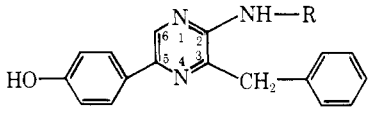
Mechanism of the Luminescent Intramolecular Reaction of Aequorin†

Osamu Shimomura,* Frank H. Johnson, and Hiroshi Morise

ABSTRACT: The photoprotein aequorin emits blue light by an intramolecular reaction when Ca^{2+} is added. The structure of the light-emitting chromophore prior to the reaction has now been deduced from various data, including the structure of the chromophore after light emission, analyses for CO_2 and HCOOH in the reaction product, analysis of carbonyl compounds in acid-treated aequorin, properties of a compound (AF-400) obtained by $NaHSO_3$ treatment of aequorin, absorption spectrum of the functional chromophore of unreacted aequorin, and structure–absorption spectrum correlation of model compounds. Evidence is described indicating that addition of Ca^{2+} leads to light emission of aequorin through reaction(s) between the chromophore and both a yellow compound ("YC") and H_2O_2 in the same molecule. The bioluminescent reaction depends on the presence of SH group(s), probably representing cysteine residue(s) of the protein part. The yellow compound, first found in the present study, is regarded as a hydrogen acceptor. All reactants necessary for light emission, except Ca^{2+} and solvent water, are self-contained in the photoprotein aequorin molecule.

The photoprotein aequorin (mol wt 30,000), isolated from the bioluminescent jellyfish *Aequorea*, emits blue light by an intramolecular reaction when Ca^{2+} is added in either the presence or absence of molecular oxygen (Shimomura *et al.*, 1962, 1963; Kohama *et al.*, 1971).

Although native aequorin is nonfluorescent, both urea-denatured aequorin and the product of the Ca^{2+}-triggered bioluminescent reaction of aequorin fluoresce blue, and the latter product, designated blue fluorescent protein or BFP,[1] shows a fluorescence emission spectrum identical with that of the bioluminescence spectrum (Shimomura and Johnson, 1970a). The blue fluorescent moiety in the urea-denatured aequorin, designated AF-350, was isolated and its structure determined as I (Shimomura and Johnson, 1972; Kishi *et al.*, 1972). Further study revealed that the blue fluorescent moiety present in BFP has the structure II, and also that II, when bound to the protein part of BFP, is the light emitter in the bioluminescence reaction (Shimomura and Johnson, 1973a).

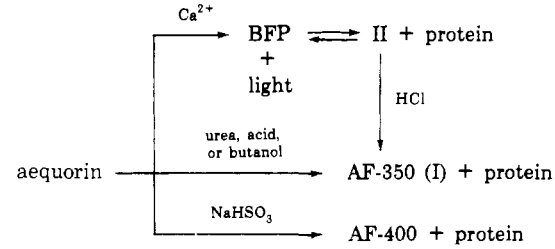


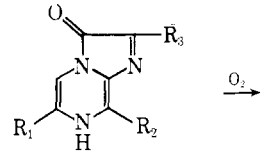
I, R = H
II, R = *p*-$COCH_2C_6H_4OH$

The relationship involving four compounds, namely, three mentioned above (aequorin, AF-350 and II) and the blue-fluorescent substance AF-400 described in an earlier publication (Shimomura and Johnson, 1969, 1973a), is diagrammed in Scheme I.

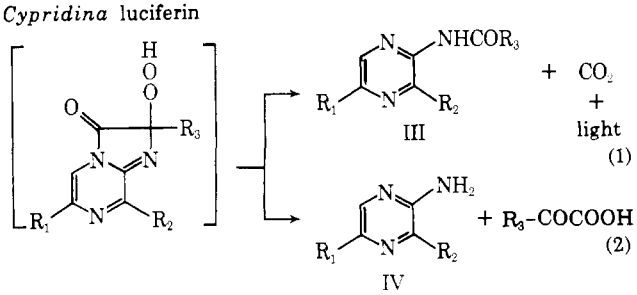
Because urea would not hydrolyze the NH–CO bond of II, I must have been produced by some kind of mechanism induced by the denaturation of the protein moiety of aequorin. This situation regarding the splitting of the NH–CO bond, as well as the structures of I and II, bear a resemblance to the oxidation products of *Cypridina* luciferin shown in the following equations, wherein R_1, R_2, and R_3 are, respectively, β-indolyl, γ-guanidinopropyl, and α-methylpropyl. In this reaction, IV is not produced from III by hydrolysis, but is formed by an oxidative degradation mechanism directly from *Cypridina* luciferin (Shimomura and Johnson, 1971).

SCHEME I





Cypridina luciferin



The resemblance between the aequorin system and the *Cypridina* luciferin system outlined above suggests that aequorin also might contain an imidazopyrazine skeleton related to V, and, considering that aequorin does not require molecular oxygen to luminesce, the functional group in aequorin might be

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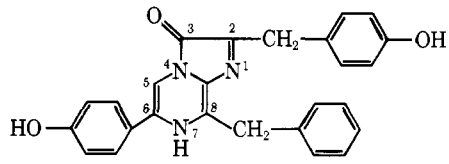
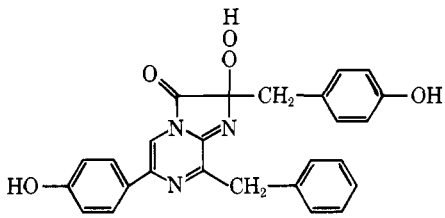
[1] Abbreviations used are: BFP, blue fluorescent protein; YC, yellow compound.

a stabilized form of VI (Shimomura and Johnson, 1973a). Recently, similar suggestions concerning the possible involvement of V and VI in aequorin bioluminescence have been made, and it has been found that *Renilla* luciferin is closely similar to V (McCapra *et al.*, 1973; Hori and Cormier, 1973a,b; Hori *et al.*, 1973). In the present study, we have critically examined the possible presence of V and VI in aequorin, and have also endeavored to make clear the mechanism of the intramolecular bioluminescent reaction, based on the properties and reactions of this photoprotein.



V



VI

Materials and Methods

Aequorin was extracted and purified as previously described (Shimomura and Johnson, 1969; Johnson and Shimomura, 1972a), and only the purified preparations which showed a single protein band or practically a single band in gel electrophoresis (pH 9.4) were used in the present study. BFP was prepared from aequorin solutions by adding 10 mM calcium acetate in an amount to complete the luminescent reaction in approximately 15 sec. AF-400 was prepared as previously described (Shimomura and Johnson, 1969). However, instead of using gel filtration to separate AF-400 from the protein fraction, the product was first washed with ethyl ether to remove AF-350; then AF-400 in the aqueous layer was extracted with *n*-butyl alcohol, and finally the butanol solution was concentrated *in vacuo*. *Cypridina* luciferase was of the same purity as used previously (Shimomura *et al.*, 1969), and *Cypridina* luciferin was the colorless crystalline dihydrobromide (Shimomura and Johnson, 1970b). 2-Methyl-6-(*p*-hydroxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one was synthesized from 2-amino-5-(*p*-methoxyphenyl)pyrazine as described (Inoue *et al.*, 1969; Hori and Cormier, 1973a). Mass spectra were obtained on a Hitachi–Perkin-Elmer Model RMU-6D at the Morgan-Schaffer Corp., Montreal.

