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IDENTIFICATION OF THE LUCIFERASE-BOUND FLAVIN-4A-HYDROXIDE AS THE PRIMARY EMITTER IN THE BACTERIAL BIOLUMINESCENCE REACTION

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Summary

The luciferase light-emitting reaction was carried out at 1°C by mixing purified luciferase-FMN-4a-hydroperoxide with long chain aldehyde (decanal). Simultaneous kinetic measurements of bioluminescence and absorbance showed that light emission decayed more rapidly than oxidized FMN appeared, indicative of a transient intermediate species subsequent to light emission. The same species was found in reaction mixtures examined immediately after light emission was completed. Both its absorption spectrum (λ max, 360 nm) and its fluorescence emission (λ max, 490 nm) are consistent with the hypothesis that the chromophore is the luciferase-bound flavin-4a-hydroxide, the ground state of the primary emitter in the reaction. It has a relatively short lifetime (7 min at 9°C) and decays to the stable product, FMN, by losing water. The activation energy for this step was determined to be 83 kJ mol^{-1}.

Introduction

In the bacterial bioluminescent reaction the electronically excited light emitting species has not been previously identified (1,2,3). FMN, a product and a highly fluorescent molecule, could be the emitter in the

reaction, but its fluorescence emission is centered at 530 nm while the luciferase-mediated bioluminescence peaks around 490 nm (4). Moreover, luciferase-bound FMN is non-fluorescent (5,6). Nevertheless, from studies with active flavin analogs having different fluorescence properties, it was concluded that the emitter is a flavin species (3,4,7).

The luciferase-bound flavin 4a-hydroperoxide (8,9,10) has a half lifetime of about 1 hour at 2°C in the absence of aldehyde (11, 12) and, under appropriate conditions, exhibits a high quantum yield fluorescence with an emission closely matching that of the bioluminescence (13). However, this peroxyflavin cannot be the emitter either, since it has not yet reacted with aldehyde in the catalytic pathway. The luciferase flavin-4a-hydroxide should have a similar fluorescence and, as suggested earlier (1,14), could be formed in the singlet excited state. However, this species might be elusive, since it would be expected to lose water and form FMN. The experiments described below provide evidence for the occurrence and emitter role of the hydroxyflavin chromophore (15).

Materials and Methods

Luciferase was extracted and purified from Vibrio harveyi, M-17 (16). A commercial spectrophotometer (Kontron, Uvikon, model 820) was modified so as to measure absorbance and bioluminescence simultaneously, with automatic corrections (50 times/sec) for any changes in real or apparent (e.g., bioluminescence) dark current. Fluorescence measurements were made with a Perkin-Elmer MFP-44 fluorescence spectrofluorometer. The luciferase flavin hydroperoxide intermediate was prepared and purified by molecular sieve column chromatography at 1°C (11). Spent reaction mixtures were prepared by running the luminescent reaction on an aldehyde (0.01% v/v sonicated decanal)-equilibrated Sephadex G-25 column (1 x 15 cm) in 0.35 M phosphate buffer at 1°C, pH 7.0 (15). Light emission had disappeared prior to the elution of the protein fraction (0.4 ml), which was collected (elution time, 10 min) after the void volume (4.7 ml) and transferred to a cuvette for measurement of either fluorescence or absorbance spectra.

Results and Discussion

In the absence of added aldehyde the decay of the flavin hydroperoxide is exponential and the spectral absorbance changes occur isosbestically with the appearance of oxidized FMN, much of it still enzyme bound (11). This decay (as monitored by absorbance increase at 440 nm) is paralleled by the decay of a very weak "endogenous" light emission from the preparation as well as by the loss of its capability to emit bioluminescence upon the addition of long chain aldehyde.

