Chemical Nature of Bioluminescence Systems in Coelenterates
(photorprotein/luciferin/luciferase/Aequorea/Renilla)

OSAMU SHIMOMURA AND FRANK H. JOHNSON

Department of Biology, Princeton University, Princeton, New Jersey 08540

Communicated by Arthur B. Pardess, February 10, 1975

ABSTRACT Analysis of substances involved in light-emitting reactions among bioluminescent coelenterates has revealed a pronounced uniformity in the structural features of initial reactants, i.e., "luciferins" and photoprotein chromophores, as well as the light-emitter product. This product is structurally identical among the different classes of coelenterates: Hydrozoa (the jellyfish, Aequorea), Anthozoa (the sea cactus, Cavernularia; sea pansy, Renilla; and sea pen, Leioptilus), and very likely also the Scyphozoa (the jellyfish, Pelagia). In each of these instances the reaction product, namely, 2-(p-hydroxyphenylacetyl)aminino-3-benzyl-5-(p-hydroxyphenyl)pyrazine, is the actual light-emitter, whether it occurs in a Ca²⁺-triggered photoprotein type of luminescence, or in a "luciferin-luciferase" type. The evidence indicates that in certain coelenterates, e.g., Cavernularia, these two types are equally significant, whereas in others (Renilla and Leioptilus) the "luciferin-luciferase" type predominates over the Ca-triggerable photoprotein type, and finally that only the photoprotein type functions in the luciferaseless jellyfish, Aequorea. In all instances investigated, the structure of the light-emitter prior to the luminescence reaction appears to be essentially the same as that of the chromophore of unreacted aequorin. The product of the luminescence reaction is absent in extracts of nonluminescent species. However, a product very similar to that of luminescent coelenterates occurs also in representatives of other phyla, including the cephalopod molluscs, e.g., the "firefly squid" Watasenia and probably various ctenophores as well.

In the bioluminescence of the hydrozoan jellyfish, Aequorea aequorea, the photoprotein aequorin reacts with Ca²⁺, either in the presence or absence of molecular oxygen, resulting in the emission of blue light by an intramolecular reaction (1, 2). The light-emitting molecule involved has been identified as Ia (Fig. 1 and ref. 3). In Aequorea, a further process of an intermolecular energy transfer takes place in vivo between the excited state of Ia and a green fluorescent protein, the latter ultimately emitting a green light (4). The present study, however, is limited to the initial light-emitting reaction of Aequorea, as well as all other luminescent species of coelenterates that have been investigated from this point of view.

In the aequorin reaction, the structure of the light-emitting molecule (Ia) prior to the luminescence reaction has been elucidated (Ila, i.e., the enolized form of Ila bound to a protein), and the intramolecular reaction involved in this luminescence was postulated to occur as shown in the following overall scheme (5) wherein an essential component, H₂O₂, is tentatively assumed to be in the form of an α-hydroxyhydroperoxide and YC refers to a yellow chromophore which has been isolated but has a yet unknown structure.

In the bioluminescence of the anthozoan Renilla (sea pansy) an unstable luciferin (IIIb), having an unknown group (X), reacts with molecular oxygen in the presence of Renilla luciferase, producing light, CO₂, and compound I (6, 7). Renilla luciferin (IIIb) is stored in the organism in the form of a derivative, termed "luciferyl sulfate" (IIb), which is more stable than IIIb, but which can be converted to IIIb either by 3',5'-diphosphodenosine in the presence of luciferin sulfokinase, or by treatment with acids (8-10).

Many of the luminescent coelenterates have been reported to contain one or more of the following factors: luciferyl sulfate which is closely similar in chemical nature to that found in Renilla, luciferase, luciferin sulfokinase, and a photoprotein (11, 12). Since the anthozoans Renilla, Cavernularia (sea cactus), and Leioptilus (sea pen) contained all of these factors, the involvement of both types of luminescence mechanisms are implied, i.e., photoprotein and luciferin-luciferase types in these species. In addition to the photoprotein aequorin, the jellyfish, Aequorea, was found to contain luciferyl sulfate but no luciferase (11). In Cavernularia, the light-emitting molecule has been identified to be Ia (13), the same as in Aequorea.

Recently, a new protein in Renilla has been reported (14). This protein, which when bound with luciferin releases the bound luciferin on addition of Ca²⁺, was termed "luciferin binding protein," and the existence of analogous proteins among other luminescent anthozoans has been presumed (14). Thus, the photoprotein activity in Renilla (11, 12) could be accounted for, at least to some extent, by the presence of the luciferin binding protein. However, the existence of any photoproteins in Renilla and other anthozoans still remains to be proved.