Absorption Spectrum of the Functional Chromophore Present in Aequorin. Aequorin (*ca.* 3 mg) was dissolved in a buffer solution (1.3 ml of 60 mM glycylglycine–NaOH containing 3 mM EDTA (pH 7.8)), and two portions of 0.6 ml each were taken out of this solution. The first 0.6-ml portion was diluted with distilled water to make the weight equal to 1.1 g, and transferred into a sample cell. The second 0.6-ml portion was added to 10 μl of 0.5 M $CaCl_2$. After the luminescent reaction was completed in about 30 sec, the product solution (BFP) was washed five times with ethyl ether to remove the chromophore. The aqueous layer, after removing dissolved ether by an aspirator, was diluted with distilled water to make the total of 1.1 g, and transferred into a reference cell. The difference absorption of the sample solution against the reference solution was measured.

Absorption Spectrum of Cypridina Luciferin Bound to Cypridina Luciferase. Absorption cells for the sample and reference solutions were both fitted with Teflon stoppers each equipped with two stainless steel syringe needles through the stopper. One syringe needle of each stopper was connected to a 1-ml syringe and the other to a Tygon tubing ($\frac{1}{32}$ in. i.d., 50 cm in length), and the whole system was made sure to be air tight. Luciferase (2.8 mg, 0.05 μmol) dissolved in 1 ml of buffer (0.05 M sodium phosphate containing 0.1 M NaCl (pH 7.3)) was added into each cell, luciferin dihydrobromide (28 μg, 0.05 μmol) dissolved in 0.2 ml of water was added into the syringe on the sample side, and also 0.2 ml of water was added into the syringe on the reference side. The plungers were set at 0.8-ml marks. The ends of the Tygon tubings were connected to a vacuum pump through a Dry Ice–acetone trap; then both cells were evacuated for 30 min with occasional swaying. After recording a control curve, the contents of the syringes were added to the respective cells. A flash of light observed in the sample cell showed the presence of residual oxygen. However, the solution became nonluminescent in a few seconds indicating no leakage of air from the outside. Thus, the spectrum was finally recorded.

Examination for CO_2 in the Reaction Product of Aequorin Bioluminescence. A specially made Y-shaped reaction vessel equipped with two outlet stopcocks (Shimomura and Johnson, 1973b), and having a total volume of 44 ml, was used for this purpose. One of the stopcocks which was not used was clamped in the "closed" position, and the other stopcock (A) was connected through a Y tube to a McLeod gauge and to another stopcock (B). The opposite end of stopcock B was connected to a vacuum pump through a Dry Ice–acetone trap. All connections were by ground glass joints or by fusion.

Aequorin (9 mg, 0.3 μmol) dissolved in 3 ml of buffer solution was placed into the bottom of the vessel and 0.1 ml of 0.2 M $CaCl_2$ was placed into the side arm. The vessel was evacuated through stopcocks A and B intermittently for 10 min, with occasional swaying to aid equilibration of dissolved gases with the gas phase. The solutions were frozen in a Dry Ice–acetone bath for 30 min with stopcock A closed. Initial reading of the McLeod gauge was now taken by reopening stopcock A after closing stopcock B (usually 3–4 μ of Hg). The solutions in the reaction vessel were thawed and equilibrated at 20° with stopcock A closed; then calcium chloride solution in the side arm was added to the aequorin solution, resulting in a bright luminescence which ceased in 30 sec. The mixture was equilibrated with the gas phase for 15 min; then it was again frozen in a Dry Ice–acetone bath for 30 min. After closing stopcock B, a second reading on the McLeod gauge was taken with stopcock A open.

In the calculation of the total amount of CO_2 formed by the luminescent reaction, it was assumed that the frozen solution contained CO_2 and HCO_3^- in the same amounts as in the equilibrated solution at 20°. The lower temperature of the lower part of the reaction vessel, as well as the diffusion of CO_2 between the frozen solution and the gas phase, were ignored. The solubility of CO_2 in buffer solutions at 20° was assumed to be 0.93 mol/l. when equilibrated with 1 mol/l. of the gas phase. The hydration of CO_2 and the ionization of H_2CO_3, both in the liquid phase, were estimated by the following relationships: $\log([CO_2]/[H_2CO_3]) = 2.59$ and $\log([H_2CO_3]/[H^+][HCO_3^-]) = 3.77$ (both at 25°; Edsall, 1969) without corrections for the temperature of 20° instead of 25°. The inner volume of the apparatus, which includes the inside of the McLeod gauge, was obtained from the pressure change when a known small volume of air was injected after the evacuation of

the apparatus. The buffer solutions employed were 0.06 M glycylglycine-NaOH (pH 7.8), 0.1 M Tris-HCl (pH 7.2), and 0.01 M sodium phosphate (pH 6.5 and 5.9), each containing 0.5 mM EDTA.

Examination for HCOOH in the Reaction Product of Aequorin Bioluminescence. BFP solution (4 mg in 2 ml) was washed with ethyl ether and then added to a mixture of 5 ml of ethanol and 0.2 ml of concentrated HCl. The precipitate that resulted was removed by centrifugation, and the supernatant was concentrated to 2 ml under reduced pressure and then tested for HCOOH by chromotropic acid and H_2SO_4 with or without preliminary reduction with magnesium (Eegriwe, 1937; Shimomura and Johnson, 1968; Shimomura *et al.*, 1972).

2,4-Dinitrophenylhydrazones of the Product by Acid Treatment of Aequorin. Aequorin (7.8 mg, 0.26 μmol) in 2 ml of 0.2 mM EDTA (pH *ca.* 7.0) was added to 0.1 ml of concentrated HCl, followed by incubation at 40° for 30 min with 4 ml of the reagent which was prepared by dissolving 40 mg of 2,4-dinitrophenylhydrazine and 1 ml of concentrated HCl in 100 ml of methanol (Spectroquality, Matheson Coleman and Bell). After evaporating about half of the methanol, the mixture was extracted with ethyl ether and the ether extract was concentrated and chromatographed by tlc (silicic acid, toluene). The separate bands were each extracted with methanol, and each of the extracts was again chromatographed by tlc (silicic acid, water-saturated ethyl ether). Individual spots were separately extracted with 4 ml each of methanol, and the absorption spectra of the extracts were recorded. The amount of hydrazone in the extract was calculated assuming an ϵ value of absorption peak maximum between 350 and 400 nm as 20,000.