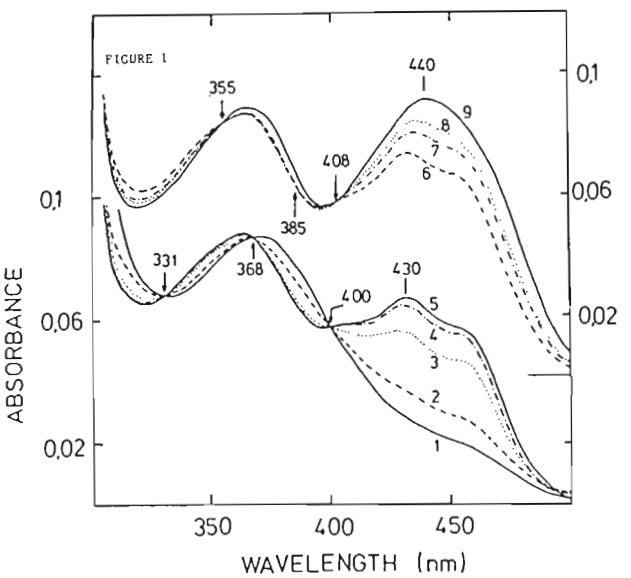


Figure 1
ABSORBANCE CHANGES WITH TIME OCCURRING UPON REACTION OF THE PURIFIED LUCIFERASE FLAVIN HYDROPEROXIDE WITH DECANAL
The hydroperoxide (8×10^{-6} M) in 0.35 M phosphate buffer pH 7.0 (trace 1, already corrected for dilution) was mixed (mixing time, <10 s) with decanal (1.6×10^{-4} M) at 1°C. Recording of trace 2 was started immediately after mixing; the scan (500 nm/min) required 55 sec for one cycle. The subsequent spectra of (traces 3-8) were started at 2, 4, 5, 10, 20 and 30 min respectively. The final spectrum (trace 9) was recorded at 1°C after warming the sample to 25°C and recooling. Arrows are used to indicate the isosbestic points.

In the present study, we measured simultaneously the changes in absorbance and in bioluminescence intensity following the addition of decanal to the isolated luciferase-flavin hydroperoxide at 1°C (Figs. 1 & 2). The absorbance changes (at 440 nm) are indicative of the appearance of oxidized FMN, but they may be divided into an early phase (traces 1-5; 0-300 sec), and a late phase (traces 6-9), characterized also by different isosbestic points.

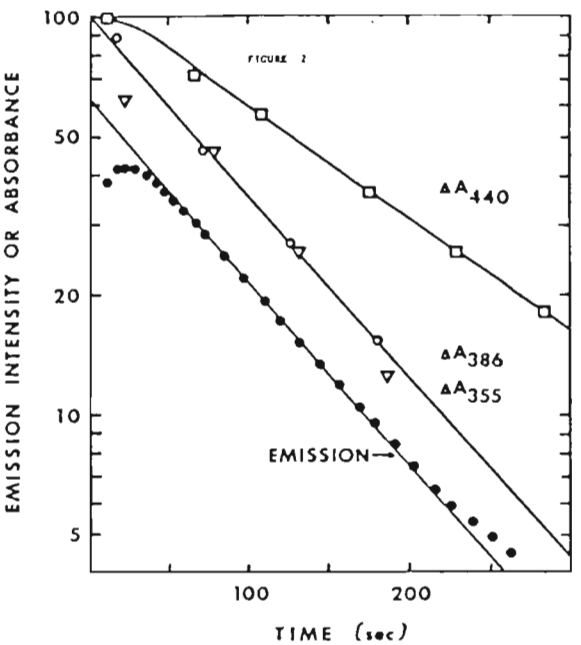


Figure 2
COMPARISON OF THE KINETICS OF SPECTRAL CHANGES AND BIOLUMINESCENCE EMISSION FOLLOWING REACTION OF LUCIFERASE HYDROPEROXIDE WITH DECANAL
The data from the experiment of Fig. 1 plotted on a semilogarithmic scale. Open symbols, absorbance changes at 440 nm (squares), 386 nm (circles), and 355 nm (triangles). The decay of bioluminescence emission (solid circles; $k = 0.62\ min^{-1}$) parallels the spectral conversions at 355 nm and 386 nm whereas the changes in absorbance at 440 nm (reflecting formation of oxidized flavin) proceed at a slower rate ($k = 0.39\ min^{-1}$). Absorbance changes were normalized so that they all extrapolated to about the same value at zero time. Emission intensity in arbitrary units.

