET/BET and CT/BCT and the efficiency of the chemiexcitation process [50]. So, it appears that as they are now formulated, both CIEEL and CTIL theories cannot be applied to imidazopyrazinone-based systems. Efficient chemiexcitation by neutral dioxetanone was explained by attractive electrostatic interactions between the CO_2 and oxyluciferin moieties of *Cypridina* luciferin, which allow for the reacting molecules to spend time in the PES region of S_0-S_1 degeneracy [50].

While our previous study provided valuable information regarding imidazopyrazinone-based chemiluminescence [50], as the experimental data obtained is directly related to a single molecule (*Cypridina* luciferin), it is valid to question if all conclusions can be really expanded to more imidazopyrazinone-based molecules. Herein, the chemiexcitation