Acid Treatment of AF-400. AF-400 prepared from 15 mg of aequorin was dissolved in water, and the solution was acidified with HCl to make 0.15 N; then the absorption spectrum was recorded. This solution was heated in a boiling water bath for 3 min, and the absorption spectrum was recorded after cooling the solution in a cold water bath. This procedure involving 3 min of heating and measurement of absorption was repeated several times until an initially strong, sharp absorption band (λ_{max} 395 nm) changed to a lower broader band (λ_{max} 400–410 nm) having an absorbance approximately one-half that of the initial peak. The solution obtained in this manner was extracted with ethyl ether, and the ether layer, after washing first with 1% $NaHCO_3$ and then with 0.1 N HCl, was concentrated for mass spectral analysis. The 1% $NaHCO_3$ layer was acidified with HCl, and reextracted with ether; then the ether layer was also concentrated for mass spectrometry.

Separation of a Yellow Compound from Aequorin. Aequorin (8 mg) in 3 ml of 50 mM sodium phosphate buffer (pH 6.0), containing 5 mM EDTA, was added to 0.3 ml of 20 mM $NaHSO_3$ and left standing at 20°. When the Ca^{2+}-triggered luminescence capacity of this mixture decreased to 5–10% of the initial value (in *ca.* 30 min), the mixture was extracted three times with ethyl ether. The ether extracts containing the yellow compound (YC) were combined and concentrated and then chromatographed by tlc (silicic acid, water-saturated ethyl ether), developing until the solvent front reaches only to 5–7 cm above the origin, to minimize the spontaneous decomposition of the compound. A yellow band, containing YC, was well separated from the band of AF-350 and YC was eluted with ethyl ether–ethanol (10:1).

Results and Discussion

Outline. Various attempts to separate an unreacted functional chromophore from aequorin have always resulted in a nonluminescent intramolecular reaction of the protein, leading to the formation of AF-350 (I) as the main product. Thus, the structural elucidation of the functional chromophore had to be done on the basis of data indirectly related to the structure, except for the ultraviolet (uv) absorption spectrum which is the only direct source of structural information. The following paragraph outlines the approach adopted toward solution of this problem; the details are described in the text thereafter.

First, the carbon–nitrogen skeleton of the functional chromophore of unreacted aequorin was established on the basis of (1) analysis of CO_2 and HCOOH in the product of the bioluminescent reaction, (2) identification of carbonyl compounds produced in acid treatment of aequorin, (3) structural information concerning AF-400 and its acid-treatment product, and (4) the structures of I (AF-350) and II. Subsequently, the presence of a peroxide group in aequorin, as well as the nature of the group, are discussed. Finally, the structures of the light-emitting chromophore prior to the reaction are deduced in accordance with the known C–N skeleton, mainly from the study of the uv absorption spectrum of the functional chromophore of unreacted aequorin. During the course of investigating the uv absorption, the presence of a second chromophore (YC; see Materials and Methods section) was discovered. The mechanism of the intramolecular luminescent reaction was inferred from various lines of evidence, including the behavior of aequorin on treatment with various inactivating reagents.

Analysis for CO_2 and HCOOH in the Reaction Mixture after Aequorin Bioluminescence. If the aequorin reaction involves a *Cypridina* bioluminescence type of reaction, the production of 1 mol of CO_2 per mol of reacted aequorin is to be expected (Stone, 1968).

The amounts of CO_2 (including dissolved CO_2 and HCO_3^-) actually found after aequorin was luminesced by addition of Ca^{2+} at the pH values of 7.8, 7.2, 6.5, and 5.9 were, respectively, 0.1, 0.17, 0.6, and 0.75 (±0.05 in each case) mol per mol of aequorin reacted. An addition of 0.2 ml of 6 N HCl to the spent solution initially of pH 6.5 yielded a further 1.8 mol of CO_2/mol of aequorin, whereas a control experiment at pH 5.9 with 10 mg of bovine serum albumin instead of aequorin yielded no detectable CO_2. In contrast to these results, *Cypridina* bioluminescence catalyzed by luciferase yielded almost equimolar CO_2 (0.9 mol) per mol of luciferin at pH 7.8 (Shimomura and Johnson, 1973d).

These results appear neither to support nor to disfavor the possible occurrence of a *Cypridina* type of reaction (eq 1) in aequorin bioluminescence, because the CO_2 found might be derived solely from bound CO_2 which was initially present in the aequorin sample and was loosened in the reaction. On the other hand, the CO_2 might also have been actually produced and then subsequently remained bound to the protein (BFP), especially at the higher pH values. Although there seems to be no simple way to estimate the effect of binding HCO_3^- by Ca^{2+}, the concentrations of CO_2 in these experiments are far less than that which would be precipitated by Ca^{2+} under the condition involved. We conclude such an effect due to Ca^{2+} is not significantly large.

A possibility that HCOOH was produced instead of CO_2 can be rejected on the basis of a very faint response of BFP samples to the test for HCOOH. The slight response is probably due to glucose present in the protein (Shimomura and Johnson, 1969), because the same response was also obtained when the reduction step with Mg was omitted.

Analysis of Carbonyl Compounds in Acid-Treated Aequorin. Treatment of aequorin with acid or other denaturants yields AF-350 (I). Thus, if a *Cypridina* type of reaction (eq 2) is involved in the luminescent reaction of aequorin, the produc-

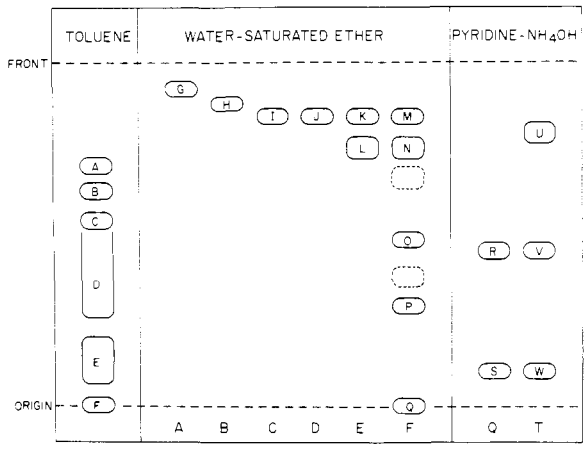


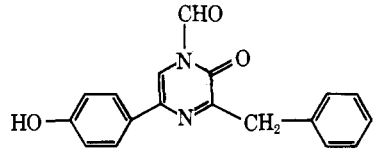
FIGURE 1: Thin-layer chromatography of 2,4-dinitrophenylhydrazones obtained from acid-treated aequorin. Two blue-fluorescent spots shown in broken lines were, respectively, from the top, AF-350 (I) and compound II. Spots L, N, and U were 2,4-dinitrophenylhydrazine. Other spots are as described in the text.

