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Crossreactivity between the light-emitting systems of distantly related organisms: Novel type of light-emitting compound

(bioluminescence/dinoflagellate/euphausid/luciferin/luciferase)

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ABSTRACT **Dinoflagellate luciferin has been found to crossreact and emit light with euphausid photoprotein; and euphausid fluorescent substance gives luminescence with dinoflagellate luciferase. Luciferin and the fluorescent substance, both highly unstable and fluorescent compounds, are biochemically similar but not identical. Preliminary spectral and chemical data suggest that both compounds contain an open-chain polypyrrole structure, novel among compounds so far known to be involved in light emission in any biological system. The dinoflagellates and euphausids are phylogenetically distant; the possibility that the latter obtain the molecule nutritionally from the former is suggested.**

Bioluminescence is ubiquitous in the oceans of the world, and the biochemistry of this phenomenon has been the object of extensive research for over 100 years. During purification of the luciferin of the dinoflagellate *Pyrocystis lunula* (1–3), it was noted that its stability and spectral properties were similar to those reported (4, 5) for the fluorescent substance (F) active in the bioluminescence of the euphausid shrimp *Meganyctiphanes norvegica*. We have found that these two substances crossreact in the two light-emitting systems, a finding that seems remarkable in view of the phylogenetic distance between the two groups. A possible explanation is that the euphausid shrimps do not synthesize the fluorescent substance but instead obtain it nutritionally from the phytoplankton.

The crossreactivity appears to be even more remarkable in light of the fact that the biochemical mechanisms are apparently distinctly different in the two systems. In dinoflagellates there is a classical enzyme–substrate reaction in which the substrate luciferin (LH_2) (1, 6, 7) is oxidized and light emission results. Among the several dinoflagellate systems thus far examined, the light-emitting components appear to be fully crossreactive (8–10). By contrast, the *Meganyctiphanes* system has been characterized as a special type of "photoprotein" system in which F catalyzes the oxidative decomposition of photoprotein (PP) with light emission coming from F (4, 5).

We report here the activity of F with dinoflagellate luciferase and of dinoflagellate LH_2 with *Meganyctiphanes* PP, and we provide preliminary data suggesting that both F and LH_2 contain an open-chain polypyrrole structure.

MATERIALS AND METHODS

Unialgal but not axenic cultures of the dinoflagellates *Pyrocystis lunula* (clone T37 isolated in the South Atlantic by E. Swift) and *Gonyaulax polyedra* (clone Gp 70 isolated from a red tide off La Jolla, CA) were grown at 17–21°C in 2.8-liter Fernbach flasks containing 1500 ml of f/2 medium (11) without the silicate but with 0.5% soil extract. *P. lunula* was grown with constant illumination (cool and warm white fluorescent lamps, 100–150 microeinsteins m^{-2} sec^{-1}, measured with a LiCor LI-192S quantum sensor (LiCor, Lincoln, NB) and harvested in stationary phase (15,000–20,000 cells per ml). *Gonyaulax* was grown on alternating light/dark cycles (12 hr each) and harvested in late logarithmic phase (12,000–14,000 cells per ml) during the middle of the dark period.

Pyrocystis LH_2 was extracted at 95°C in 2 mM potassium monohydrogen phosphate/5 mM 2-mercaptoethanol, partially purified by DEAE-cellulose ion exchange chromatography (6), and stored under argon at −80°C for later use. During this and subsequent steps, all solvents were rigorously degassed, saturated with argon, and kept ice cold. Just prior to use, this LH_2 preparation was concentrated under reduced pressure and further purified by utilizing alumina chromatography (to remove the mercaptoethanol) followed by DEAE-cellulose chromatography, by a method similar to one previously described (4). *Gonyaulax* luciferase was extracted in buffer at pH 8 (6) and purified to >95% purity by utilizing blue agarose (Bio-Rad Affigel Blue) chromatography (3). *Gonyaulax* LH_2-binding protein was partially purified from cells extracted at pH 8 by a modification of described methods (6, 12). After precipitation by ammonium sulfate the material was subjected to gel filtration on Sephadex G-200 and the active fractions were pooled and concentrated.

F was extracted from frozen *Meganyctiphanes* and purified by using alumina and DEAE-cellulose chromatography (4). *Meganyctiphanes* PP was purified from aqueous extracts by DEAE-cellulose chromatography as reported (4), but with the following modification: instead of the Sephadex G-25 gel filtration step, the PP eluted from the DEAE-cellulose column was precipitated with 40% saturated ammonium sulfate and purified on a Sephadex G-200 column.

