PEROXIDE-LINKED BIOLUMINESCENCE CATALYZED BY A COPPER-CONTAINING, NON-HEME LUCIFERASE ISOLATED FROM A BIOLUMINESCENT EARTHWORM*

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Received March 30, 1971

Summary

The earthworm, *Diplocardia longa*, requires luciferin, luciferase, and H_2O_2 for *in vitro* bioluminescence. The purified luciferase does not contain a heme prosthetic group but contains a nearly stoichiometric amount of tightly bound copper.

Several years ago this laboratory reported the existence of a large (about 10 in. in length) bioluminescent earthworm in Georgia and a stimulation of the luminescence from this worm by H_2O_2[1]. It is shown here that in addition to H_2O_2, luciferin and luciferase are required for light emission from this organism and some of the properties of the highly purified luciferase are presented. Although the luciferase catalyzes the bioluminescent peroxidation of luciferin to produce a bluish-green emission (500 nm) this enzyme does not contain a heme prosthetic group. Inhibition data suggest that luciferase is a metalloprotein and atomic absorption spectroscopy data indicate luciferase contains copper.

Materials and Methods

The worms were collected in South Georgia where they are widespread and were identified as *Diplocardia longa* from the original description of J. Percy Moore[2] (P. Kreiss and G. E. Gates, personal communication). No mention of luminescence was made in the original description since the author received

* This work was supported in part by grants from the National Science Foundation and the U.S. Atomic Energy Commission. During a portion of this work R.B. was an N.I.H. predoctoral fellow. M.J.C. is a Career Development Awardee No. 1-K3-6M-3331-06 of the U.S. Public Health Service.

the worms in a preserved condition.

As in other bioluminescent earthworms[3,4], the luminescence arises from the coelomic fluid exuded by the worm upon mechanical or electrical stimulation. The coelomic fluid is exuded from the mouth and dorsal pores along the body of the worm and light emission occurs when cells suspended in the coelomic fluid lyse to produce a luminous slime. The coelomic fluid was collected by electrically shocking the worms in 0.1 M EDTA. In the EDTA solution the cells do not lyse and no light emission occurs. An acetone powder was prepared from the suspension of coelomic cells in the EDTA. One gram of coelomic fluid acetone powder was obtained from 20 worms by this procedure. Luciferase and luciferin were stabilized in the acetone powder when stored at -20°C.

Luciferin and luciferase were extracted by homogenizing 1.5 g. portions of the acetone powder with 80 ml. of 0.1 M sodium borate buffer (pH 7.6) with a tissue grinder equipped with a motor driven teflon pestle. The homogenate was centrifuged at 16,000 x g for 5 min. and the pellet rehomogenized. This process of homogenization was repeated a total of four times. Three separate 1.5 g portions of the acetone powder were carried through the above procedure and the supernatants pooled. This amount of acetone powder (4.5 g) corresponds to approximately 90 worms. All operations were carried out between 0-4°. The luminous pooled supernatants were immediately poured onto a DEAE-cellulose column, 9 x 14 cm, which had been previously equilibrated with the extraction buffer. The luciferin did not adsorb and passed through the column free of luciferase activity. The luciferin was extracted into methyl acetate and further purified by column chromatography through alumina in the same solvent. The yield of luciferin from the DEAE-cellulose column was determined by comparing the total quanta produced *in vivo* from the luminous slime of an "average" earthworm to the total quanta obtained from the isolated luciferin measured in the *in vitro* reaction. The total quanta produced from luciferin off the DEAE column was found to be 1.8×10^{17} quanta as determined in the *in vitro* assay. This corresponds to 2×10^{15} quanta per worm. The luminous slime from

an "average" earthworm yields about 5 x 10^{15} total quanta suggesting a luciferin yield of about 40 percent.

Luciferase was adsorbed on the DEAE-cellulose during the above procedure. It was eluted with 0.1 M Na_2HPO_4, concentrated by ammonium sulfate precipitation, and further purified by Sephadex G-150 chromatography. The luciferase yields one band which corresponds to the activity on cellulose acetate strip electrophoresis at pH 7.5 and also appears homogeneous as judged by sedimentation equilibrium analysis (Bellisario and Cormier, unpublished data).

Luminescence was measured with a MacNichol photometer[5]. The light intensity was calibrated in quanta sec^{-1} by use of the luminol chemiluminescence secondary light standard as described by Lee *et al.* (1966)[6]. The assay for luciferase consisted of recording the flash of light obtained by injecting H_2O_2 into a mixture of luciferin and luciferase. The peak height of luminescence was used as a measure of the initial rate of reaction. The total light was determined by mechanical integration of the flash of light recorded on a Texas instrument recorder. The complete assay system consisted of 0.04 mmoles of potassium phosphate (pH 7.5), 0.2 ml of luciferin, 0.011 to 0.096 mg luciferase and 4.4 x 10^{-3} mmoles H_2O_2 in a final volume of 1.0 ml.

Table 1

Requirements for *in vitro* Light Emission

Components	Maximal Light Intensity Quanta Sec^{-1} x 10^{-7}
Complete System	4000
Without H_2O_2	0.13
Without Luciferin	<0.03
Without Luciferase	<0.03

Conditions: The complete assay system is described under Methods. Each reaction mixture contained 0.011 mg of luciferase.