tion of *p*-hydroxyphenylpyruvic acid as the counterpart of AF-350 would be expected. Consequently, evidence was sought as to the formation of this acid by the following experiment. Acid-denatured aequorin (0.26 μmol) was treated with 2,4-dinitrophenylhydrazine, and the resulting hydrazone was chromatographed by two steps of tlc. One of the components that separated was compared with 2,4-dinitrophenylhydrazone prepared from an authentic sample of *p*-hydroxyphenylpyruvic acid in exactly the same manner as for aequorin, as shown in Figure 1. In tlc (silicic acid–pyridine containing 2% volume of 28% ammonium hydroxide), sample Q originating from aequorin gave two spots (R and S) exactly corresponding to two spots (V and W) given by sample T which was made from the authentic sample of *p*-hydroxyphenylpyruvic acid. The spectral characteristics of the extract of spot R (λ_{max} (50% MeOH) 384 nm in neutral solution, 377 nm in 0.04 N HCl, 384 nm in 0.04 N NaOH) and of spot S (λ_{max} (50% MeOH) 369 nm in neutral solution, 358 nm in 0.04 N HCl, 373 nm in 0.04 N NaOH) were identical with that of the extract of spot V and spot W, respectively. The total amount of the hydrazone in sample Q was estimated as 0.18 μmol (0.7 mol/mol of aequorin) approximately corresponding to the amount of AF-350 found in sample F. Thus, it is concluded that *p*-hydroxyphenylpyruvic acid is formed by acid treatment of aequorin.

In the course of the experiments just described, the presence of unexpected carbonyl compounds, mostly aliphatic aldehydes, in the aequorin preparation was revealed (Figure 1). The combined extract of spots I, J, K, and M contained a total of 0.37 μmol of 2,4-dinitrophenylhydrazone (1.4 mol/mol of aequorin) which, according to mass spectrometry, consisted of hydrazones of mostly acetaldehyde with some acetone and a trace of formaldehyde. Similarly, extracts of spots H and G, respectively, contained 0.18 μmol (0.7 mol/mol of aequorin) of the hydrazone of propionaldehyde and 0.075 μmol (0.3 mol/mol of aequorin) of the hydrazones of aliphatic aldehydes having four–six carbons. Two minor spots, O (0.04 μmol) and P (0.003 μmol), were not identified. The composition of 2,4-dinitrophenylhydrazone prepared from BFP was much the same as that prepared from acid-treated aequorin, except that, as expected, BFP resulted in much less (less than one-tenth) *p*-hydroxyphenylpyruvic acid than acid-treated aequorin.

A part or all of the detected aliphatic aldehydes plus acetone (total 2.4 mol/mol of aequorin) might be simply impurities originally contained in the organisms and subsequently adsorbed on aequorin molecules, inasmuch as none of the aldehydes, nor the corresponding alcohols, nor acetone was used in the process of purification of aequorin. For the time being, however, the possibility that the aldehydes play a significant role in the bioluminescent reaction of aequorin cannot be completely excluded.

Properties of AF-400. A blue-fluorescent compound obtained from aequorin by $NaHSO_3$ treatment and designated AF-400 (Shimomura and Johnson, 1969) is insoluble in ethyl ether. It changes to an ether-soluble form by treatment with cold 0.1 N HCl, with very little change in the absorption spectrum. When AF-400 is heated in 0.15 N HCl, some changes in the absorption spectrum occur and the main product isolated from the reaction mixture has the mass spectrum shown in Figure 2 and the absorption spectrum shown in Figure 3E, curve 3. Considering that this compound, as well as AF-400, have apparently been derived from the same structure that yielded AF-350, the molecular formula of this compound is most likely $C_{18}H_{14}N_2O_3$ (M + 1: calcd, 20.5%, obsd, 22.3%), and the fragmentation pattern of the mass spectrum appears most favorably interpreted by structure VII. The intense peaks at *m/e*



VII

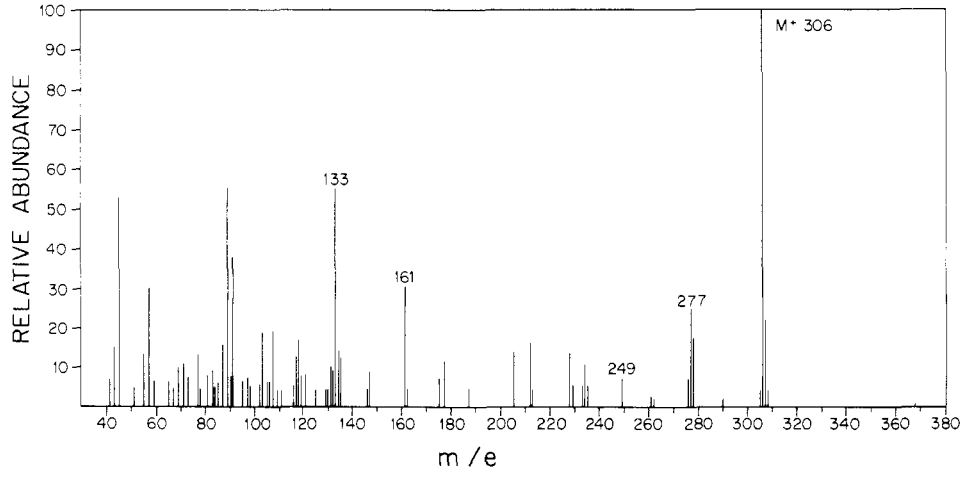


FIGURE 2: Mass spectrum (70 eV) of an acid-treatment product of AF-400. The values of relative abundance at *m/e* 205, 212, and 228 were not quite reproducible, probably due to impurities, at least to some extent.