Under the conditions used at 0°C, the half-life of pure LH_2 (and also of F) was approximately 6 hr; it was about 2 hr for PP and several days for luciferase. Assays utilizing luciferase were carried out at room temperature (25°C) in 2 ml of 0.35 M potassium hydrogen phosphate/0.1 mM EDTA, pH 6.3, containing 0.1% bovine serum albumin. PP was assayed in 5 ml of 20 mM Tris/150 mM NaCl, pH 7.6, at 6°C ± 1°C. Luminescence was measured with a photometer operated in the integration mode and calibrated in quanta with *Cypridina* bioluminescence (13).

RESULTS AND DISCUSSION

The absorption spectrum of purified *Pyrocystis* LH_2 closely matched that of F of *Meganyctiphanes* (Fig. 1). In addition, the fluorescence emission spectra, which have been published for both substances (5, 6), are essentially indistinguishable. As

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Abbreviations: LH_2, dinoflagellate luciferin (substrate); F, fluorescent substance in bioluminescence of shrimp (*M. norvegica*); PP, photoprotein.

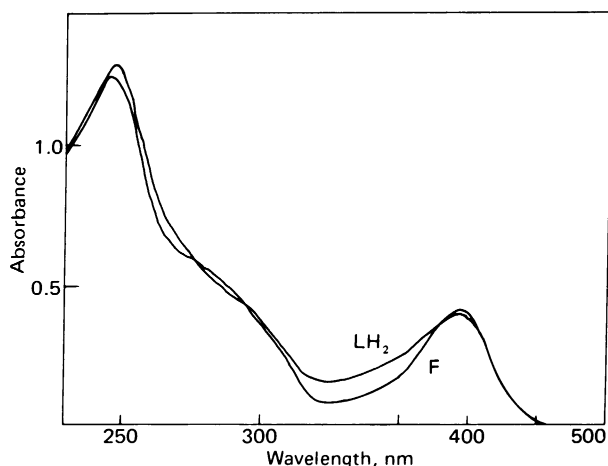

FIG. 1. Absorption spectra of purified dinoflagellate (*Pyrocystis*) LH_2 (solid line) and euphausid (*Meganyctiphanes*) F (dashed line) in 10 mM potassium arsenate/130 mM NaCl/50% ethanol, pH 7.4. There was no detectable absorption above 500 nm.

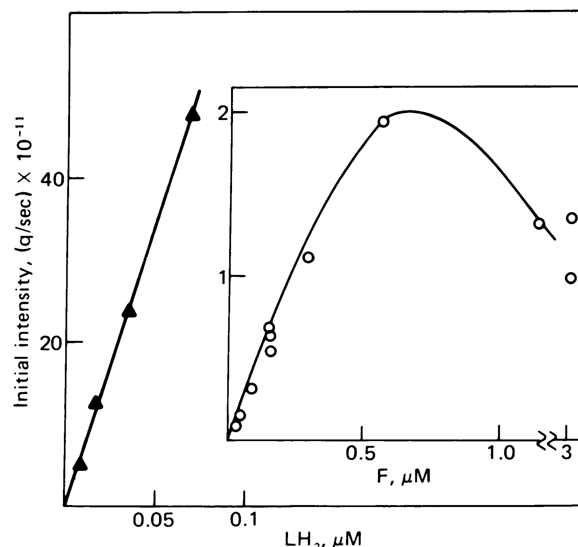

FIG. 2. Bioluminescent reactions of *Pyrocystis* LH_2 (▲) and *Meganyctiphanes* F (O), both with 0.4 μg of purified *Gonyaulax* luciferase. For each experiment, the initial light intensity is plotted against the final concentration of LH_2 or F in the reaction mixture. Note the different scales for the two substances.

also previously noted, both substances are very unstable and are readily inactivated by autooxidation as well as by acidic conditions. By titrating with potassium ferricyanide, the extinction coefficient of *Pyrocystis* LH_2 was found to be 2.76×10^4, assuming a two-electron transfer oxidation; the reported (4) value for *Meganyctiphanes* F was 2.84×10^4.

The preliminary indications are that both of these compounds contain some type of polypyrrole structure, possibly resembling a bile pigment. Both gave a positive Schlessinger test (14), diagnostic of dipyrrylmethane structures: treatment with zinc acetate and iodine yielded a complex with an absorption maximum at about 480 nm and a green fluorescence. Upon prolonged storage without a reducing agent, even at −80°C, both substances yielded a blue pigment (absorption maximum, 620 nm) as a result of slow oxidation. Samples of both compounds were also subjected to degradation by chromate oxidation according to the method of Rudiger (15). In both cases, low-resolution mass spectra of the degradation products revealed fragments of m/e consistent with maleimides and therefore diagnostic of a bile pigment-type structure. The molecular weight of LH_2 (about 550 by gel filtration) and its positive Ehrlich reaction (16) are also consistent with an open-chain polypyrrole-type structure.