In the earlier phase the isosbestic points (331, 368 and 400 nm) are similar to those previously reported (329, 368 and 401 nm) for the dark decay of the V. harveyi flavin hydroperoxide (10). Also, the increase in absorbance is more rapid at 355 nm ($t_{1/2}$ = 67 s; k = 0.62 min^{-1}) than at 440 nm ($t_{1/2}$ = 106 s; k = 0.39 min^{-1}), with the decay in bioluminescence paralleling the former (Fig. 2). At the same time, only about 55% of the final absorbance at 440 nm (thus oxidized FMN) has developed during this first phase even though the major fraction (70%) of the light has been emitted.

The later phase, with isosbestic points at 355 and 408 nm, involves only small absorbance changes in the 300-400 nm range, but continued changes at 440 nm. This phase may therefore involve an intermediate flavin species and its conversion to oxidized FMN. A spectrum for this species (postulated to be the luciferase-FMN-4a-hydroxide; see below) can be obtained (Fig. 3) by subtracting from an intermediate spectrum (trace 5) the appropriate amounts of both oxidized FMN (the endpoint spectrum) and the amount of still unreacted flavin-4a-hydroperoxide (trace 1), as deduced from the amount of light still to be emitted.

A preparation of the hydroxy FMN species without the peroxy species was obtained by running the reaction at 1°C on a Sephadex G-25 column preequilibrated and eluted with decanal-containing buffer. Two forms of flavin were bound to eluted luciferase, as deduced by absorbance and fluorescence spectra. A major part was in the oxidized form, with absorbance maxima in the 370 nm and 440 nm regions and fluorescence emission peaking at 530 nm. A second form was evident from its fluorescence emission in the 490 nm range (Fig. 4). Its absorbance (Fig. 3, trace C), determined by correcting the absorbance of the freshly eluted reaction mixture for the amount of luciferase-bound oxidized FMN calculated to be present, is similar to that obtained by the previous method. Its fluorescence emission spectrum (λmax, 495 nm; Fig. 4, trace F) is similar to the bioluminescence emission and to that of the fluorescence of the luciferase flavin hydroperoxide (13, 17).

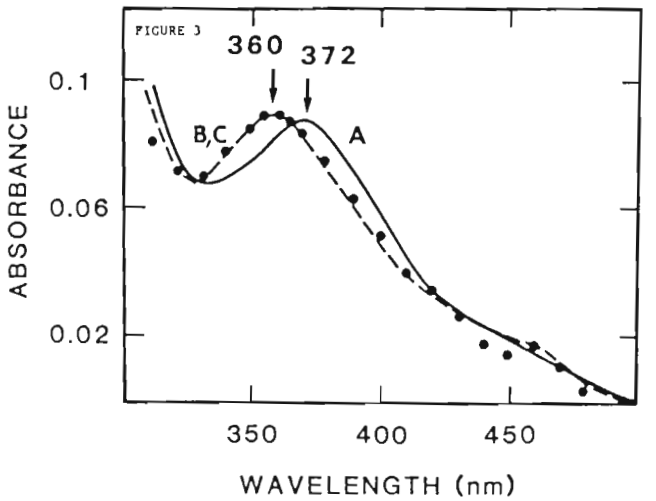
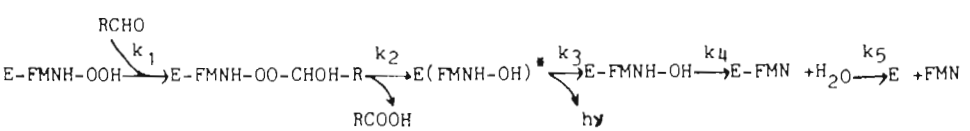