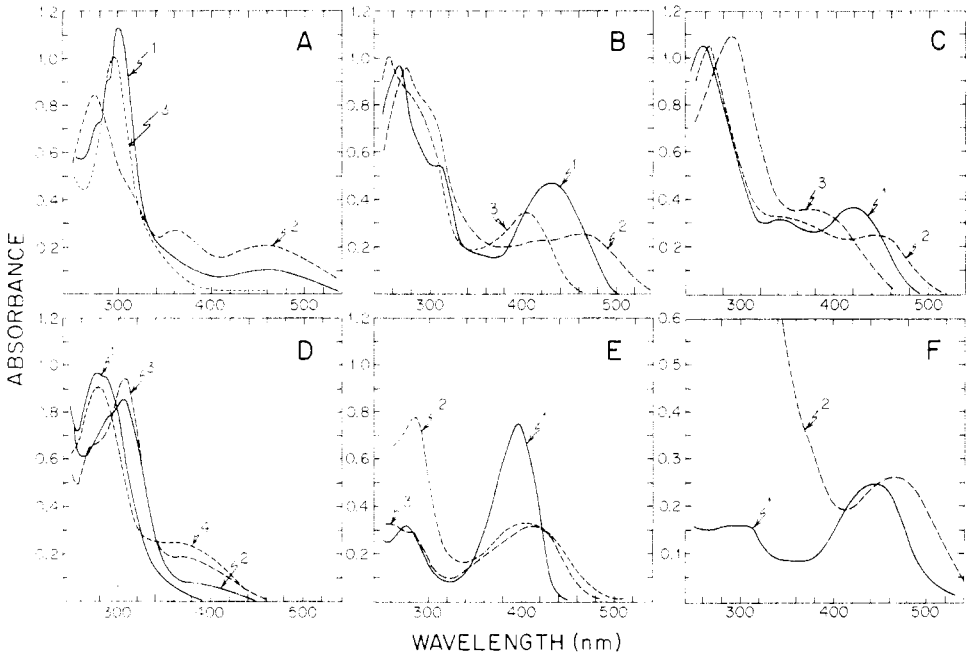


FIGURE 3: Absorption spectra of various compounds related to the functional chromophore of aequorin. (A) Functional chromophore of aequorin (1); *Cypridina* luciferin bound to *Cypridina* luciferase, containing some oxyluciferin (λ_{max} 360 nm) formed by residual oxygen (2); *Renilla* luciferyl sulfate at pH 12, replotted from Figure 2 (Hori *et al.*, 1972) (3). (B) *Cypridina* luciferin in aqueous solutions; at neutral pH (1), in 0.1 N HCl (2), and in 0.01 N NaOH (3). (C) Compound X in aqueous solutions; at neutral pH (1), in 0.1 N HCl (2), and in 0.01 N NaOH (3). (D) *Cypridina* benzoylluciferin in methanol (1), *Cypridina* benzoylluciferin in methanol containing HCl (2), *Cypridina* luciferin in methanol containing HCl (3), and compound X in methanol containing HCl (4). (E) AF-400 in water (1), AF-400 after heating in 0.15 N HCl (2), and a product isolated from the reaction mixture of curve 2, measured in ethyl ether (3). (F) The yellow compound in ethyl ether (1), and aequorin in a pH 6.0 buffer solution (2).

278, 277, 161, and 133 can be considered as, respectively, ($M^+ - CO$), ($M^+ - CHO$), ($HOC_6H_4CCHNCHO^+$), and (*m/e* 161 − CO).

Although a final confirmation of this structure must await the synthesis of this compound, nevertheless structure VII suggests that the N atom of AF-350, which corresponds to the N at position 1 of V, had been bound to a C atom in the functional chromophore of aequorin.

Carbon-Nitrogen Skeleton of the Functional Chromophore of Aequorin. From the foregoing data we believe it is reasonable to deduce the presence of a C–N skeleton of an imidazopyrazine in aequorin, as in V. This deduction is based on (1) the structure of II, (2) the production of *p*-hydroxyphenylpyruvic acid by acid treatment of aequorin, and (3) the possible structure of the acid-treatment product of AF-400 (VII).

Presence of a Peroxide Group in Aequorin. In essentially all bioluminescent reactions, the energy requirements for light emission at the observed wavelengths (60,853 cal/einstein at 470 nm; *cf.* Johnson *et al.*, 1974, p 142ff) are quite high relative to that of an ordinary, nonluminescent reaction. Thus, even though the aequorin reaction is one of the very rare instances wherein neither added oxygen nor hydrogen peroxide is necessary for luminescence (*cf.* summary, Johnson and Shimomura, 1972b, p 317), it is only natural to assume that oxygen as such, or possible in the form of a peroxide, is somehow involved. Moreover, certain resemblances between the aequorin system and that of the oxygen-requiring system of *Cypridina*, as already mentioned, favor this assumption.

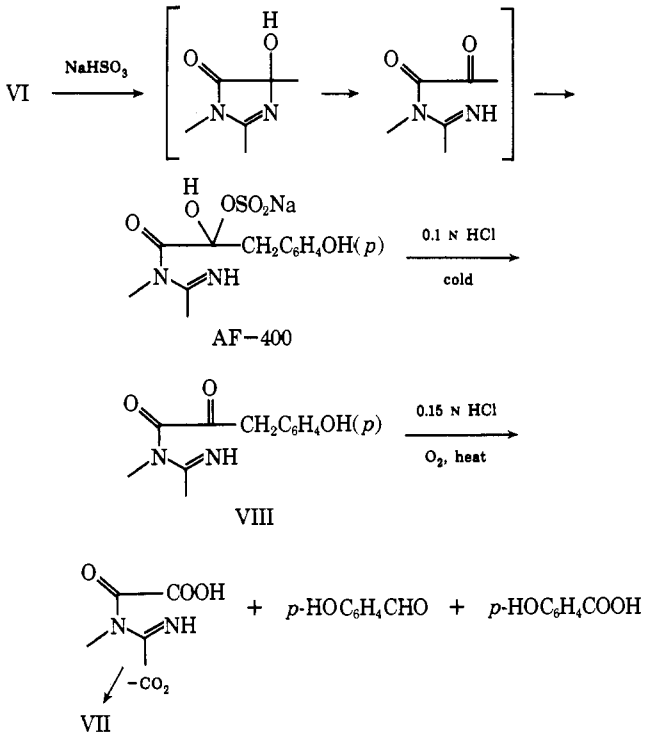
Although gaseous oxygen is not required for light emission of the aequorin reaction (Shimomura *et al.*, 1962), the presence of strongly adsorbed oxygen molecules on the aequorin molecule in solution seems within the realm of possibility, especially on taking into account the difficulties encountered in removing all traces of molecular oxygen from the luciferase solutions obtained from *Cypridina* and *Latia* (Shimomura *et al.*, 1966). Such a possibility, however, must be judged to be highly unlikely because we have found that freeze-dried aequorin, as well as aequorin before drying, shows exactly the same light-emitting capacity on adding Ca^{2+} in a thoroughly evacuated reaction vessel. The freeze-dried aequorin was prepared in the presence of small amounts of EDTA and $(NH_4)_2SO_4$ to prevent inactivation of the capacity for light emission (Shimomura and Johnson, 1969).

Thus, if adsorbed oxygen in aequorin is indeed nonexistent, then the presence of a peroxide group, either H_2O_2 or some other hydroperoxide, is an all but inevitable consequence, and this is supported by the extreme sensitivity of aequorin to $NaHSO_3$ (Shimomura and Johnson, 1969). Structure VI, which postulates a hydroperoxide group at position 2, readily accounts for the predominant formation of AF-350 (I) through various methods of denaturation of aequorin, whereas a peroxide group at position 3 would not explain the formation of AF-350. Moreover, VI would be able to yield VII, *via* AF-400, in the possible reaction shown in Scheme II. In support of Scheme II, the mass spectrum of the ether-soluble form of AF-400, which was obtained by treating AF-400 with cold 0.1 N HCl, showed a small molecular peak at *m/e* 439, corresponding to the molecular weight of VIII, in spite of the poor fragmentation pattern probably due to an intensive decomposition of the compound before the analysis. Furthermore, 1% $NaHCO_3$ solution, which was used in the preparation of VII to wash the ether solution of the sample, was found to contain *p*-hydroxybenzoic acid, according to a mass spectrum obtained from the ether extract of the acidified solution.

