CALCIUM-TRIGGERED LIGHT EMISSION IN RENILLA. A UNITARY BIOCHEMICAL SCHEME FOR COELENTERATE BIOLUMINESCENCE.

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Summary

A system which emits light upon calcium addition was obtained from the bioluminescent sea pansy Renilla by extracting in EDTA. The activity has properties similar to photoproteins isolated from other coelenterates, but differs by virtue of being associated with a particulate material. A unitary biochemical scheme in coelenterates is proposed, in which the oxidation of a luciferin is postulated to form a stable protein (enzyme)-bound peroxide intermediate, whose further reaction to form a dioxetane ring is triggered by the Ca++-Protein interaction. The concerted cleavage of the dioxetane ring yields one of the carbonyl fragments in the excited state.

Among the different classes of coelenterates there have been described two bioluminescent systems which have appeared to be quite different biochemically. The experiments described below indicate that the two systems are actually very similar; that in one a relatively stable enzyme-bound intermediate was isolated, while in the second a more complete system was studied without, however, involving the separate isolation of this intermediate.

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Its isolation is reported here.

The second of the two systems, which we believe to be the more complete one, has been isolated and studied by Cormier and his associates from the anthozoan, Renilla reniformis, the common sea pansy.[1,2,3,4,5]. From whole animal extracts they isolated a substrate (Renilla luciferin) and enzymes, and demonstrated two steps which they represented[6] as follows:

(1) LH_2-X $\xrightarrow[\text{Enz 1}]{\text{Ca++}}$ LH_2+X
DPA

(2) LH_2+O_2 $\xrightarrow[\text{Enz 2}]{}$ light

In the first step the activation of luciferin (LH_2) involves the removal of some group, X, tentatively identified as sulfate, stimulated by the cofactor 3', 5' diphosphoadenosine (DPA). The second step is the energy yielding oxidative reaction which ultimately leads to an excited state and the emission of light. A requirement for calcium was also found.

From the hydrozoans Aequorea and Halistaura, Shimomura et al.[7,8,9]. isolated and characterized an unusual type of protein, one which emits light when it is simply mixed with calcium ions.

(3) Protein $\xrightarrow{\text{Ca++}}$ light

The emission is rapid, occurring as a flash lasting about 1 sec at 20° ($k = 1\ sec^{-1}$), and the quantum yield is quite high, about 0.14[10]. Aside from the protein and calcium, no other factors, including oxygen, are required. We have reported the occurrence of similar luminescent proteins in several other hydrozoans, and in the ctenophore Mnemiopsis as well, which falls into a different, though closely related, phylum[11]. We have also isolated

a similar protein from the scyphozoan Pelagia noctiluca. Adopting the term photoprotein as defined by Shimomura and Johnson[12] we propose that this whole class of luminescent proteins be referred to as calcium activated photoproteins[13].

It is evident from this information that the Renilla reaction could, in a terminal set of reactions following (1) and (2), involve an enzyme intermediate whose light emission is triggered by calcium in a fashion similar to the other coelenterates. Thus, reaction (2) would form an enzyme-bound intermediate of a relatively stable nature which would correspond to the calcium-activated photoprotein of reaction (3). We therefore extracted Renilla in a fashion designed to demonstrate such an intermediate and found that it does contain a calcium-activated system whose properties are similar, though not identical, to those obtained from other coelenterates.

Renilla koellikeri was obtained from the Santa Barbara, California area through the courtesy of Dr. James Case. The luminescent autozooids in the expanded condition were excised in sea water, rinsed briefly in distilled water and extracted in 10 mM Tris buffer with 40 mM EDTA at pH 8.9 using a tissue homogenizer. The debris was removed by filtration with Whatman #1 paper; the addition of excess calcium to the supernatant resulted in luminescence with a half decay time of about 4 seconds at 20° C

The activity was partially purified by Sephadex G-75 gel filtration, using the extraction solution for equilibration and elution. The activity eluted in the exclusion volume, indicating an unexpectedly large size: all other calcium-activated photoproteins so far tested (+) have eluted from this column at a

(+) Aequorea aequorea; Obelia geniculata; Obelia longissima; Mnemiopsis leidyi.

position corresponding to a molecular weight in the vicinity of 20,000[11,14]. Upon centrifugation for 20 minutes at 30,000 x g in the SS-34 head of the Sorvall, the activity was fully recovered in the resuspended pellet, indicating that the activity is associated with some cell (possibly membrane) fragment or particle. The activity is not affected by osmotic shock or by ultrasonication for 1 to 2 minutes. Some activity was lost after longer periods of sonication, but all the remaining activity could be pelleted by centrifugation as described above.

Among several divalent cations tested, Ca++ is the most effective in stimulating light emission. Some luminescence occurs with Pb++, Ba++, Sr++ and Mn++, but they also inhibit the response to Ca++ added secondarily. Mg++ evokes no luminescence but is an inhibitor of the calcium triggered emission. This has also been shown to be the case in the Aequorea aequorea reaction[14].

The following additional points have been established with the Renilla system, all of which indicate its similarity to calcium-activated photoproteins from other coelenterates.

(a) The activity is heat labile: all activity is lost after 2 min at 100° C. The activity is relatively stable at temperatures between 4° and 25°C.; a substantial amount remains after 12 hours.

(b) The activity cannot be obtained a second time by the removal of calcium and its subsequent addition.

(c) As judged by dilution experiments, only a single component in the extract is involved in the activity; light emission is directly proportional over a 10-fold range to the quantity of extract present in the reaction mixture. Moreover, after dialysis for 16 hours at 4°C., 24% of the activity remained inside the bag, compared to 20% in an undialyzed control. The retention of activity after gel filtration or pelleting is further evidence in support of this conclusion.

(d) The presence of molecular oxygen is not required for this activity. After the removal of oxygen by evacuation,(+) calcium was mixed with the photoprotein

(+) The oxygen concentration was estimated to be less than 0.005% by using the oxygen-dependent in vitro bacterial bioluminescence as a test.

and the luminescence was the same as in the presence of oxygen.

(e) The luminescence intensity is temperature-dependent over the range 5° - 20° C., but the total photon yield is invariant. A dimmer but longer lasting luminescence occurs at lower temperatures.

It thus seems evident that in the Renilla bioluminescent system there do occur steps beyond (1) and (2) which involve a calcium activated reaction similar to (3). In the complete Renilla system the requirement for calcium, as well as the similar (though not identical) effects of other divalent cations, is consistent with this conclusion. The striking difference is that the calcium stimulated activity in Renilla extracts is associated with a particulate fraction. Although the nature of this association has not been determined, it is believed that the relationship has functional significance in vivo.

It seems equally evident that reactions similar to (1) and (2) must occur in the formation of photoproteins in Aequorea and other coelenterates.

Light emission has been shown to occur in the absence of oxygen in many of these forms [15,16,17.], and it has long been postulated that this is attributable to some stable (enzyme) intermediate which is beyond the step where oxygen enters, possibly a peroxide of some sort [18,19,20]. This is fully consistent with the present evidence for the coelenterates. Moreover, the bioluminescent reaction mechanisms in both Cypridina[21,22,23] and the firefly [24,25.] have now been shown to involve peroxide intermediates and to proceed via the four membered peroxide (dioxetane) ring, whose concerted cleavage to the carbonyl containing fragments leads to the typical McCapra type chemiluminescence[26,27,28,29] Based on its lifetime ($k=100\ sec^{-1}$) the intermediate in the aequorin system which is capable of emission

after free calcium has been removed [30] is postulated to correspond to the protein bound dioxetane.

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