Identity of the Emitter in the Bacterial Luciferase Luminescence Reaction: Binding and Fluorescence Quantum Yield Studies of 5-Decyl-4a-hydroxy-4a,5-dihydroriboflavin-5′-phosphate as a Model†

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ABSTRACT: The excited state of 4a-hydroxy-4a,5-dihydroFMN has been postulated to be the emitter in the bacterial bioluminescence reaction. However, while the bioluminescence quantum yield of the luciferase emitter is about 0.16, chemiluminescence and fluorescence quantum yields of earlier flavin models mimicking the luciferase emitter were no more than 10^{-5}. To further examine the proposed chemical identity of the luciferase emitter, 5-decyl-4a-hydroxy-4a,5-dihydroFMN was prepared as a new flavin model. Both the wild-type *Vibrio harveyi* luciferase and a catalytically active αC106A mutant formed complexes with the flavin model at a 1:1 molar ratio with K_d values at 2.4 and 1.2 μM, respectively. This flavin model inhibited the activity of both luciferases, suggesting that it was bound to the enzyme active center. While the free flavin model was itself only very weakly fluorescent, its binding to either luciferase species resulted in markedly enhanced fluorescence, peaking at 440 nm. The fluorescence quantum yields of 5-decyl-4a-hydroxy-4a,5-dihydroFMN bound to wild-type and αC106A luciferases were 0.08 and 0.05, respectively, which are about 50% of the respective emitter bioluminescence quantum yields of these two luciferases. The present findings clearly demonstrated that the luciferase active site was suitable for marked enhancement of fluorescence of 4a-hydroxyflavin and, hence, provides a strong support to the proposed identity of 4a-hydroxy-4a,5-dihydroFMN, in its exited state, as the luciferase emitter.

The $\alpha\beta$ dimeric bacterial luciferase, a flavin-dependent monooxygenase, has been extensively studied with respect to its structure and reaction mechanism. This enzyme catalyzes the oxidation of reduced riboflavin-5′-phosphate ($FMNH_2$)[1] and a long-chain aliphatic aldehyde (R−CHO) to generate the corresponding fatty acid (R−COOH), FMN, water, and bioluminescence ($\lambda_{max} \sim 490$ nm). A proposed chemical mechanism for this reaction is shown in Scheme 1.

Following Scheme 1, luciferase-bound N1-deprotonated reduced FMN ($FMNH^-$) intermediate **I** (*1*) reacts with oxygen to generate the 4a-hydroperoxy-4a,5-dihydroFMN (HFOOH) intermediate **II**, which has been isolated and extensively characterized (*2*−*7*) and its absorption spectrum and light-emitting activity successfully mimicked by a synthetic 4a-hydroperoxyflavin model (*8*). Intermediate **II** subsequently combines with the aliphatic aldehyde to form intermediate **III** detected by absorption spectroscopy and kinetic analyses and proposed to be 4a-peroxyhemiacetal-4a,5-dihydroFMN (HFOOCH(OH)R) (*9*). After a still not fully understood mechanism, intermediate **III** decays to form an exited enzyme-bound emitter. One proposed pathway involves the formation of a caged pair of a carboxylic acid radical anion and 4a-hydroxy-4a,5-dihydroFMN radical cation ($HFOH^{+\bullet}$) intermediate $\mathbf{IV}^{+\bullet}$ (*10*−*12*). A subsequent radical annihilation generates a carboxylic acid and the excited state of 4a-hydroxy-4a,5-dihydroFMN (HFOH*) intermediate **IV*** as the emitter. Relaxation of **IV*** to the ground state is accompanied by bioluminescence emission. The formation of the ground state intermediate **IV** immediately after bioluminescence has been detected by absorption and fluorescence spectroscopy (*13*−*15*). Finally, the proposed 4a-hydroxy-4a,5-dihydroFMN (HFOH) intermediate **IV** decays to form water and FMN to complete the reaction.

In Scheme 1, the true identity of the emitter (**IV***) is of critical importance to our understanding of the luciferase mechanism. The proposed HFOH* structure for the luciferase emitter has received some experimental support but has never been fully established. One supporting line of evidence is that the absorption and fluorescence spectra of intermediate **IV** are both similar to those of a 5-ethyl-4a-ethoxy-3-methyl-4a,5-dihydrolumiflavin model compound (EtFOEt in Figure 1) measured at 77 K (*16*). A second supporting evidence is that one-electron oxidation of a similar model 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin (EtFOH in Figure

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[1] Abbreviations: $FMNH_2$, reduced riboflavin 5′-phosphate; HF−OH and HF−OH*, ground state and excited state, respectively, of 4a-hydroxy-4a,5-dihydroFMN; EtFOEt, 5-ethyl-4a-ethoxy-3-methyl-4a,5-dihydrolumiflavin; EtFOH, 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin; DFOH, 5-decyl-4a-hydroxy-4a,5-dihydroriboflavin-5′-phosphate or 5-decyl-4a-hydroxy-4a,5-dihydroFMN.