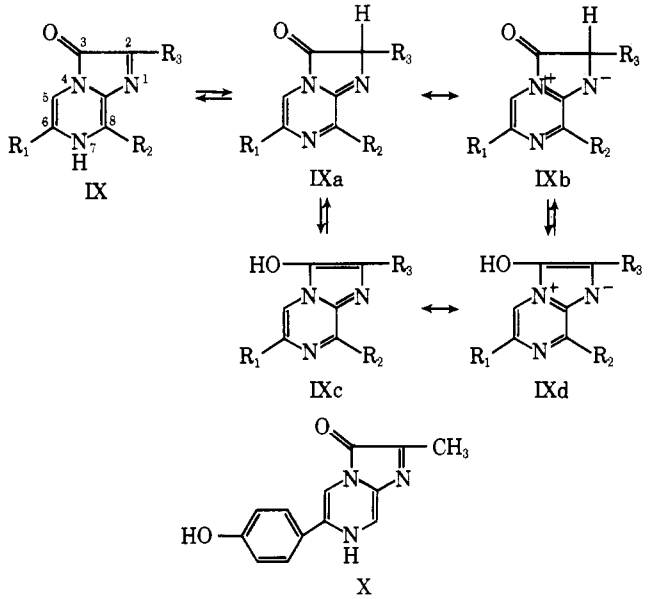
Structure of the Functional Chromophore. The absorption spectrum of the chromophore measured as the difference spectrum of native aequorin and the protein residue of aequorin (Figure 3A) shows two absorption maxima: 460 (ϵ 2400) and 300 nm (ϵ 28,500). Two small shoulders at 280 and 290 nm are probably due to the slight inequality of protein parts.

For comparative purposes, some of the principal tautomers and resonant forms of *Cypridina* luciferin (IX) are shown

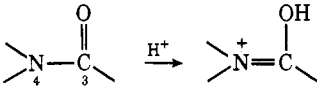
SCHEME II



below (R_1 = β-indolyl, R_2 = γ-guanidinopropyl, R_3 = α-methylpropyl). These structural forms should also be applicable to the other compounds having the same skeleton with different R_1, R_2, and R_3, including V and a model compound (X). The absorption spectra of IX and X, shown in Figures 3B–D, are distinctly similar regarding the character of the peaks of the longest wavelength.



Structure IX has been assigned for *Cypridina* luciferin in neutral solution (yellow, λ_{max} 430 nm (ε 9500), Figure 3B), and, in acidified anhydrous methanol (colorless, Figure 3D), structures IXa, IXb, IXc, and IXd all protonated at position 1, with probable predominance of the last structure, can be assigned (see Kishi *et al.*, 1966a,b). For benzoylluciferin (Figure 3D), the benzoylation must have taken place at the enolic OH of IXc and IXd, as judged from ir absorption at 1780 cm^{-1} due to the carbonyl group (Eguchi, 1963), and, in neutral solution, the benzoylated form of IXd, in which the rings of pyrazine and imidazole are both aromatic, appears predominant in view of the uv absorption. In aqueous acid solution, the 430-nm peak of *Cypridina* luciferin shifts to 460 nm accompanied by some decrease in absorbance (Figure 3B), possibly due to protonation of IX



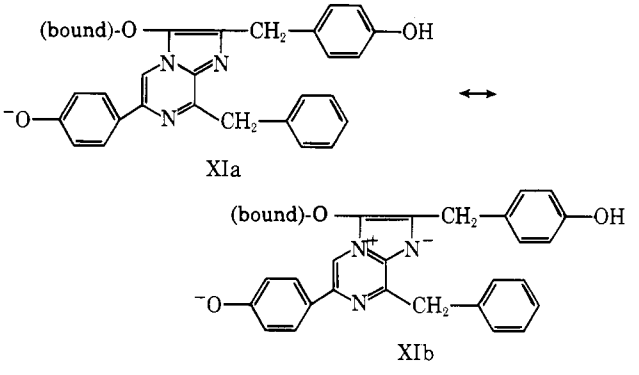
A similar shift of the 430-nm peak was also observed when *Cypridina* luciferin was mixed with *Cypridina* luciferase under anaerobic conditions (Figure 3A).

Of the two absorption peaks of the functional chromophore of aequorin, the 300-nm peak would simply indicate that the OH of the *p*-hydroxyphenyl group at position 6 is ionized. However, the peak at 460 nm, as well as the low absorbance in the 350–400-nm region, considerably complicates the interpretation of the spectrum.

The structure–spectrum correlation of various compounds shown in Figure 3 and also that of I, II (Shimomura and Johnson, 1972, 1973a), III, IV (Shimomura *et al.*, 1957; Kishi *et al.*, 1966a,b), and *Renilla* luciferin analogs (Hori and Cormier, 1973b; Hori *et al.*, 1973) almost certainly indicate that IXa and IXb, and consequently VI, have the absorption maximum of the longest wavelength band at a considerably shorter wavelength than 460 nm, and also have a moderately strong peak at 350–400 nm (ε ~10,000). Thus, structure VI, which could readily explain the degradation reaction, cannot be considered the chromophore of native aequorin.

The data of the model compounds mentioned above also indicate that compound V, in aqueous acid solution or when bound to a suitable protein, would give an absorption maximum close to 460 nm, coinciding in wavelength with the 460-nm peak of aequorin but differing in ε value by having at least double the same peak as that of aequorin, as well as the considerable absorption in the 350–400-nm region. Thus, we consider that compound V in aqueous acid solution or in a bound protein is not adequate to explain the chromophore of native aequorin.

The absorption spectrum of structure IXd, as typically seen for *Cypridina* benzoylluciferin (Figure 3D), shows very little absorption above 350 nm, and the general character of the spectrum below 400 nm appears well in accord with the spectrum of the functional chromophore of aequorin although the 460-nm peak is missing. It follows that, if a IXd type of chromophore, namely XIb, exists in aequorin, the presence of another chromophore which absorbs at 460 nm becomes also necessary.



Ultimately, as reported below, a second chromophore having a yellow color was separated from aequorin. Furthermore, *Renilla* luciferyl sulfate in alkaline solution, which is now believed to have the same chromophore as XIa (though with an

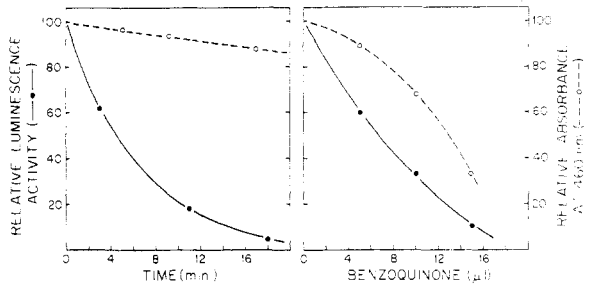


FIGURE 4: Relationship between luminescence capacity and absorption at the 460-nm peak when aequorin was slowly inactivated: (left) 50 μl of 10 mM $NaHSO_3$ was added to 10 ml of 0.05 M sodium phosphate buffer (pH 6.0) containing 1.2 mg of aequorin and 5 mM EDTA in a 5-cm light-path cell at time zero; (right) 1 mg of aequorin in 1 ml of 0.05 M sodium phosphate buffer (pH 6.5) containing 5 mM EDTA was titrated with 5 mM *p*-benzoquinone.

unknown substituent and a sulfate group, respectively, at positions 2 and 3 of the imidazopyrazine ring), shows an absorption spectrum (Hori *et al.*, 1972) that is consistent with the spectrum of the native chromophore of aequorin (Figure 3A), taking into account the presence of the yellow compound and probably some influence of the protein part. Thus, we conclude that structure XIb, resonating with structure XIa, is the most probable structure present in native aequorin. In native aequorin, the enolic O at position 3 must be in a state that is stabilized in some manner, such as by hydrogen bonds with the protein part, or as an ester with a COOH group of the protein part, or possibly as a sulfate, such as has been found for *Renilla* luciferin. No information has yet been obtained regarding the condition of the OH of the *p*-hydroxybenzyl group at position 2.