The euphausid F reacted with dinoflagellate (*Gonyaulax*) luciferase to give light but with less than 1% of the activity of dinoflagellate (*Pyrocystis*) LH_2 (Fig. 2). This lower light yield could be due to a lower quantum yield in the reaction or to a decreased velocity or to a combination of both. With dinoflagellate LH_2 the V_{max} was not reached in these experiments, so the K_m cannot be estimated. With *Meganyctiphanes* F a maximal rate (V_{max}) was reached at 0.6 μM, indicative of a K_m of about 3×10^{-7} M. This compares with a previous estimate of $K_m = 4.7 \times 10^{-7}$ M for *Pyrocystis* LH_2 with *Gonyaulax* luciferase (17). With F at greater than the optimal concentration, the reaction was inhibited, possibly due to the presence of some inhibitory substance, such as a breakdown product, usually present in less-purified preparations of this compound.

The test in the converse sense gave a stronger crossreaction (Fig. 3). With a fixed amount (100 μg) of *Meganyctiphanes* PP, dinoflagellate LH_2 stimulated the reaction at a rate equal to about 30% of that achieved with euphausid F. Again, it is not known what the apparent difference in efficiency should be attributed to but, in any case, the euphausid PP evidently is less fastidious than is the *Gonyaulax* luciferase with regard to the structure of the compound with which it reacts. In the activity tests there was a more marked deviation from linearity with dinoflagellate LH_2 at higher concentrations.

Both of the crossreactivity tests reported above relate to the ability of the molecules to function in a light-producing reaction. A different test for crossreactivity was carried out by using a second protein from *Gonyaulax*, one not directly involved in

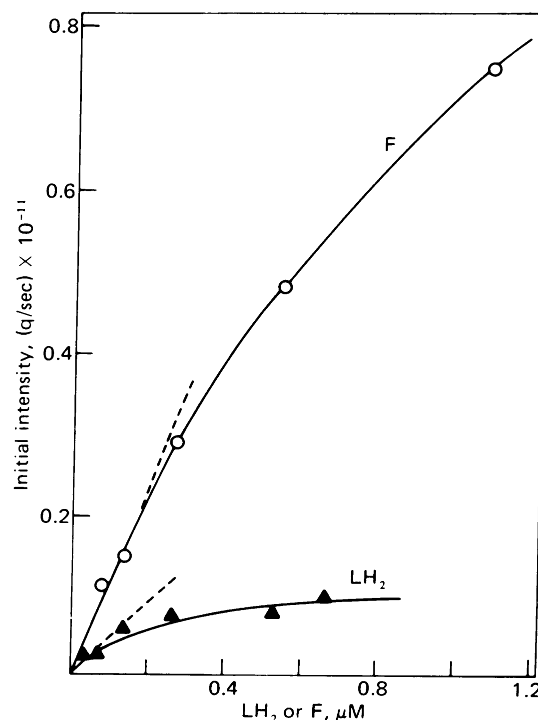

FIG. 3. Bioluminescent reaction of *Pyrocystis* LH_2 (▲) and *Meganyctiphanes* F (O), both with 100 μg of partially purified *Meganyctiphanes* PP and plotted on the same scale. In each experiment, the initial light intensity is plotted against the final concentration of LH_2 or F in the reaction mixture.

catalyzing light production. This is the specific LH_2-binding protein (6, 12); it occurs in extracts of some dinoflagellates, including *Gonyaulax polyedra*, but not in some other species such as *Pyrocystis lunula* (10). This protein (molecular weight, approximately 110,000) binds LH_2 at pH 8 (but not at pH 6) with a high affinity such that upon gel filtration (Sephadex G-25, pH 8) of a mixture of the two the LH_2 is bound and elutes with the higher molecular weight protein (12). In this same test, F was similarly bound to and eluted with the LH_2-binding protein. With the small molecules in large excess over the binding protein in both experiments, only about 15% as much F as LH_2 was bound (Fig. 4).