Figure 3
ABSORBANCE SPECTRA FOR THE LUCIFERASE-PEROXY AND -HYDROXY FLAVINS
Curve A (solid line) shows the spectrum of the luciferase flavin-4a-hydroperoxide (same as trace 1, Fig. 1). Curve B (dashed line) is the spectrum of the postulated hydroxy flavin, obtained by subtracting from trace 5 (Fig. 1) 55% of trace 9 (the amount of oxidized FMN already formed, calculated from the amount of peroxy and hydroxy flavin still present), 30% of trace 1 (the amount of flavin peroxy remaining in the mixture, calculated from the amount of light still to be emitted), and normalizing the obtained spectrum by a factor of 5.8 in order to compare with the flavinhydroperoxide. Curve C (solid circles) is also a spectrum calculated for the luciferase-hydroxyflavin, measured by the absorbance of a spent reaction mixture taken immediately after its elution from a column, subtracting the absorbance of the equilibration buffer (phosphate-aldehyde) and the luciferase-bound FMN present. The spectrum was normalized at 360 nm by multiplying by 2.35.

A minimal mechanism which, in our opinion, best fits the present data is shown in Scheme 1. On the assumption that k_2 and k_4 are the limiting steps, and about equal in value, the kinetic behavior of ΔA_{355} will be first order but that for ΔA_{440} will exhibit a lag. The data (Fig. 2) are consistent with this.



Scheme 1

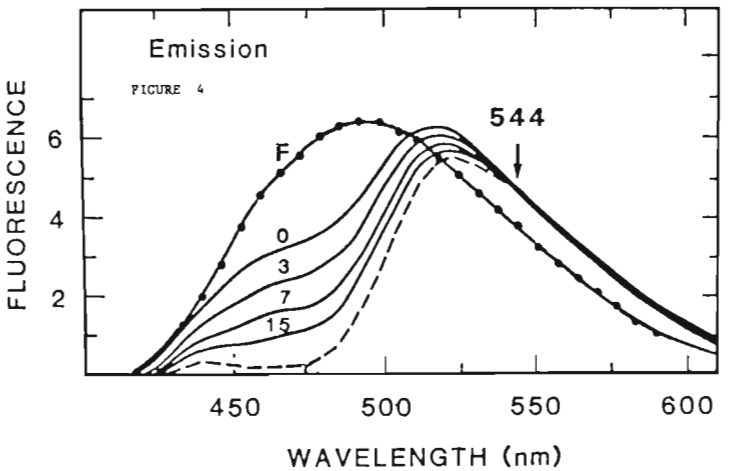


Figure 4
TIME DEPENDENT CHANGES OF THE FLUORESCENCE EMISSION SPECTRA OF THE FLAVIN INTERMEDIATE AFTER THE COMPLETED LIGHT REACTION
A solution of 50 µl luciferase (3.1×10^{-4} M), 25 µl FMN (4.5×10^{-3} M) was reduced by sodium dithionite and applied to a G-25 column, equilibrated with 0.01% v/v decanal in 0.35 M phosphate buffer, pH 7.0. The protein fraction (0.4 ml) was eluted and fluorescence spectra were recorded at 9°C. Curve o shows the first emission spectrum (excitation at 380 nm) immediately after the elution. The other curves, as marked, were recorded after 3, 7 and 15 min. A final spectrum (dashed line) was recorded also at 9°C after warming the sample to 25°C. The corrected fluorescence emission spectrum for the intermediate (solid points) was calculated by subtracting the fluorescence attributable to oxidized FMN and then applying the corrections for the fluorimeter. Excitation, 380 nm; sensitivity, 1; slit, 6 nm; scan speed, 120 nm/min.

The proposed catalytic cycle for the bacterial luciferase reaction is shown in Fig. 5. The conversion of the peroxyhemiacetal to the flavin-4a-hydroxide and RCOOH (Step k_2) may be written as a hydride shift (Baeyer-Villiger; 18) or equally well as a proton loss (15). Although the proposed reaction scheme does not in itself demand the formation of the hydroxide in an excited state, it accounts for the energetic requirements of light emission: the labile O-O bond disappears in the reaction step in which the postulated luminophore is created. Moreover this mechanism is in agreement with the studies of Bruice and coworkers (19) with flavin-4a-hydroperoxide model systems, and the spectra are consistent with previous work (20). The identity of the new intermediate as the hydroxide

also follows from comparisons with studies of p-hydroxybenzoate hydroxylase (21), phenol hydroxylase (22), and N, S-monooxygenase (23). The spectrum of the postulated luciferase-4a-hydroxide is also consistent with the absorbance of authentic FAD-4a-hydroxide bound to p-hydroxybenzoate hydroxylase (24).