The present study was undertaken in an effort to identify the structures of light-producing compounds, such as luciferin, and the light-producing chromophore of a native photoprotein, as well as the structures of light-emitters derived therefrom, in representative coelenterates. Comparisons have also become possible regarding the quantitative extents of
photoprotein activity, as well as content of luciferyl sulfate and light-emitting compounds produced by in vivo luminescence.

**MATERIALS AND METHODS**

*Aequorea aequorea*, *Leioptilus*, and a nonluminescent pen- nutulid resembling *Stylatula* were obtained at or from the Friday Harbor Laboratories of the University of Washington, and *Cavernularia obesa* at the Uozu Aquarium and Amakusa Marine Laboratory, Japan. *Renilla mulleri* and *Leptogorgia virgulata* were purchased from Gulf Specimen Co., Panacea, Fla., and *Renilla kolikeri* from the Pacific Bio-Marine Supply Co., Venice, Calif. The animals were generally kept in aerated (artificial) sea water at 15–15°C until ready for use.

Before extraction and measurement of various factors, *Cavernularia* (whole body), *Leioptilus* (after removing the nonluminescent stalk and horny axis), and *Renilla* (after removing stalk), all in contracted states, were cut to slices 3 mm thick with a sharp razor blade. *Stylatula*-like species (also after removing the horny axis) and *Leptogorgia* were chopped into pieces 1 cm in length. With *Aequorea*, thin strips (2–3 mm thick) which contained luminescent organs were cut off from the margin of the umbrella, and only these strips were used. Most extractions were carried out at night, between 8 and 12 p.m., due to insufficient knowledge concerning the day–night rhythm of some of these bioluminescent systems.

**Measurement of In Vivo Luminescence.** To a test tube containing 1 ml of sea water and a small piece of test sample taken from various parts of the specimen body, 2 ml of 1 M KCl was added at 20°C, and the resulting luminescence was measured until the light finally ceased, usually within 10 min. The emitted light was measured by means of a photomultiplier-light integrating amplifier-recorder assembly which was calibrated in absolute units at various wavelengths.

**Measurement of Ca²⁺-Triggered Luminescence Activity.** Slices or pieces of the specimen (5 g) were added to 20 ml of 0.05 M EDTA (pH 9.0 at 20°C, adjusted with Tris) at 0°C. After leaving 10 min, the pieces in the solution were repeatedly pressed and squeezed with a spatula to discharge most of the potential light-emitting activity into the solution. This activity was then measured by adding 2.5 ml of 0.02 M calcium acetate to 0.5 ml of the test solution at 20°C.

**Isolation of Compound Ia after Stimulating Specimens to Luminescence In Vivo.** The method described here is in principle the same as that which was previously used for *Cavernularia* (13), although a few improvements in purification procedures were made because of greater amounts of impurities encountered in *Leioptilus* and *Renilla*.

Slices or pieces of specimen (80 g) were put into 80 ml of 1 M KCl containing 0.01 M CaCl₂ to stimulate in vivo luminescence. The pieces in the solution were pressed and squeezed using a spatula and a pair of pliers until the luminescence finally died away. The resulting mixture was added to 450 ml of methanol, briefly homogenized in a Servall Omni-mixer merely to disintegrate further the original pieces, then filtered. The filtrate was concentrated under reduced pressure to about 20 ml, washed four times with benzene to remove compound Ib (or Ia) as well as lipids, and then the water layer was further concentrated to 3–5 ml.

The content of luciferyl sulfate (IIb) in this aqueous concentrate was estimated as follows. The concentrate (0.1 ml) in a light-measurement tube was acidified with 10 μl of 1 N HCl, and heated in boiling water for 1 min to hydrolyse luciferyl sulfate to free luciferin (10) which is chemiluminescent in dimethylsulfoxide or in dimethylformamide (7, 15). The tube showing a blue fluorescence, was finally evaporated to dryness. The dried residue was extracted with less than 1 ml of methanol, the insoluble matter discarded, and the methanol-soluble material was further purified by two steps of thin-layer chromatography on silica gel, first with water-saturated ether (R₂ of Ia; 0.5) and then with water-saturated chloroform-ethyleacetate (1:1) (R₂ of Ia, 0.36).

The amount of compound Ia obtained by the above procedure was estimated from the absorption spectrum measured in methanol, taking an ε value of 13,500 for the 333 nm absorption peak.

**Extraction of Luciferyl Sulfate (Iib), Estimation of the Amount of Luciferyl Sulfate by Chemiluminescence after Acid-Treatment of the Sample, and Isolation of the Product of the Chemiluminescence Reaction.** To avoid luminescence of living specimen, specimens in sea water, except for *Aequorea*, were cooled in advance slowly to near 0°C, before the specimens were cut into slices. Slices containing the luminescent organs of *Aequorea*, or slices of other species prepared after cooling (about 80 g), were added into 300 ml of methanol at 0°C. After leaving 15 min, the pieces in methanol were pressed and squeezed using a spatula and a pair of pliers, then these pieces were broken down to smaller pieces by brief use of a Servall Omni-mixer, and the mixture was finally filtered. The filtrate was concentrated under reduced pressure to about 20 ml, washed four times with benzene to remove compound Ib (or Ia) as well as lipids, and then the water layer was further concentrated to 3–5 ml.

