Peroxidized coelenterazine, the active group in the photoprotein aequorin
(bioluminescence/calcium/functional group/luciferin)

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ABSTRACT The photoprotein aequorin emits light by an intramolecular reaction when Ca\(^{2+}\) is added under either aerobic or anaerobic conditions. Previously reported evidence has indicated two possibilities: (i) the functional group of aequorin is coelenterazine itself, a compound that plays key roles in the bioluminescence of various other types of organisms; or (ii) it is the enolized form of this compound. Present data rule out both of these possibilities, through elucidation of the structure of the yellow compound that is split off aequorin by treatment with NaHSO\(_3\). The yellow compound is now shown to be a tertiary alcohol of coelenterazine on the basis of chemical reactions, mass spectral data, and relationships to known derivatives of coelenterazine. From this structure and the method of forming the yellow compound from aequorin, aequorin evidently contains a peroxide of coelenterazine as the active group. The presence of such a peroxide is consistent with the fact that aequorin yields free coelenterazine upon treatment with Na\(\text{HSO}_3\). Although there is no applicable technique at present to determine with assurance the specific state of the peroxide in the protein, a study with \(^{18}\)O tracer indicates that a linear peroxide structure is more likely than the alternative possibility of a dioxygenate structure.

In aqueous solution, the photoprotein aequorin extracted from the bioluminescent jellyfish Aequorea emits blue light by an intramolecular reaction when a trace of Ca\(^{2+}\) is added, in the presence or absence of molecular oxygen (1, 2). In this Ca\(^{2+}\)-triggered reaction, nonfluorescent aequorin is converted into a blue-fluorescent protein product (BFP). The molecule of BFP is readily dissociable into the protein part plus coelenteramide (I), a compound that is responsible for the blue fluorescence of BFP (3). Our data have shown that, prior to the luminescence reaction, native aequorin can be considered to be the extremely stable intermediate of an enzyme reaction in which the substrate is coelenterazine (II). This consideration is based in part on evidence that aequorin can be regenerated from the protein part of BFP plus II by incubation under aerobic conditions (4). Compound II is chemically identical to the luciferins of various types of luminescent organisms, including those of the decapod shrimp Oplophorus and the sea pansy Renilla (5).

Because aequorin can luminesce in the absence of molecular oxygen, it has been widely postulated and believed (3, 6–9) that this protein contains a peroxide as a necessary source of energy. The published reports, however, have been unclear and, in fact, confusing in regard to the binding site of such a peroxide in the aequorin molecule. By 1974, various investigators had postulated (3, 8, 9) that in the aequorin molecule a peroxide is covalently bound to compound II, at the imidazolone carbon to which the \(p\)-hydroxybenzyl group is attached, although only a partial structure of II had been established at that time.

In 1974 we inferred from our results (2) that, for a light-emitting reaction, the molecule of aequorin must contain four components: (i) coelenterazine (II) in the colorless enolized form; (ii) a peroxide; (iii) a yellow chromophore of unknown structure (designated YC) as an electron acceptor; and (iv) SH group(s). In that report, the possibility that aequorin contains the peroxide of II was thought unlikely, mainly on the basis of comparison of absorption spectra.

In 1975 Cormier and associates reported (10, 11) that, in effect, aequorin contains compound II plus a separate hydroperoxide group on the basis that they "extracted" compound II from aequorin. They did not demonstrate, however, that the process of "extraction" did not cause the formation of II from a precursor present in aequorin.

The present study indicates that the structure of a "yellow compound" (YC), produced by treatment of aequorin with Na\(\text{HSO}_3\) (2), is a tertiary alcohol of coelenterazine. Moreover, this and other evidence strongly indicates that aequorin contains a peroxide of II, thus clarifying the confusing situation referred to above.

MATERIALS AND METHODS
Aequorin was extracted and purified as reported (12, 13). BFP was prepared from aequorin solutions by adding 10 mM calcium acetate in an amount to complete the luminescent reaction in about 15 sec. Oplophorus luciferase, for the luminescence test of II in pH 8.3 buffer, was a part of the material purified recently (14). Samples of synthetic compound II and two kinds of derivatives of II were gifts from S. Inoue. Dehydrocoelenterazine was synthesized as described (15). \(^{18}\)O\(_2\) gas was purchased from Prochem, Summit, NJ. Mass spectra were measured by Morgan-Schaffer Corporation, Montreal, on a Hitachi-Perkin Elmer model RMU-6D mass spectrometer. Thin-layer chromatographic separations were carried out on Gelman ITLC type SA silicic acid sheets with CH\(_3\)Cl/ethanol, 100:6 (vol/vol), as the developing solvent, except where otherwise noted.