Results and Discussion

The requirements for *in vitro* bioluminescence are listed in Table I. As indicated in the table, no light is detected if either luciferin or luciferase is omitted from the assay mixture. However, there is a low steady state level of light emission from the mixture of luciferin and luciferase in the absence of any added H_2O_2. This effect is attributed to the presence of a small amount of H_2O_2 in the luciferin preparation since the light emission is abolished when the endogenous H_2O_2 in the luciferin preparation is decomposed with MnO_2[7] or catalase. As illustrated in Figure 1, catalase also inhibits the flash of light in the complete assay system of luciferin, luciferase and H_2O_2. A second addition of H_2O_2 partly overcomes the catalase inhibition.

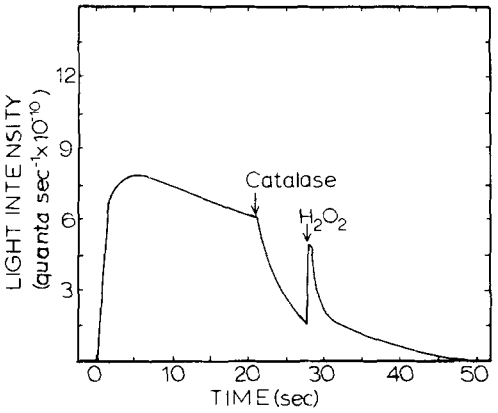

Figure 1. Effect of catalase on *in vitro* light emission. Assay conditions were as outlined under Methods except that the reaction was initiated at zero time by injecting 0.1 ml of 1.1 x 10^{-2} M H_2O_2 into the reaction mixture which contained 0.096 mg of luciferase. At the times indicated, two times recrystallized Sigma catalase (4 µg) and 0.2 ml of 8.8 x 10^{-2} M H_2O_2 were injected into the reaction mixture.

Oxygen does not appear to be necessary for *in vitro* light emission since evacuation of the assay mixture and flushing with hydrogen does not inhibit the light emission. Although these conditions were sufficiently anaerobic to maintain FMN in the reduced state, it is difficult to rule out the presence of small amounts of oxygen that may have been generated from the H_2O_2 present in the reaction mixture.

From these results, the requirements for *in vitro* light emission are expressed in the following reaction scheme consisting of a "luciferin-luciferase" type of bioluminescent reaction utilizing H_2O_2 instead of molecular oxygen:

$$\text{LUCIFERIN} + H_2O_2 \xrightarrow{\text{LUCIFERASE}} \text{LIGHT} + \text{PRODUCTS}$$

The bluish green light emission *in vitro* has a maximum emission around 500 nm (20,000 cm^{-1}). This is essentially identical to the *in vivo* light emission from the secreted luminous coelomic fluid of the earthworm. In contrast to the *in vitro* system, however, oxygen is required for *in vivo* light emission. No light is emitted from a worm stimulated to eject its coelomic fluid under an argon atmosphere. A viscous slime is produced from the lysed coelomic cells as usual, but no luminescence occurs. The admission of air results in a brilliant flash of light which can be again quenched with argon.

However, the endogenous light emission from a crude extract of the fresh coelomic fluid is not dependent upon oxygen. Evacuation of the homogenate and flushing with hydrogen does not inhibit the light emission. This endogenous light emission from crude extracts is attributed to the presence of H_2O_2 in the crude extracts since the light emission is abolished by the addition of catalase[1]. Moreover, it has been previously shown that the addition of H_2O_2 to the crude extract results in 100 fold stimulation of luminescence intensity suggesting H_2O_2 was limiting[1]. It is therefore assumed that an oxidase is present *in vivo* to generate H_2O_2 under aerobic conditions as has been postulated for myeloperoxidase[8] and thyroid peroxidase[9]. However, such a peroxide generating system has not been isolated or identified in *D. longa*.

Luciferin has not been isolated in sufficient quantity for structural determinations, but it is a water soluble non-volatile compound. Furthermore it is dialyzable and behaves as an aromatic or heterocyclic compound since it is retarded on Sephadex G-10 chromatography and elutes after sodium chloride. It is resistant to oxidation and can be boiled under an oxygen atmosphere for 30 min. with no loss of activity. It has been stored frozen for a year with no loss of activity.

The highly purified luciferase has an absorption spectrum of a typical protein with a maximum at 278 nm and a 280:260 nm ratio of 1.8. At a protein concentration of 5 mg/ml the colorless enzyme shows no evidence of any characteristic heme Soret absorbance. A Soret peak of about 1.5 absorbancy units would be expected at this protein concentration if luciferase contained heme in an equimolar ratio to enzyme. Although luciferase catalyzes the bioluminescent peroxidation of luciferin, luciferase shows no activity towards a number of typical peroxidase substrates[10] such as guaiacol, O-dianisidine, or pyrogallol The enzyme has a molecular weight of 320,000 as judged from sedimentation equilibrium data assuming a $\bar{V}$ of 0.73. Luciferase appears to be a metalloprotein since low concentrations of a variety of metal binding agents such as KCN, O-phenanthroline, and diethyldithiocarbamate strongly inhibit the enzyme. In addition atomic absorption spectroscopy shows the presence of 0.8 mole of copper per mole of enzyme.

These observations are of interest since they represent one of the few cases[11] in which a luciferase has been postulated to be a metalloprotein. Furthermore the demonstration that luciferase behaves as a non-heme peroxidase and in fact contains copper is also of considerable interest.

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