The content of luciferyl sulfate (IIb) in this aqueous concentrate was estimated as follows. The concentrate (0.1 ml) in a light-measurement tube was acidified with 10 μl of 1 N HCl, and heated in boiling water for 1 min to hydrolyse luciferyl sulfate to free luciferin (10) which is chemiluminescent in dimethylsulfoxide or in dimethylformamide (7, 15). The tube
was quickly cooled, the contents were neutralized with solid NaHCO₃; then finally the light emitted by chemiluminescence was measured when 3.5 ml of dimethylsulfoxide was added to the tube. Calculation of the content of luciferyl sulfate was based on the light emission of chemiluminescence when an amount of an authentic sample of IIIa in 0.1 ml of water was treated in exactly the same manner as described above; 0.1 nmol of IIIa resulted in $3 \times 10^{18}$ photons, and the photon yield was closely proportional to the amount of IIIa.

The remainder of the aqueous concentrate was acid-treated, neutralized, and chemiluminesced in a proportionally increased scale to that described above, but with dimethylformamide in place of dimethylsulfoxide as solvent. The spent solution of chemiluminescence was evaporated under reduced pressure nearly to dryness, the residue was taken up in ether, and compounds in this ether solution were purified by the NaOH extraction method and by thin-layer chromatography as described in the isolation of compound Ia after in vivo luminescence.

RESULTS AND DISCUSSION

Light-emitting compounds extracted and purified from *Leioptilus, Renilla mülli*eri, and *Renilla kolikeri*, that had been exhaustively stimulated to in vivo luminescence in KCl solution before the extraction, were all identified to be Ia; this coincides with the previously obtained results from *Aequorea* (3) and from *Cavernularia* (13). The present identifications were based on comparison of the purified compounds with an authentic, synthetic sample of Ia, in regard to the behavior in thin-layer chromatography with various solvent systems (see the *Materials and Methods section*), UV absorption spectra ($\lambda_{max}$ in methanol at 277, 292, and 333 nm; in water at 272 and 330 nm; in 0.01 N NaOH at 302 and 364 nm) and the mass spectrum (m/e 411, 304, and 277), although in the case of *Renilla mülli*eri, the mass spectrum of the purified compound was not obtainable, due to an accidental loss of the sample. No other compound of the general structure of Ib, or similar to Ib, was found either in the purified samples, on the basis of mass spectra, or in fractions set aside during the course of purification, as judged by UV absorption spectra of components separated by thin-layer chromatography with various developing solvents.

The preparations of luciferyl sulfate extracted from *Aequorea, Leioptilus*, and the two species of *Renilla* were all chemiluminescent in dimethylsulfoxide or in dimethylformamide after acid-treatment of the samples, as expected from previous reports (7, 10, 11, 15). The products of chemiluminescence of these luciferyl sulfate samples, prepared and purified as described in the *Materials and Methods section*, were all identified as consisting of a mixture of Ia and a small amount of Ic on the basis of the behavior in thin-layer chromatography, UV absorption characteristics, and mass spectra, coinciding with the result recently obtained from *Cavernularia* (13); the compound Ic had been originally obtained from aequorin and referred to as AF-350 (2, 16). The evidence just described indicates that the luciferyl sulfate extracted from the above species, including *Cavernularia*, has the structure of Ic, namely, the enol-sulfate of IIIa. Moreover, a synthetic sample of IIIa was found to give a strong luminescence in the presence of partially purified *Renilla* luciferase.

Data providing a comparison of the approximate content of Ca²⁺-triggerable luminescence, compound Ic, and also compound Ia that is obtainable after the specimens had been stimulated to in vivo luminescence, are shown in Table 1. With *Aequorea*, the light yield of in vivo luminescence stimulated with KCl solution can be quantitatively attributed in full to the amount of photoprotein (aequorin).

In *Cavernularia*, the Ca²⁺-triggered luminescence activity and the amount of Ic were approximately equivalent. The amount of Ic was overwhelmingly predominant in *Leioptilus* and in the two species of *Renilla*, in contrast to the situation in *Aequorea*. Because both species of *Renilla* gave a very poor luminescence response in KCl solution, the unexpectedly low amounts of Ia obtained from these species of *Renilla after in vivo luminescence can be considered to be the consequence of an incomplete response of luminescence to the stimulation.