Preparation of YC from Aequorin. YC was obtained by the Na\(\text{HSO}_3\) treatment of aequorin as described (2) but with a shortened reaction time (ca. 15 min). The purification was done abbreviated.
by twice repeated thin-layer chromatography, developing each time until the solvent front extended to about 10 cm from the origin. The YC band (RF 0.6) was eluted with ethyl ether. About 50% loss of YC occurred in each chromatography due to instability of this compound.

Oxidation of Coelenterazine (II) to YC. Coelenterazine (0.1–0.5 mg) dissolved in 1 ml of ethanol was mixed with 2 drops of 50 mM sodium phosphate buffer (pH 6.0) containing 5 mM EDTA and 1 drop of 30% H2O2. To this mixture, several microliters of a diluted aqueous CrO3 solution was carefully added so as to result in a change in the color of the fluorescence from bright yellow to blue within 1–2 min. The product was immediately mixed with 5 ml of pH 6.0 buffer (the same as used above) and then quickly extracted three times with ethyl ether (9 ml total). The ether extracts were combined, washed three times with water (2 ml total), and dried with anhydrous Na2SO4. The dried ether solution was concentrated and then purified by twice repeated thin-layer chromatography in the same manner as described above.

Reduction of YC with Na2S2O4. YC prepared from 15 mg of aequorin was dissolved in 0.5 ml of ethanol and this solution was mixed with 2 ml of freshly prepared 0.1% Na2S2O4. After 1 min the mixture was extracted with ethyl acetate. The ethyl acetate extract was quickly evaporated under reduced pressure and the residue was taken up in methanol and purified by thin-layer chromatography (silicic acid; MeOH/CH2Cl2, 1:9, vol/vol). A yellow band having yellow fluorescence (RF ca. 0.3, compound II) was extracted with methanol.

Regeneration of Aequorin in 18O2 Gas. BFP (10 mg) in 20 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 5 mM 2-mercaptoethanol was placed in the bottom of a specially designed reaction vessel, and 0.7 ml of methanol containing II (2 mg) and 1 ml of water were placed in the side arm of the same vessel. The vessel was evacuated with a vacuum pump for 5 min and then filled with 18O2 (99 atom %) to a pressure of 150 mm Hg (see ref. 16 for the detailed technique). The two solutions inside the reaction vessel were mixed by tilting and the vessel was incubated for 2 hr at 20° and then overnight at 10°. The vessel was opened and the reaction mixture was saturated with (NH4)2S2O4, and centrifuged. The precipitate, which contained 9 mg of regenerated aequorin, was dissolved in 1 ml of 10 mM EDTA (pH 7.0) and passed through a column of Sephadex G-25 previously equilibrated with 50 mM sodium phosphate containing 5 mM EDTA (pH 6.0) to separate the regenerated aequorin from the other substances (i.e., I, unreacted II, and 2-mercaptoethanol).

Assay of Cysteine Residue. One-half milligram of aequorin or of BFP in 0.5 ml of 10 mM EDTA containing Tris (pH 7.8) was treated with 0.1 ml of 4 mM N-ethylmaleimide for 15 min. Unreacted N-ethylmaleimide was inactivated by the addition of 40 µl of 10 mM 2-mercaptoethanol, and then this mixture was dialyzed overnight at 4° against 2 liters of distilled water. The dialyzed solution was mixed with an equal volume of concentrated HCl and hydrolyzed at 105°–110° for 48 hr in an evacuated sealed tube. The hydrolysate was evaporated to dryness and analyzed for S-succinylcysteine on a Beckman amino acid analyzer. The content of modified cysteine residue was calculated based on the serine, threonine, and valine contents which are known and closely similar to each other (12).

RESULTS AND DISCUSSION

Properties and Reactions of YC. Purified YC is very unstable. The stability in solutions decreases in the order: ethyl ether, ethanol, methanol, water. YC can be kept for 2–3 days at −30° in dry ethyl ether, in which YC is most stable, but it rapidly decomposes in water, even at 0° and at neutral pH. YC left standing in ether solution for an extended period, or dry YC decomposed by heating in vacuo at 130°, consisted of approximately 50% each of coelenteramine (III, formally AF-350; see refs. 17 and 18) plus an unknown blue fluorescent compound, according to thin-layer chromatography.

When a trace of HCl was added to an ethanolic solution of YC, the yellow solution (λmax (EBOH) 445 nm) instantly and irreversibly changed to colorless (λmax 385 nm) (Fig. 1). When a trace of NaOH was added to a solution of YC, the solution turned to red (λmax 530 nm). The absorption peaks at 385 and 530 nm could be reversibly shifted to each other by simply readjusting the pH of the solution.