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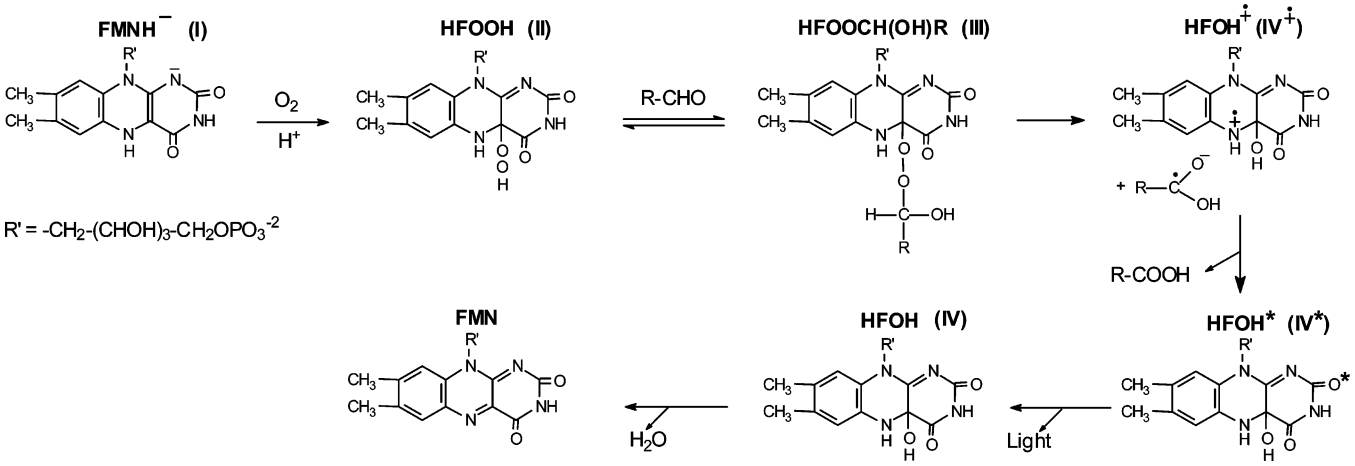
Scheme 1



1) to the corresponding flavin radical cation followed by one-electron reduction gives rise to chemiluminescence (*17*−*20*). However, the observed chemiluminescence is in a longer wavelength range ($\lambda_{max} \geq 530$ nm) (*8*) and has extremely low quantum yields (10^{-4} to 10^{-7}, depending on the reaction medium) in comparison with luciferase bioluminescence ($\lambda_{max} \sim 490$ nm, and quantum yield = 0.1−0.16) (*6*, *21*). Moreover, at room temperature, the fluorescence quantum yield of EtFOEt was not quantified but reported to be very low (*16*) and that of EtFOH was estimated to be no more than 10^{-5} (*19*). Hence, both the chemiluminescence and the fluorescence quantum yields derived from 4a-hydroxyflavin models are $\geq 10^4$-fold lower than the bioluminescence quantum yield of the luciferase emitter. Such a huge discrepancy in the quantum yield must be reconciled before the structural assignment of 4a-hydroxy-4a,5-dihydroFMN for intermediate **IV** can be firmly established. Although no quantitative data are available, qualitatively, it has been observed that the fluorescence quantum yield of EtFOEt is higher in a frozen state at 77 K than at room temperature (*16*). Conceivably, the luciferase active site could bind the HFOH intermediate **IV** tightly and rigidly to greatly enhance its emission quantum yield. Unfortunately, synthetic HFOH cannot be obtained in a stable form, and luciferase does not bind either EtFOEt or EtFOH to any detectable extent. This discrepancy between the extremely low fluorescence and chemiluminescence quantum yields of flavin models and the ∼0.16 bioluminescence quantum yield of the wild-type luciferase emitter has remained unresolved.

To gain more definitive information about the chemical nature of the luciferase emitter, we have synthesized and isolated 5-decyl-4a-hydroxy-4a,5-dihydroFMN (DFOH in Figure 1) as a new model for luciferase intermediate **IV**. This flavin model compound was sufficiently stable for studies in aqueous media and was found to bind to *Vibrio harveyi* luciferase in a 1:1 molar ratio. In addition to other characterizations, measurements of the fluorescence quantum yield of the luciferase-bound DFOH strongly support the assignment of 4a-hydroxy-4a,5-dihydroFMN as the identity for luciferase intermediate **IV**.

