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Combined experimental and theoretical study of Coelenterazine chemiluminescence in aqueous solution

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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 light-emission. Besides bioluminescence, Coelenterazine is also known to emit chemiluminescence in aprotic solvents. We report here the study of Coelenterazine chemiluminescence in aqueous solution. Water inhibits light-emission even in mixtures with water content as low as 20%. Moreover, we provide convincing spectroscopic evidence that the presence of water affects the ground state (S_0) chemical reaction, and not the excited state processes (as chemiexcitation and the fluorescent quantum yield). However, the energetics of the S_0 chemical reaction is not affect by addition of water, which points to the inhibition being caused by the reduced lifetime of superoxide anion in water, which is an intermediate in the luminescent reactions of Coelenterazine. This finding indicates that one of the catalytic roles of bioluminescent enzymes is to extend the lifetime of this radical.

1. Introduction

Bioluminescence consists on the conversion of thermal energy into excitation energy, thereby leading to light emission [1–4], and has been attracting attention from the research community due to high quantum yields and high signal-to-noise ratio [5–8]. Furthermore, in bioluminescent 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 features make bioluminescent systems very 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].

More than 700 *genera* have been found to produce bioluminescence, in organisms as diverse as the fireflies, fungi, earthworms, fishes and bacteria, among others [15–18]. However, about 80% of all bioluminescent organisms can be found in the ocean [19,20]. Moreover, the majority of the marine organisms react with the same bioluminescent substrate: Coelenterazine (Scheme 1) [3,4,10,21]. The bioluminescent reaction involving Coelenterazine proceeds as follows (Scheme 1) [22–24]: the first step is the oxygenation of the imidazopyrazinone scaffold of Coelenterazine, which leads to the quick formation of the dioxetanone intermediate; the second step is the thermolysis of dioxetanone, which allows for a thermally-activated ground state (S_0) reaction to produce a reaction product (Coelenteramide) in its first singlet excited state (S_1). Typical Coelenterazine-based bioluminescent reactions involve the presence of an enzyme named luciferase, which catalyzes the oxygenation step of Coelenterazine [3,4]. As Coelenterazine, these luciferases can be found in a great variety of marine organisms, such the decapod shrimp *Oplophorus gracilirostris*, the anthozoan *Renilla reniformis*, and of the copecods *Gaussian princeps* and *Metridia longa* [3,4]. Currently, the *Gaussian* and *Renilla reniformis* luciferases are the ones with the highest number of practical applications [3,4].

It should be noted that the imidazopyrazinone core of Coelenterazine is a common link among marine luminescent substrates, and so, its bioluminescent mechanism is the same for many other marine organisms. In fact, the same imidazopyrazinone core can be found in *Cypridina* luciferin (present in the sea firefly *Cypridina hilgendorfii*), dehydrocoelenterazine (present in the squid *Symplectoteuthis oualaniensis)* and in *Watasenia* luciferin (present in the squid *Watasenia*

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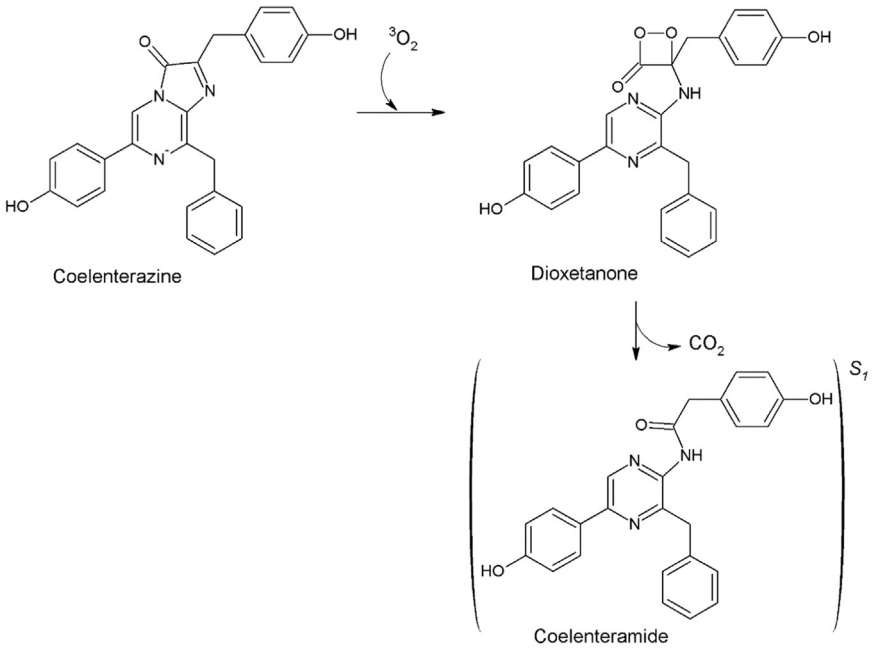

Scheme 1. Reaction mechanism of Coelenterazine luminescence.

scintillans) [3,25,26].