The mass spectrum of YC was indistinguishable from that of dehydrocoelenterazine (IV) (15) and also closely resembled the spectrum of coelenterazine (II) (Fig. 2). Thus, YC is possibly structurally similar to II and IV, although these three compounds are evidently all different as judged by their UV and visible spectra (Figs. 1 and 3), fluorescence (II, yellow; IV, deep red; YC, nonfluorescent), and their distinctly different chromatographic behavior. Measurement of the nuclear magnetic resonance spectrum and the infrared spectrum was not successful because of the instability and insufficient quantity of this compound.

Treatment of YC with Na2S2O4 resulted in the formation of coelenterazine (II); the identity of II was established by mass spectrum, UV spectrum, and thin-layer chromatography. Formation of II from YC was also observed in a smaller yield (ca. 10% of the former case) when YC was incubated for 15 min with 10% 2-mercaptoethanol (cf. ref. 10). The yields of II were measured with Opilophorus luciferase which catalyzes the luminescence oxidation of II (14) but does not react with YC or IV. The effect of NaBH4 was poor in the formation of II from YC, and NaHSO3 did not produce II from YC or II.

Dropwise addition of concentrated H2SO4 into a solution of
YC in anhydrous ethyl ether with stirring at 0° resulted in a red coloration. A similar red color was also observed when perchloric acid was added to a dioxane solution of YC. In both cases, the red reaction mixture was first neutralized with NaHCO₃, and then the organic layer, now purple, was separated from inorganic precipitate, concentrated, and finally subjected to thin-layer chromatography. The purified pigment was proved to be dehydrocoelenterazine (IV) by mass spectrometry, thin-layer chromatography, and absorption spectrometry, in comparison with an authentic sample. The yield of this compound was low by either method, presumably due to the occurrence of side reactions caused by the use of strong acid. Compound IV can be reduced to II with NaBH₄ (15) but not with Na₂S₂O₄.

Synthesis of YC. Oxidation of II with various oxidants nearly always produced some YC, which was recognizable by means of thin-layer chromatography, but it usually decomposed before isolation, presumably due to the presence of some decomposition-catalyzing by-product. The method given in Materials and Methods was developed by trial and error. This method consistently yielded purified YC, although the final yield was only approximately 5%. The product was indistinguishable from YC obtained from aequorin according to UV spectrometry, mass spectrometry, thin-layer chromatography, and the chemical reactions described above.

Structure of YC. Based on the properties and reactions described above, we assign structure V to YC, as shown in the following scheme together with the summary of its reactions.

Structure V appears to be consistent with the decomposition of YC to coelenteramine (III) and with the reduction of YC to II by Na₂S₂O₄, considering the presence of the N—C—OH bond and the tertiary alcohol of allyl type. Identical mass spectra of YC and dehydrocoelenterazine (IV) (Fig. 2) are interpreted as indicating a strong tendency of YC to dehydrate in a mass spectrometer, by the loss of the tertiary alcoholic OH and one H of the β-carbon, similarly to the dehydration caused by the action of H₂SO₄ in ether or by the action of perchloric acid in dioxane.

An isomer of V, having a secondary alcoholic OH (instead of the tertiary alcoholic OH) at the methylene carbon of the p-hydroxybenzyl group and with the same imidazopyrazine skeleton as II, cannot be the structure of YC; this compound, obtainable by treatment of IV with aqueous alkali (unpublished data), showed an UV spectrum, yellow fluorescence, and ability to chemiluminesce all resembling those of II but differing from those of YC.

The colorless product (λₑₒ₂max 385 nm) obtained from YC by a trace of acid and the red product (λₑₒ₂max 530 nm) similarly obtained by alkali were both clearly different from I, II, III, and IV, as judged by absorption spectra and thin-layer chromatography. We could not determine the structures of those compounds, mainly because of their highly labile nature. According to presently available evidence, however, the colorless compound might be an isomeric compound of V, with the tertiary alcoholic OH of V shifted to the pyrazine-carbon having one H (cf. structure X, ref. 19), and the red compound could be the dianion of this isomeric compound.

**FIG. 2.** Mass spectra of YC and dehydrocoelenterazine (IV) at 190° (coincide with each other) (A) and of coelenterazine (II) at 180° (B), all at 70 eV, by direct introduction of samples.
The Active Group of Aequorin. The presence of YC (V) in native aequorin is a highly unlikely possibility on the following basis: (i) V is apparently not an intermediate in the luminescent oxidation of II, (ii) YC did not emit light in the presence of BFP plus H₂O₂ plus Ca²⁺ under aerobic or anaerobic conditions, (iii) attempts to extract YC from dry aequorin with various organic solvents (ethanol, ether, CHCl₃, dioxane, etc.) in the absence of NaHSO₃ uniformly failed.