Presence of a Second Chromophore in Aequorin. A careful examination revealed that, when aequorin was slowly inactivated under controlled conditions, *e.g.*, by suitable concentrations of such potent inactivators as $NaHSO_3$ or *p*-benzoquinone (Figure 4), the decrease of luminescence activity is considerably faster than the decrease of the 460-nm absorption peak. With $NaHSO_3$, the difference between decrease of luminescence activity and the decrease of the 460-nm peak was especially great. Moreover, shaking the test solution with ethyl ether, as soon as luminescence activity and absorption at 460 nm had decreased to 5–10 and 80–85%, respectively, of their original values, an unstable yellow compound (λ_{max} 440 nm) became evident together with AF-350 (I) in the ether layer. The absorption spectrum, after purification, of the yellow compound (YC) (Figure 3F) is distinctly different from those of AF-350 and its close derivatives.

Under the conditions employed, the yields of AF-350 and the ether-soluble yellow compound, assuming the ϵ value for the 440-nm peak to be the same (2400) as that for the 460-nm peak of aequorin, were both approximately 0.8 mol/mol of aequorin. Either increasing the concentration of $NaHSO_3$, or lengthening the reaction time, resulted in the formation of AF-400 at the expense of AF-350, accompanied by some decreases in the amount of YC. Under the conditions used to prepare AF-400, the yields of AF-350 and YC were, respectively, 0.1 and 0.4 mol/mol of aequorin.

In ethanol, YC was easily reduced to a colorless form by addition of $NaBH_4$, β-mercaptoethanol, or sodium hydrosulfite plus a small amount of water. The yellow compound, in an amount equivalent to approximately 0.05 μmol of aequorin from which it had been prepared, showed a weak but clear response in the test for quinones and α-diketones by catalytic acceleration of the formaldehyde-*o*-dinitrobenzene reaction (Feigl, 1960).

TABLE I: Light Emission of Aequorin Initiated by Various Reagents.[a]

Reagent	Concn	pH	Max Rate[b]	Total Light[c]
Ca^{2+} (control)	10 mM	6.5	100	100
p-Benzoquinone	10 μM	6.5	0.02	6
Br_2	3 μM	6.5	0.06	4
I_2	10 μM	6.5	0.2	5
N-Bromosuccinimide	3 μM	6.5	0.2	5.5
N-Ethylmaleimide	0.4 mM	8.1	0.02	5
Iodoacetic acid	0.1 mM	8.1	0.002	>1
p-Hydroxymercuribenzoate	10 μM	6.5	0.015	5

[a] At 25°, 2 ml of 50 mM sodium phosphate (pH 6.5) or 50 mM Tris-HCl buffer (pH 8.1), both containing 5 mM EDTA and one of the reagents, was added to 25 μg of aequorin. [b] Maximum (= initial) rate in units wherein 1 unit corresponds to approximately 2.5×10^{11} photons/sec. [c] Final values in units wherein 1 unit equals approximately 10^{12} photons. "Final" represents the time for near completion of the reaction, amounting to more than 1 hr for the last three reagents.

Luminescence of Aequorin Initiated by Various Reagents Other than Ca^{2+}. The luminescent reaction of aequorin can be triggered to various extents by some metal ions other than Ca^{2+} (Izutsu *et al.*, 1972; Shimomura and Johnson, 1973c) and also by *p*-benzoquinone (Y. Kohama, 1971, unpublished results).

We have found in the present study that some of the reagents which inactivate the luminescence capacity of aequorin also induce a limited luminescence. As apparent from the examples shown in Table I, the luminescence reactions induced by such inactivators are characterized by very slow rates of light emission which continue for a considerable period after the Ca^{2+}-triggered light-emitting capacity of aequorin is completely lost (see Table I). The quantum yield, as well as the rate, vary widely with the concentrations of both reagent and aequorin. Under optimum conditions, the yield generally exceeded 10% of that of bioluminescence triggered by Ca^{2+}. The inactivation of aequorin by urea, guanidine hydrochloride, sodium dodecyl sulfate, or $NaHSO_3$ did not induce any luminescence. Thus, in conjunction with the data of Table I, it is reasonable to deduce the participation of SH groups(s) in the Ca^{2+}-triggered light-emitting reaction.

Mechanism of the Ca^{2+}-Triggered Bioluminescent Reaction. The reactants in the aequorin molecule which are necessary for the bioluminescence reaction would be expected to include YC, in addition to XI and a peroxide group already discussed, as judged by the fact that, when aequorin was caused to luminesce very slowly by adding a sufficiently small amount of Ca^{2+}, the residual capacity to luminesce, on a later addition of Ca^{2+}, was always strictly proportional to the residual absorption at 460 nm.

In regard to the SH group(s), the following experiment using *N*-ethylmaleimide, which causes no visible denaturation of aequorin, as judged by the absence of cloudiness, helped the interpretation.

When aequorin (0.5 mg) was dissolved in 1 ml of 0.05 M Tris-HCl buffer (pH 8.1), containing 1 mM each of *N*-ethylmaleimide and EDTA, the luminescence activity on adding Ca^{2+} was completely lost in 3–4 min without any change in the absorption spectrum of the solution, thus indicating that XI and the yellow compound both remained intact. Addition of Ca^{2+} to this solution resulted in no light emission but in the disappearance of the 460-nm peak and the almost quantitative formation of AF-350 (I) as evidenced by the appearance and height of the 345-nm peak. The rate of this reaction was similar to that of the normally fast calcium-triggered bioluminescence reaction. When Ca^{2+} was not added, the solution was spontaneously and weakly luminescent, as expected from the data of Table I, and after a long period of 1 hr when the luminescence of the solution became negligibly weak, the quantum yield of this sample finally amounted to 10% of the Ca^{2+}-triggered, fast luminescence.

The results described above indicate that aequorin contains at least one SH group which is essential for Ca^{2+} to trigger luminescence, and that, when this SH is blocked, a spontaneous luminescent reaction and a Ca^{2+}-triggered nonluminescent reaction involving XI and the yellow compound can still take place. It follows that the SH group is not required in these reactions. Thus, it seems evident that the SH group does not itself change after the Ca^{2+}-triggered luminescent reaction.

