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CHEMICAL NATURE OF THE LIGHT EMITTER IN BIOLUMINESCENCE OF AEQUORIN

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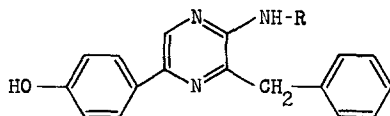
The photoprotein aequorin (M.W. ca. 30,000), isolated from the bioluminescent jellyfish Aequorea (1,2), emits blue light in aqueous solution by an intramolecular reaction when Ca^{2+} is added, and becomes transformed into a product BFP (blue fluorescent protein) which shows a fluorescence spectrum almost exactly corresponding to the bioluminescence spectrum (3,4). A compound designated "AF-350" which contains the chromophore of the light-emission was previously separated from the photoprotein (3), and the structure of this compound has been determined (5,6). We now find, however, that the actual light emitter is not the excited state of AF-350 (M.W. 277) itself nor that of AF-350 covalently bound to the protein part, but is the excited state of a substituted AF-350 (M.W. 411). The structure of this substituted AF-350 is reported herein. Moreover, the structure of the chromophore prior to the luminescence reaction is discussed on the basis of evidence found in this same study.

Aequorin was prepared as previously reported (3,4), then converted quantitatively to BFP by the addition of an excess of $CaCl_2$. Synthetic AF-350 (I) was a generous gift from Dr. Y. Kishi, Nagoya University, and acetyl AF-350 (II) was prepared by treating the synthetic AF-350 with acetic acid plus acetic anhydride at 70°C for 30 minutes, followed by tlc (silicic acid, water-saturated ethyl ether) to remove by-products.



I: R = -H

II: R = $-COCH_3$

III: R = $-CO-R_x$

Treatment of BFP (0.7 μmol) in 1 N HCl at 25°C for a few minutes, followed by tlc (silicic acid, water-saturated ethyl ether) of n-butanol extract of the product, afforded a blue fluorescent compound designated as III. The compound III was further treated with

1 N HCl at room temperature for 3 days, and the product, after neutralization with $NaHCO_3$, was repeatedly extracted with ethyl ether to remove highly fluorescent AF-350 (I) produced by hydrolysis, as well as to remove the unreacted part of compound III. The residual aqueous layer was acidified with HCl, then extracted with ethyl ether yielding an acid $R_x \cdot COOH$.

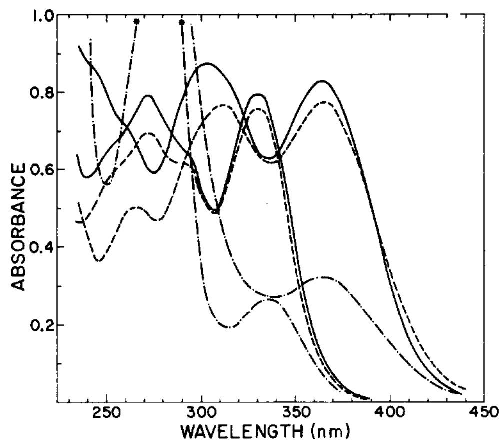


Fig. 1

Absorption spectra of BFP (—— - —— -), II (-----), III (———), in neutral aqueous solution, and spectra shifted to longer wavelength in 0.01 N NaOH (II, III) or at pH 11.2 (BFP).

Similarity in UV spectra of BFP and acetyl AF-350 (II) suggests that, in BFP, the amino group on the pyrazine ring is acylated, and this is supported by a good agreement in UV spectra between II and the product of acid treatment (III) (Fig. 1).

The mass spectrum of III (Fig. 2) further supports the view of an acylated amino group on the pyrazine ring in III, and consequently in BFP, as indicated by the presence of peaks at m/e 107 (R_x^+), 134 (COR_x^+ - H), 277 (M^+ - COR_x + H) and 304 (M^+ - R_x) in addition to peaks observed in the mass spectrum of AF-350 (5). Moreover, the M + 1 peak (28.8% of M^+ peak) indicates that the R_x group in III must have the formula of C_7H_7O or $C_6H_7N_2$, suggesting the presence of an aromatic ring or a heteroaromatic ring.

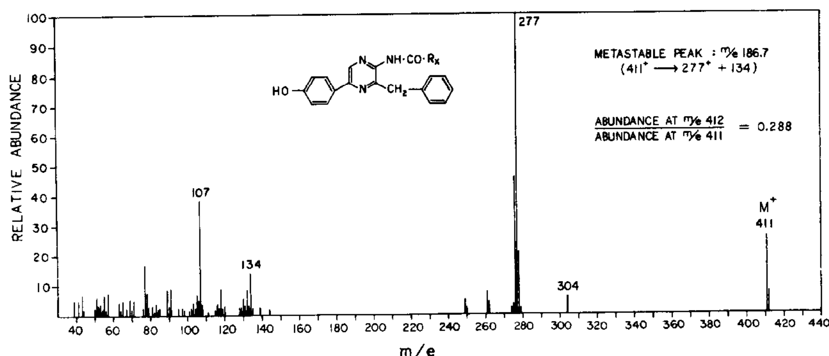


Fig. 2. Mass spectrum of the compound III (direct introduction), at 70 eV.

