

SPECIFICITY OF AEQUORIN LUMINESCENCE TO CALCIUM

Osamu Shimomura and Frank H. Johnson

*Biology Department
Princeton University
Princeton, New Jersey*

SUMMARY

The presence of Pb^{++} , Co^{++} , Cu^{++} , and Cd^{++} , each of which possesses a certain luminescence-triggering activity of aequorin, potentially interferes with the specificity of the aequorin luminescence response to Ca^{++} . Interference by the above cations can be eliminated, without influencing the sensitivity of the luminescence of aequorin to Ca^{++} , by adding 1 mM of sodium diethyldithiocarbamate.

INTRODUCTION

The isolation of a substance involved as an active principle in the luminescence of *Aequorea aequorea*, a hydromedusan jellyfish indigenous to coastal waters of the Pacific Northwest, was reported some years ago (Shimomura et al., 1962). The purified substance proved to be a conjugated protein for which the name aequorin was introduced. Later studies on other bioluminescent organisms, *Chaetopterus* and *Meganyctiphanes* (Shimomura and Johnson, 1966; 1968), indicated that aequorin was the first example of a type of luminescence system that was given the collective name "photo-protein," characterized by the properties that (1) under optimum conditions the total amount of light emitted was proportional to a specific protein, and (2) the light-emitting process did not directly involve any enzyme, at least in the usual sense of the term.

In aqueous solution, aequorin was found to have the unique property of being triggered by traces of Ca^{++} , and to a lesser extent by Sr^{++} , to emit light, either with or without molecular oxygen. The sensitivity of the luminescence response to Ca^{++} is estimated to extend to as little as a small volume of 10^{-7} M solution (Shimomura et al., 1963a; Shimomura and Johnson, 1973). The light-emitting reaction seems not susceptible to inhibition by any compounds commonly occurring in biological systems. Moreover, no evidence has been found that aequorin has any toxic properties. The speed of response to Ca^{++} is fast; maximum intensity is reached in a few milliseconds (Hastings et al., 1969; Loschen and Chance, 1971).

On the basis of sensitivity together with the favorable properties just mentioned and a seemingly satisfactory specificity, the use of aequorin luminescence as a means of the microdetermination of Ca^{++} in biological systems was suggested (Shimomura et al., 1963b). The method was soon applied to advantage in the detection of changes in Ca^{++} concentration during the contraction of single muscle fibers (Ridgway and Ashley, 1967; Ashley and Ridgway, 1968), and, in a number of other physiological processes including metabolic activity of mitochondria (Azzi and Chance, 1969), activity of single neurons as well as action of inhibitors thereon (Baker et al., 1971), and rhythmic discharge of single ganglionic cells (Chang et al., 1974). In studies of Ca^{++} inside single cells, one would be most likely dealing with 10^{-17} to 10^{-15} mole ions of ionic calcium, assuming a cell size of $100 \mu\text{m}$ in diameter.

In regard to specificity, extended research on the influence of various cations (Izutsu et al., 1972; Shimomura and Johnson, 1973) have shown that, under certain conditions of concentration and pH, cations other than Ca^{++} and Sr^{++} can trigger the luminescence of aequorin. Such cations include those of the rare earth elements and also Pb^{++} , Cd^{++} , Co^{++} , and Cu^{++} . In the present study, we tried to improve further the specificity of aequorin reaction of Ca^{++} by masking these cations other than Ca^{++} through the use of various chelating agents.

MATERIALS AND METHODS

Purified aequorin of approximately 95 percent purity (Shimomura and Johnson, 1969; Johnson and Shimomura, 1972) was desalted by a small column of Sephadex G-25 equilibrated with 0.1 mM ethylene diaminetetra acetate (EDTA), pH 7.5. The solution was kept frozen until ready for use. CH_3COONa , $\text{Pb}(\text{CH}_3\text{COO})_2$, CuO , CoO , $\text{Cd}(\text{HCOO})_2$, LaCl_3 , SrCl_2 (all ultrapure grade) and Y_2O_3 (99.999 percent) were obtained from Alpha Inorganics, and all other chemicals were reagent grade, with care taken to choose those containing a minimum amount of Ca. Deionized distilled water having a resistance of more than $10 \text{ M}\Omega$ was used, and all solutions were prepared and kept in polypropylene containers from which they were dispensed with plastic pipets and were not allowed contact with glass throughout the experiments, except for dissolving the metal oxides with HCl in Vycor test tubes as previously described (Shimomura and Johnson, 1973).