The flavin-4a-hydroxide intermediate is a clear candidate as the primary excited state and emitter in the bacterial luciferase reaction (14, 15). Such a species would also argue against proposed flavin structural rearrangements in the reaction (25, 26, 27). While the exact steps responsible for populating the excited state have not been established, both the proposed Baeyer-Villiger mechanism (18) and a postulated free-radical mechanism (28) are compatible with the formation of the flavin-4a-hydroxide.

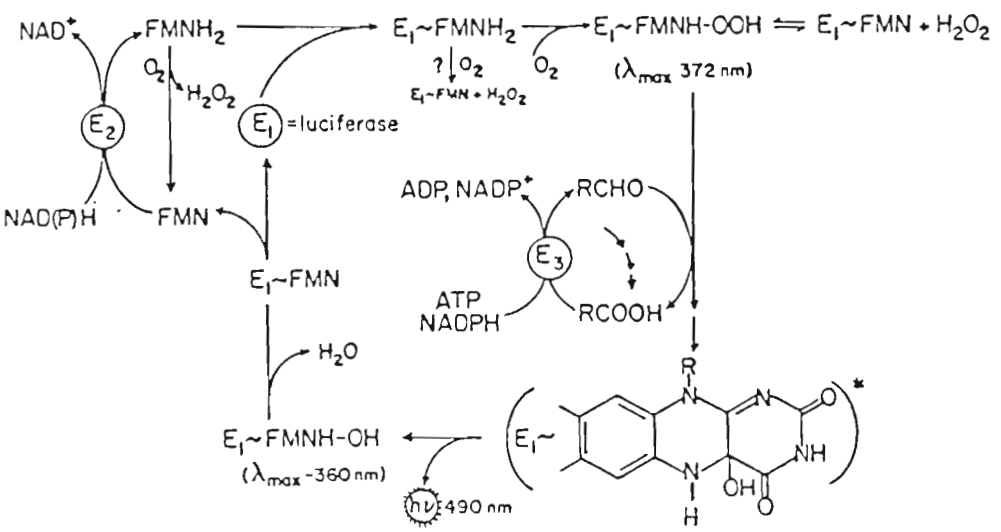


Figure 5
Scheme representing the catalytic pathway of bacterial luciferase. The reaction of the flavin peroxy with aldehyde results in the formation of the peroxyhemiacetal (not shown), followed by its conversion to the excited state hydroxide. E_1 represents luciferase; E_2, FMN reductase and E_3, fatty acid reductase.

The intermediate reported here may be the same as a transient fluorescent species reported by Matheson and Lee (29). Although they did not identify it as a flavin, they noted its occurrence during the luciferase reaction initiated by mixing $FMNH_2$ with aldehyde and oxygen, and proposed a model in which the fluorescent species is formed in the reaction, but not initially as an excited state. In our model, by contrast, the transient which exhibits fluorescence in the 490 nm region is the reaction product, namely the luciferase-bound FMN-4a-hydroxide, formed in the reaction initially in the excited state, and therefore the emitting species in the bioluminescent reaction. Emission from secondary chromophores (ones not involved in the chemical reaction per se) has been shown to occur in several bioluminescent systems, including certain coelenterates (30) and bacteria (31). In the bacterial system chromophores such as lumazine (32) and oxidized flavin (33), bound to distinct separate proteins, are postulated to be secondary emitters, populated by energy transfer from a primary excited state. The identity of the primary excited state as the FMN-4a-hydroxide in our scheme should be contrasted to the model of Matheson and Lee (29, 34), which assumes that the primary excited species is still unidentified and, in particular, that the flavin does not play any role whatsoever in this respect.

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