Taking into account the nature of all components known to be involved, as well as the resemblances between the *Cypridina* and the aequorin systems, the peroxide postulated to be involved in aequorin luminescence is most likely H_2O_2, even though in aequorin the H_2O_2 may exist in a stabilized form which can easily yield free H_2O_2 as discussed below. Thus, on the basis of available evidence, the calcium-triggered luminescent reaction can be reasonably represented according to the following reaction scheme.

$$\mathrm{XI} + \mathrm{H_2O_2} + \mathrm{YC} \xrightarrow{Ca^{2+},\ \mathrm{RSH}} [\mathrm{VI}] + \mathrm{YC{-}H_2}$$
$$[\mathrm{VI}] \rightarrow \mathrm{II} + \mathrm{CO_2} + \text{light}$$

Formation of AF-350 (I) in the Ca^{2+}-triggered nonluminescent reaction of *N*-ethylmaleimide denatured aequorin could be caused by involving H_2O instead of H_2O_2 in the reaction, rather than by the reductive decomposition of the intermediate VI in a manner similar to eq 2.

In the aequorin molecule, all functional components except Ca^{2+} are tightly bound to the protein part without any recognizable dissociation, and they are assumed to be located close to each other. To have a bond to the protein part, and also to increase the stability, H_2O_2 could be bound to a CHO or COOH group of the protein part forming, respectively, α-hydroxy hydroperoxide and peroxy acid, which should easily release H_2O_2 as needed. A possibility that H_2O_2 is bound to the yellow compound is a moot question at this moment. The group(s) of SH can be attributed to any of at least three cysteine residues in aequorin protein (Shimomura and Johnson, 1969), although an experimental verification for this has not been attempted as yet.

XI seems to be attached to the protein part at two binding sites, at least, namely, the O at position 3 and the OH of the *p*-hydroxyphenyl group at position 6, based on the discussion already given. Although the latter binding is ionic, the binding site is considered to be not exposed to the solvent, because changing the pH of the solution from 6 to 9.5 does not shift the 300-nm peak of aequorin. In contrast to this, the same OH group is not bound but is exposed to the solvent in the bioluminescence product of aequorin (BFP) (Shimomura and Johnson, 1972).

Investigation of the structure of the yellow compound as well as the more detailed mechanism of the reaction is presently in progress in this laboratory.

Acknowledgment

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Nuclear Magnetic Resonance Study of the Complexes of Manganese(II) and Fully Adenylylated Glutamine Synthetase (*Escherichia coli* W). Frequency, Temperature, and Substrate Dependence of Water Proton Relaxation Rates†

Joseph J. Villafranca* and Fred C. Wedler

ABSTRACT: A study of the longitudinal, $(1/T_{1p})_b$, and transverse, $(1/T_{2p})_b$, proton relaxation rates (prr) of water was conducted as a function of frequency (6–48 MHz) and temperature (1–40°) for the binary complex of Mn(II) and fully adenylylated glutamine synthetase ($E_{\overline{12}}$) isolated from *Escherichia coli* W. The prr data display a maximum in plots of $(1/T_{1p})_b$ *vs.* the reciprocal of the absolute temperature $(1/T)$, with the maximum shifting to lower temperatures as the frequency is lowered from 48 to 6 MHz. When these data were analyzed according to the Solomon–Bloembergen–Morgan (SBM) scheme the data are in accord with the predicted dipolar relaxation mechanism for proton (H_2O)-electron (Mn(II)) relaxation involving a correlation time, τ_c, in the range (2–10) $\times 10^{-9}$ sec. The $(1/T_{1p})_b$ values were higher at 24 MHz than at 48 MHz as expected from the SBM scheme but the values at 12 and 6 MHz were lower than the 24-MHz relaxivity data. The explanation for this observation is that the τ_c values are themselves frequency dependent which establishes that the electron spin relaxation time, τ_s, is the dominant correlation time for the electron–proton dipolar interaction. The $(1/T_{2p})_b$ data also fit the SBM scheme with contributions from dipolar relaxation processes and from τ_m, the exchange of water molecules from the primary coordination sphere of $E_{\overline{12}}$-bound Mn(II). The overall analysis led to the conclusion that there are three water molecules exchanging from the $E_{\overline{12}}$-Mn(II) complex with a lifetime of $\tau_m = 1.3 \times 10^{-7}$ sec (300°K). In the presence of L-glutamate the same features are seen in the $(1/T_{1p})_b$ and $(1/T_{2p})_b$ data for the ternary $E_{\overline{12}}$-Mn(II)-glutamate complex as in the binary $E_{\overline{12}}$-Mn(II) complex with the exception that all relaxivity values are lower. Analysis by the SBM scheme was consistent with a reduction in the number of exchanging water molecules ($\tau_m = 1.0 \times 10^{-7}$ sec) from three to two upon formation of the ternary complex. These data suggest that L-glutamate is coordinated to the enzyme-bound Mn(II). The substrate ATP does not produce the same effect as L-glutamate but instead the major effect of ATP seems to be to decrease the concentration of $E_{\overline{12}}$-Mn(II) by formation of an ATP-Mn(II) complex. The following activators or inhibitors of glutamine synthetase activity, L-tryptophan, L-alanine, L-histidine, glycine, glucosamine-6-P, and carbamyl-P, had no effect on the $(1/T_{1p})_b$ values in solutions of $E_{\overline{12}}$-Mn(II). GDP and CTP both lowered the relaxivity values in solutions of $E_{\overline{12}}$-Mn(II) in a similar manner to ATP and an interpretation was made that ATP, GDP, and CTP did not interact with enzyme-bound Mn(II) in the same manner as did L-glutamate. The role of the metal ion in the high affinity and intermediate affinity Mn(II) binding sites is discussed in relation to previous kinetic, binding, and isotope exchange data.

Recognition of the central role for glutamine synthetase in nitrogen metabolism has produced considerable recent interest in all aspects of this key enzyme. Regulation by feedback mechanisms of the reaction

$$\text{L-Glu} + NH_3 + \text{ATP} = \text{L-Gln} + \text{ADP} + P_i \quad (1)$$

is observed to varying degrees in enzymes from various sources, most notably the case of *Escherichia coli.* The catalytic mechanism as well as the modes of modifier action have been investigated in some detail (Wedler and Boyer, 1972a,b) for the *E. coli* enzyme. In addition to feedback inhibition, other regulato-

† From the Chemistry Departments of The Pennsylvania State University, University Park, Pennsylvania 16802 (J. J. V.), and Renssalaer Polytechnic Institute, Troy, New York 12181 (F. C. W.). *Received January 23, 1974.* This work was supported in part by grants from the National Science Foundation (GB-34751 (F. C. W.) and GB-34291) (J. J. V. for instrumentation) and the Petroleum Research Fund, administered by the American Chemical Society.