The luminescence reaction was initiated by rapid addition of 4 ml of buffer solution containing the metal salt to 5 μl of aequorin solution containing 10 μg of aequorin placed in a polycarbonate test tube. Light (λ max 470 nm) was measured by a photomultiplier-amplifier-recorder assembly with a pen response time of approximately 20 ms for the full scale.

RESULTS

Among various chelating agents tested, sodium diethyldithiocarbamate (DDC) was found to be far more effective than 8-hydroxyquinoline, 8-hydroxyquinoline-5-sulfonic acid, salicylic acid, oxalic acid, or thiourea, in regard to the masking of Pb^{++} , Cd^{++} , Co^{++} , and Cu^{++} to improve specificity of the aequorin reaction to Ca^{++} . The data for DDC, summarized in table 1, indicate that 1 mM of DDC completely suppressed the activities of 0.1 mM each of Pb^{++} , Cd^{++} , Co^{++} , and Cu^{++} , but had little effect on the activities of Ca^{++} , Sr^{++} , and the representatives of the rare earth metals, La^{+++} and Y^{+++} . Trials with a higher concentration of DDC (5 mM) resulted in a considerable decrease in the total light emitted with Ca^{++} , indicating the partial destruction of aequorin; thus, approximately 1 mM is considered to be the optimum concentration for DDC.

In the absence of DDC, the effects of heavy metal ions are often enhanced in the presence of thiol (SH) compounds. For example, Pb^{++} and Cd^{++} , each at a concentration of 10^{-4} M in a buffer solution (pH 8) containing 1 mM of cysteine or 1 mM of 2-mercaptoethanol, both induced a luminescence of 1.5×10^{12} photons/s. Such luminescence, however, could be completely suppressed by addition of 1 mM of DDC, the same as without SH compounds as shown in table 1.

The activities of 0.1 mM of La^{+++} and Y^{+++} were suppressed by 8-hydroxyquinoline-5-sulfonic acid (1 mM) to a level lower than the activity of Sr^{++} , although this reagent was found to be relatively ineffective in masking Pb^{++} and Cd^{++} .

In regard to the microdetermination of Ca^{++} by the luminescence of aequorin, in general this method evidently has some unique advantages from the points of view of speed and of harmlessness to biological materials. Furthermore, it is satisfactorily specific to Ca^{++} at a pH of 8 in the absence of rare earth elements and of high concentrations of Sr^{++} . Although the activity of Pb^{++} , Co^{++} , Cu^{++} , and Cd^{++} can be completely eliminated by adding DDC, the occurrence of these ions in biological systems is normally unlikely; if DDC is used, however, control tests for possible harmful effects are obviously required.

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Table 1

Influence of Sodium Diethyldithiocarbamate (DDC) on the Maximum Intensities* of Aequorin† Luminescence Induced by Various Metal Ions‡

Metal ion added (0.1 mM)	In 0.01 M Na-acetate pH 6.0		In 0.01 M glycylglycine-NaOH pH 8.0	
	Without DDC	With 1.0 mM DDC	Without DDC	With 1.0 mM DDC
None	< 0.02	< 0.02	< 0.02	< 0.02
Ca ⁺⁺	36.	32.	40.	26.
Sr ⁺⁺	4.	4.	9.	7.
Pb ⁺⁺	15.	< 0.02	1.§	< 0.02
Co ⁺⁺	0.05	< 0.02	1.§	< 0.02
Cu ⁺⁺	0.05	< 0.02	0.5	< 0.02
Cd ⁺⁺	18.	< 0.02	0.05	< 0.02
Y ⁺⁺⁺	34.	28.	14.	12.
La ⁺⁺⁺	24.	20.	20.	20.

* Expressed in 10^{12} photons/s. At 298 K (25°C).

† 10 μ g aequorin (potentiality = 42×10^{12} photons) was used in each test.

‡ In the present tests, those cations which have been found inactive in eliciting a luminescence reaction of aequorin, namely, K⁺, Be⁺⁺, Mg⁺⁺, Ba⁺⁺, Mn⁺⁺, Ni⁺⁺, Fe⁺⁺, Fe⁺⁺⁺, and Zn⁺⁺ (Shimomura and Johnson, 1973) were omitted.

§ A momentary flash took place at the start of the reaction (1.5×10^{12} photons in 0.1 to 0.2 s).

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