The fact that YC has been obtained from aequorin by NaHSO₃ treatment suggests that the structure of its precursor present in aequorin would most likely be VI or VII—namely, peroxides of coelenterazine (II). The absorption spectra of these peroxides (VI or VII) would be expected to be similar to that of V (λ_max 445 nm), absorbing in the same region (2) as aequorin (λ_max 465 nm). The suggestion by Hori et al. (11) that aequorin contains a tautomeric form of II (equivalent to V with the tertiary OH replaced by H) now becomes understandable inasmuch as the tautomer of II is spectrometrically equivalent to VI.

In an effort to determine which of the above two structures might be present in aequorin, we prepared active aequorin from BFP and coelenterazine under an atmosphere of 99% ¹⁸O₂ gas and then prepared YC from this aequorin. Aequorin prepared in this manner should contain a peroxide group nearly completely labeled with two ¹⁸O atoms, but the YC prepared from this aequorin can contain an ¹⁸O-labeled carbonyl group only when aequorin contained VII. The mass spectrum of this YC sample showed no signal at m/e 423, indicating that the carbonyl group had not been labeled with ¹⁸O. Although this result tends to support the presence of VI in aequorin, rather than VII, no conclusion can be reached until detailed knowledge is available concerning the reaction of NaHSO₃ on both structures. In any event, the final confirmation of the peroxide structure would be achievable only through physical methods that do not induce any chemical change in the aequorin molecule, and no such method is available for aequorin at present; x-ray crystallography is not applicable because aequorin has not been, and perhaps cannot be, crystallized.

By analogy with the formation of II from YC by Na₂S₂O₄ treatment, VI and VII, as well as aequorin which contains VI or VII, should produce II by the same treatment. To test this point, a solution of aequorin was treated with an equal volume of fresh 0.2% Na₂S₂O₄ for 30 sec. The product emitted strong light upon the addition of Oplophorus luciferase, indicating the formation of II, and thus providing support for the presence of VI or VII in aequorin. The total light emitted revealed that the yield of II from aequorin was slightly more than 50% on the basis of the number of molecules, based on a quantum yield of 0.34 for Oplophorus luminescence (14). Ward and Cormier (10)

![Fig. 3. Absorption spectra of coelenterazine (II) in ethanol (14.6 μg/ml) (curve 1), dehydrocoelenterazine (IV) in ethanol with or without 0.01 M HCl (curve 2) (cf. ref. 15), and the same compound in ethanol containing 0.01 M NaOH (curve 3). Concentrations for curves 2 and 3 were the same but not determined. Light path, 1 cm.](image1)

![Fig. 4. Postulated mechanism of luminescence and regeneration of aequorin.](image2)
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reported that they had "extracted" Renilla luciferin [i.e., II (5)] from aequorin by first treating aequorin with NaHSO₃ and then incubating the reaction mixture with 10% 2-mercaptoethanol under anaerobic conditions. We now interpret this to be not a true extraction but rather the result of a series of chemical reactions probably involving the formation of V by NaHSO₃ treatment of aequorin and the reduction of V to II by 2-mercaptoethanol treatment. In fact, II, has never been obtained by ordinary extraction with an organic solvent.

Fig. 4 summarizes present knowledge concerning the structure of the functional part of aequorin, the bioluminescent reaction triggered by Ca²⁺, and the regeneration of aequorin. The phenolic OH of the p-hydroxybenzyl group would be unessential in aequorin because BFP formed an isoaequorin in the presence of IIa plus O₂, in a fashion similar to the regeneration of aequorin with II; the product isoaequorin was yellowish (λ_max 465 nm, identical to aequorin) and showed a Ca²⁺-triggered bioluminescence reaction closely similar to that of ordinary aequorin. The phenolic OH of the p-hydroxyphenyl group was thought to be bound to the protein part, possibly by an ionic bond (2). We now believe that this OH is essential because BFP did not form an isoaequorin in the presence of IIb plus O₂.

In regard to SH groups in aequorin, our previous result by carboxymethylation (12) indicated that aequorin contains 1.9 cysteine residues (i.e., 2 SH) on the basis of the molecular weight of approximately 21,000 (ref. 20; unpublished data). In the present study, we used N-ethylmaleimide to modify SH group(s) in the assay of cysteine residues. By this method, BFP was shown to contain 2.1 cysteine residues per molecule, in agreement with the previous data on aequorin. In the case of native aequorin, treatment with N-ethylmaleimide for 15 min caused a 95% loss of Ca²⁺-triggered luminescence activity, together with an increase in the weak, spontaneous luminescence (without Ca²⁺) of the reaction mixture (2) to the highest level. The aequorin modified to such an extent was found to contain only 0.95 residue of N-ethylmaleimide-modified cysteine per molecule of aequorin. The results seem to indicate, among other things, that one SH group which readily reacts with N-ethylmaleimide has an important role in stabilizing the active moiety of aequorin. Thus, when this SH group is blocked, a spontaneous reaction of the active group results in a weak, continuous luminescence.

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