A fairly strong light-emission was observed when slices of *Leioptilus* were put into cold methanol. Moreover, the resulting methanol extract was found to contain a significantly large amount of compound Ia. This evidence, in conjunction with the amount of Ia obtained after in vivo luminescence, which is much greater than the sum of Ca²⁺-triggered luminescence activity and compound Ic (Table 1), seemingly

<table>
<thead>
<tr>
<th>Species</th>
<th>Body weight (g)</th>
<th>Luminescence in vivo (photons)</th>
<th>Ca²⁺-triggered luminescence (nmol)</th>
<th>Compound Ic (nmol)</th>
<th>Compound Ia (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aequorea aequorea</em></td>
<td>0.64</td>
<td>$9 \times 10^{18}$</td>
<td>0.7</td>
<td>0.03</td>
<td>0.6</td>
</tr>
<tr>
<td><em>Cavernularia obesa</em></td>
<td>30</td>
<td>$3.5 \times 10^{14}$</td>
<td>1.5</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><em>Leioptilus</em> (Ptilosarcus gruneyi)</td>
<td>70</td>
<td>/</td>
<td>0.7</td>
<td>2.8</td>
<td>16</td>
</tr>
<tr>
<td><em>Renilla mülli</em>eri</td>
<td>6</td>
<td>/</td>
<td>0.14</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Renilla kolikeri</em></td>
<td>5</td>
<td>/</td>
<td>0.07</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Stylatula</em> (non-luminescent)</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Leptogorgia virgulata</em> (non-luminescent)</td>
<td>10–40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Data for a single average-sized specimen of each species, in the contracted state.

* By stimulation with KCl, without mechanical stimulation.

* Luminescence due to photoproteins and luciferin binding proteins are included in these data. Quantum yields of all Ca-triggered luminescence reactions are assumed to be the same as that of aeqourin, i.e., 1 nmol emits $1.3 \times 10^{18}$ photons (2).

* This refers only to a thin strip cut off from the margin of umbrella. The weight of a whole body is approximately 50 g.

* The sample was pigment and not transparent; thus a considerable loss of photons due to absorption would be expected.

* Data not obtained due to inadequate excitation by KCl.
indicates the presence of IIc in a considerably greater amount than actually obtained, or the presence of a considerable amount of free IIIa or IIIa bound to a luciferin binding protein (but largely unmeasurable by addition of Ca²⁺ in the present procedure), or perhaps the presence of the combined effects of these in a living unstimulated Leioptilus.

Nonluminescent anthozoans, including *Leptogorgia virgulata* and specimens resembling *Stylatula*, when treated in the same manner as the luminescent species described above, did not give any indication of Ca²⁺-triggered luminescence, or the presence of compound IIe or compound Ia. The necessary conclusion is that the evidence hitherto obtained must indicate that structure Ia represents the same light-emitting molecule produced in the bioluminescence reactions of *Aequorea*, *Cavernularia*, *Leioptilus*, and at least 2 species of *Renilla*. The structure of this molecule prior to the luminescence reaction can be represented by IIIa (in the luciferin-luciferase reaction) or by its enolized structure IIa (in the photoprotein), and reveals only a superficial tautomeric difference between the photoprotein type and luciferin-luciferase type of luminescence in this context.

This conclusion is seemingly applicable also to many other bioluminescent coelenterates. In fact, Cormier et al. (11) have reported that the samples of “luciferyl sulfate” extracted from the hydrozoan, *Obelia*, the ctenophore, *Mnemiopsis*, and the anthozoan, *Renilla reniformis*, were all apparently similar to the one extracted from the species studied in the present report. The hydrozoan, *Obelia*, the scyphozoan, *Pelagia*, and other species have not been examined in the same regard, but it has been reported (12, 17) that they have a photoprotein type of luminescence system like *Aequorea*, as does also the hydrozoan, *Halostauria* (18). Recent studies indicate that photoproteins like sequorin are involved in the ctenophores, *Mnemiopsis* and *Beroe* (19).

Finally, we propose that compounds Ia, Ic and IIIa be named respectively, “coelenteramide”, “coelenteramidine” and “coelenterazine”*, because of their uniqueness and widespread involvement of these compounds in bioluminescent coelenterates. Although the first natural compound, which was isolated in a pure state and also for which structure IIIa was assigned, derived from the bioluminescent squid *Watasenia* (20), the close relationship of this compound to the other two compounds should justify the proposed terminology.

We thank Dr. T. Goto and Dr. S. Inoue for a sample of synthetic compound IIIa, and for help in various ways; Dr. T. Kikuchi and Mr. A. Sakashita for specimens of *Cavernularia*; Mr. Cleve Vandersluyys for specimens of *Leioptilus* and *Stylatula*-like sea pens; and Dr. M. J. Cormier for a sample of partially purified *Renilla* luciferase. We also thank NSF and ONR for partial support.