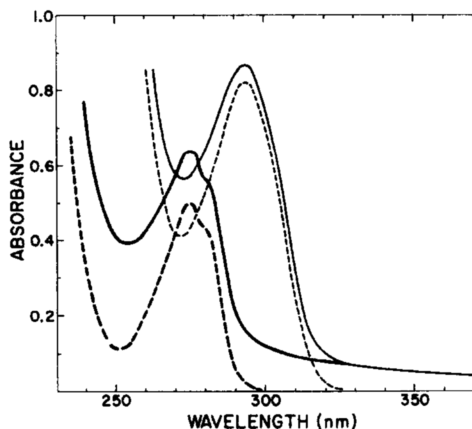
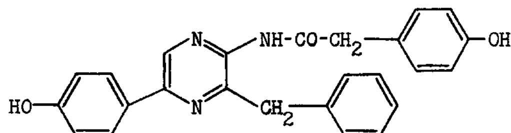


Fig. 3

Absorption spectra of R_x·COOH (———), and p-hydroxyphenylacetic acid (-----), in 0.01 N HCl, and in 0.01 N NaOH (with peaks shifted to longer wavelength).

An acidic compound, R_x·COOH, obtained by the hydrolysis of III, showed a characteristic shift of UV absorption peak by addition of alkali, suggesting the presence of a phenolic OH, and spectral curves of this compound agreed quite satisfactorily with those of an authentic sample of p-hydroxyphenylacetic acid (Fig. 3), whereas these curves were considerably different from the absorption spectra of o- and m-hydroxyphenylacetic acid.

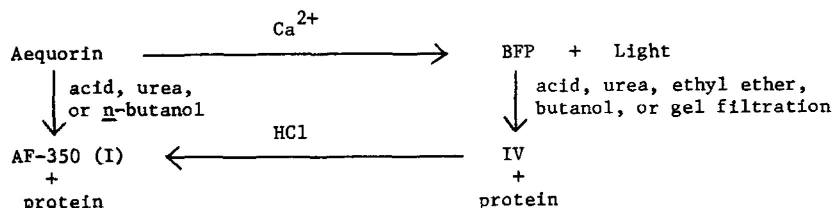
From the evidence described above, we conclude the structure of compound III to be as represented in IV.



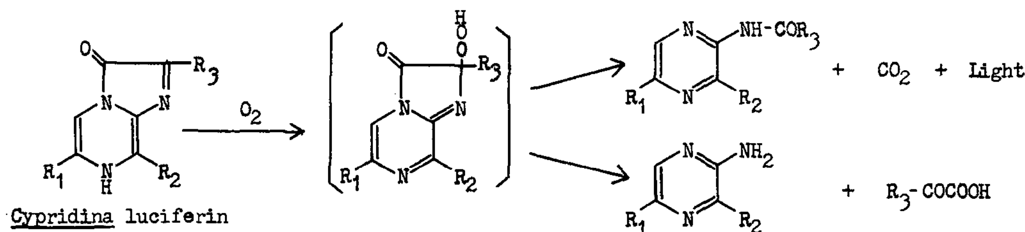
IV

Compound IV was synthesized by heating AF-350 (2 μmol) with p-hydroxyphenylacetic acid (20 μmol) at 185°C for 40 min. under a vacuum of 20 mm Hg, followed by removal of the excess acid under a higher vacuum. After purification by tlc (silicic acid, water-saturated ethyl ether), the product (ca. 50% yield) was indistinguishable from compound III in UV spectra, tlc behavior and mass spectrum, thus confirming our conclusion.

Compound IV can be separated from BFP by various methods as diagrammed on the next page. When separated by shaking with ethyl ether or by gel filtration, neither IV nor the protein residue was appreciably fluorescent in aqueous solution. However, mixing of the two aqueous solutions, in the presence of a trace of Ca^{2+}, instantly restored a bright blue fluorescence (λ_{max} 469 nm) identical to that of BFP, and therefore corresponding exactly to the bioluminescence spectrum. It follows that the excited state of IV present in BFP is the light emitter in the bioluminescence, and further that IV is dissociably bound to the protein part in BFP.



In native aequorin, a functional group, of still uncertain structure, but most likely containing all C-C and C-N linkages which exist in IV, binds tightly to the protein part, and no dissociation has yet been observed. Thus, as indicated in the above scheme, the C-N bond at the amino N on the pyrazine ring in aequorin is split by mild treatment, *e.g.*, by saturating with urea or shaking with butanol, yielding I with a free amino group. This unusual splitting, in addition to a structural resemblance, recalls the oxidation of *Cypridina* luciferin, in which splitting of an equivalent bond is observed in one of the pathways (7), as shown below.



The functional group of aequorin, however, appears not to contain a *Cypridina* luciferin type skeleton shown above, because, in addition to the fact that aequorin does not require O_2 in the luminescence reaction, the UV absorption of aequorin ($\lambda_{max}^{H_2O}$ 465 nm, ε *ca*. 2000) differs markedly from various *Cypridina* luciferin type compounds ($\lambda_{max}^{H_2O \text{ or } MeOH}$ *ca*.430 nm, ε *ca*.10,000) each with different substituents at R_1, R_2 and R_3 (8,9,10). Examination of the possibility that aequorin might contain a stabilized form of the intermediary skeleton shown above in brackets is presently in progress.

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