Besides bioluminescence, Coelenterazine and related imidazopyrazinone-based substrates are also able to emit chemiluminescence in aprotic solvents (as DMSO and diglyme) without an enzyme, in an identical mechanism to that of the bioluminescent reaction [22–24,27–29]. The most significant difference between chemiluminescence and bioluminescence is the higher quantum yield of the latter process, which is to be expected due to the absence of the catalytic luciferase in chemiluminescence [22–24,27–29]. Due to these similarities, chemiluminescence has been used as a model for the study of the bioluminescence reaction [22–24,27–29]. These studies have found that Coelenterazine presents appreciable chemiluminescence quantum yields (between 0.0021 and 0.005) in DMSO, *N*,*N*-dimethylformamide (DMF) and hexamethylphosphoric triamide, in the absence of base [21]. Addition of base and acidic buffers decreased the measured quantum yield [21].

Contrary to aprotic solvents (mainly DMSO) [21–24], the chemiluminescence of Coelenterazine in aqueous solutions has been sparsely studied, despite it being the solvent in which bioluminescence takes place (and consequently, the practical applications of this system). Nevertheless, some studies regarding other imidazopyrazinone-based substrates found the chemiluminescence in water to be extremely weak [30]. One explanation for this behavior was provided by Shimomura, who have found the fluorescence of *Cypridina* oxyluciferin to be very weak in aqueous solution [31]. Other authors have studied the chemiluminescence of different imidazopyrazinones in water/DMF and methanol/DMF mixtures, and have found that high contents of water greatly decrease the chemiluminescence efficiency [32].

These results indicate that water should affect negatively the chemiluminescence quantum yield of Coelenterazine, which is composed by three parameters: the yield of the S_0 reaction; the efficiency of the $S_0 \rightarrow S_1$ chemiexcitation; the fluorescence quantum yield of the chemiluminophore [1,2]. However, this potentially negative effect exerted by water is not in line with the known reactive oxygen species (ROS)-induced Coelenterazine chemiluminescence in water. Besides molecular oxygen ($^3O_2$), the oxygenation step of Coelenterazine chemiluminescence can also be triggered by different ROS, such as superoxide anion (O_2^-) and singlet oxygen ($^1O_2$). In fact, Coelenterazine and other imidazopyrazinone molecules have been used with good results as dynamic probes for ROS in biological media (both *in vivo* and *in vitro*) [21,33–35]. Contrary to fluorescent ROS sensors, Coelenterazine is a dynamic sensor and not an accumulation one. This means that when the chemiluminescent reaction between the probe and the target ROS species occurs and light is emitted, no more photons can be produced from those reactions. Coelenterazine also has the advantage of exhibiting chemiluminescence independently from cell-derived myeloperoxidase, and by not sensitizing O_2^- (which limits the occurrence of false-positives) [36].

Given this, the ROS-mediated chemiluminescence indicates that water by itself does not affect negatively the chemiluminescence quantum yield of Coelenterazine, and that chemiluminescence may also occur in aqueous solution (in the presence of $^3O_2$). However, that would mean that the chemiluminescence reaction of Coelenterazine would compete with its bioluminescent reaction (as this last reaction occurs in aqueous solution), which should affect the efficiency of the latter process in its various practical applications (such as bioimaging and bioanalysis). Thus, it is essential to determine if Coelenterazine can also produce chemiluminescence in aqueous solutions (in the absence of ROS), and if so, to determine the efficiency of this process. Herein, we report such study by using a spectroscopic approach to assess the potential of Coelenterazine for chemiluminescence in aqueous solution. The chemiluminescence in DMSO, an aprotic solvent typically used as a model solution for the bioluminescent reaction, was also measured so to serve as comparison. This approach allowed us to obtain valuable insight into the luminescence of Coelenterazine and related imidazopyrazinone-based ones, which can be found in about eight *phyla* of luminescent organisms.

2. Experimental methods

Coelenterazine was purchased from NanoLight™ Technology, and was dissolved in methanol and stored at −20 °C. Kinetic chemiluminescent assays were performed in a homemade luminometer using a Hamamatsu HC135-01 photomultiplier tube. All reactions took place at ambient temperature (24–27°) and were performed at least in triplicate. The reactions were carried out in pure DMSO or in DMSO/water mixtures. The reaction was initiated by the injection of the DMSO (or DMSO/water mixture) solution (380 μL) into an assay tube containing Coelenterazine (20 μL). The concentration of Coelenterazine in the final solution is of 3.0 μM. The light was integrated and recorded in 0.1 s intervals. Resulting data was analyzed by using the Graphpad software package (Version 7.03 for Windows). UV–Vis spectra were measured