It is certainly possible that the two low molecular weight molecules may have evolved independently; but it is also possible that the euphausid shrimps may utilize nutritionally derived dinoflagellate LH_2 for their fluorescent substance. If so, the situation becomes even more intriguing when the biochemical aspects are considered. A molecule that is utilized as a more or less "conventional" substrate in the dinoflagellate oxidative bioluminescent reaction is evidently adapted to function in the *Meganyctiphanes* reaction in an apparently quite different biochemical role. The type of reaction mechanism that appears to occur in the euphausid system, involving a fluorescent molecule in a catalytic role, was unknown in model chemiluminescent systems at the time the *Meganyctiphanes* reaction was first described. However, quite recently it has been shown that the breakdown of certain cyclic peroxides

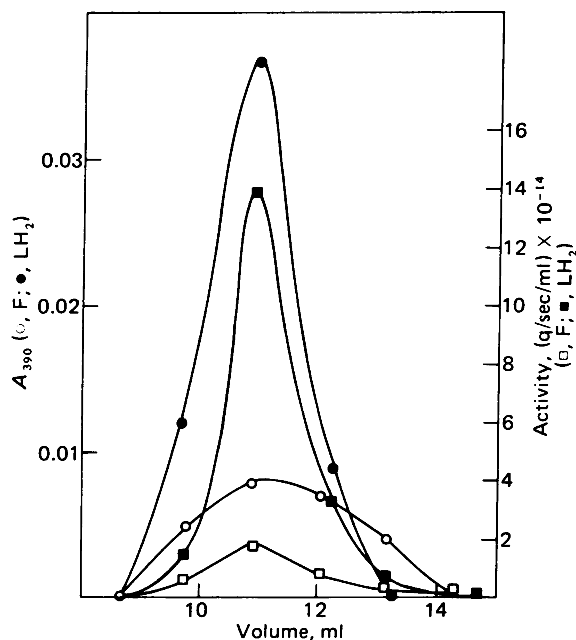

FIG. 4. Binding of *Pyrocystis* LH_2 and *Meganyctiphanes* F by *Gonyaulax* LH_2-binding protein. In two separate runs, the same amount (10 nmol, based on extinction coefficients given in text) of F and of LH_2 were combined in 0.6 ml with 0.5 mg of partially purified binding protein and subjected to gel filtration at 4°C on a 25-ml Sephadex G-25 column (0.9 × 30 cm), equilibrated with 50 mM Tris/5 mM EDTA/1 mM dithiothreitol/125 mM NaCl, pH 8.3. Some of the LH_2 and F was bound by the binding protein and eluted in the void volume as shown in the figure; much more of both (about 10-fold greater than that bound) eluted in the salt volume, peaking between 30 and 45 ml (not shown). Based on the activity of each substance with *Gonyaulax* luciferase plotted after correction for crossreactivity difference from Fig. 2 and summed over all fractions under the void volume peak, about 15% as much F was bound as LH_2. Considering absorption at 390 nm, which has some error due to absorption by the binding protein preparation, the value was 22% based on the peak tube and 36% based on the integral of all tubes.

is catalyzed by various dyes having the common property of a low oxidation potential and that the resulting light emission involves the singlet excited state of the participating dye (18, 19). The possibility that such a mechanism is involved in the breakdown of the PP by F is intriguing.

The finding that there is crossreactivity but not complete interchangeability between the molecules is most easily explained by assuming that they are chemically similar but not identical. If the sensitized oxidation of the PP involves a process such as the dye-catalyzed mechanism (18, 19) mentioned above, it would not be surprising that these two quite similar and easily oxidized compounds are partially interchangeable in this reaction. On the other hand, even with some of the more classical bioluminescence substrates used in different systems, relatively small differences in structure are known to result in large changes in light production (20–24). If the euphausids do obtain the molecule nutritionally, it is not unreasonable to assume that it might be chemically altered prior to use in the luminescent system.

Until recently it was believed that the different bioluminescent systems in different phylogenetic groups had evolved independently and consequently are biochemically distinct, a view that gained credence from the fact that luminescence exhibits no evident phylogenetic or evolutionary continuity. Indeed, a considerable number of chemically different bioluminescent reactions are now recognized (25), including bacteria (flavin, a long-chain aldehyde), coelenterates (coelenterazine, an imidazopyrazine), firefly (benzothiazole), *Latia* (sesquiterpene), and earthworm (*N*-isovaleryl-3-aminopropanol; H_2O_2). However, it has recently been shown that coelenterazine is widespread in its occurrence in biological systems, and indeed it has been suggested that the molecule is obtained by some organisms in ingested food (26). A similar nutritional origin has been suggested for an ostracod crustacean (*Cypridina*) luciferin (an imidazopyrazine) found in the fishes *Parapriacanthus* and *Porichthys* (27–29). In the present study we found yet another type of chemically distinct light-emitting molecule that, moreover, is utilized by two phylogenetically distinct groups. At the same time, the two reaction mechanisms are evidently quite different and utilize the molecule in distinctly different ways.

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