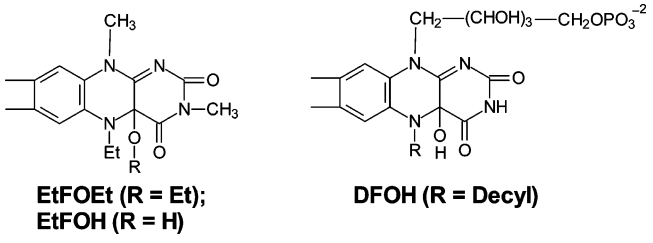


FIGURE 1: Molecular structures of flavin models.

EXPERIMENTAL PROCEDURES

Materials. FMN, decanal, and palladium on activated carbon were purchased from Sigma. The standard phosphate buffer at pH 7.0 was made of phosphates at mole fractions of 0.39 sodium monobase and 0.61 potassium dibase. *V. harveyi* luciferase and its αC106A mutant were expressed in *Escherichia coli* JM101 harboring pTH3 (*22*, *23*) and pTXA1 (*24*), respectively, and purified to >95% purity as described previously (*25*). The αC106A luciferase is catalytically active and, using decanal substrate, exhibiting a quantum yield ∼60% of that for the wild-type enzyme (*24*). For reasons to be discussed later, αC106A luciferase was used throughout this work except that, whenever specifically mentioned, wild-type luciferase was also used in some parallel studies.

Synthesis and Purification of DFOH. DFOH was prepared by adapting the method (*26*, *27*) for the similar derivative of 3-methyllumiflavin with modifications. 5-Decyl-1,5-dihydroFMN was obtained by hydrogenating FMN (0.5 g) in a mixture of acetic acid (50 mL), H_2O (30 mL), decanal (1 mL), and Pd−C (0.2 g) at room temperature and under 60 Psi of H_2 pressure for 12 h. Pd−C was removed by filtering. The filtrate was concentrated in vacuo at 50 °C, and the precipitate obtained was washed 3 times with ether and dried by air. During the treatment, 5-decyl-1,5-dihydroFMN was partially autooxidized into 5-decylFMN semiquinone (0.58 g) with a melting point of 196−198 °C. 5-DecylFMN semiquinone (0.55 g) was then oxidized under acidic conditions (in 5 mL of 2 N $HClO_4$ with the addition of 2 g of $NaClO_4$ and 0.5 g of $NaNO_2$) to yield 5-decyl-FMN$^+$. The oxidized flavin product was formed as violet precipitates, which were collected by filtration and washed twice with 2 mL of 2 N $HClO_4$. 5-DecylFMN$^+$ was finally converted into DFOH by stirring all of the precipitates in 10 mL of 0.5 M phosphate for 1 h. The DFOH product, as a yellow green precipitate, was recovered by centrifugation.

For purification, 4 mg of DFOH was vortexed in 0.3 mL of 50 mM phosphate and centrifuged. The supernatant was

loaded in a 1 × 40-cm Sephadex G-75 column preequilibrated and eluted with 5 mM phosphate. The peak at void volume was DFOH in the form of micelles, separated well from the mixture of free small molecules. The micelles were vortexed in standard phosphate buffer and loaded on the Sephadex G-75 column again. Solubilized and micelle DFOH were separated, and the former was used immediately for experiments.

Isolation of the Luciferase:DFOH Complex. Luciferase in 5.0 mM phosphate was mixed with the purified true DFOH solution. The mixture was loaded on a 1 × 40-cm Sephadex G-75 column preequilibrated with 5.0 mM phosphate and eluted with the same buffer. The complex was recovered from the fractions containing luciferase.

Fluorescence Measurements. Steady-state fluorescence was measured by using a SLM 48000S fluorometer (SLM Instrument, Inc.). Emission spectra were corrected for wavelength-dependent variations in photomultiplier tube response and monochromator (*28*). Using 360 nm excitation, fluorescence emission spectra of the isolated luciferase:DFOH complex and standard FMN, both adjusted to have 0.02 A_{360}, were measured. Areas of the corrected emission spectra were used to calculate the quantum yields of the luciferase complexes in comparison with the known quantum yield of 0.24 for FMN (*29*).

Binding Stoichiometry. The first method followed the principle of continuous variation of Job (*30*). A series of samples were set up, each in 1 mL of 5 mM phosphate, that all contained a constant total concentration of luciferase plus DFOH at 10 μM but with the mole fraction of DFOH varied from 0 to 1. Fluorescence at 430 nm (primarily associated with the enzyme:DFOH complex) was measured using excitation at 360 nm. Weaker background fluorescence signals of controls lacking one of the components were excluded for the total fluorescence signals of the samples that contain both flavin and luciferase to determine the fluorescence intensity of the luciferase:DFOH complex. When the fluorescence signals of the enzyme:DFOH complexes were plotted against the mole fractions of the flavin, linear portions from both ends of the plot can be extrapolated to reach an interception point. The flavin-binding stoichiometry can then be calculated from the mole fraction corresponding to this interception point. A second method involved the titration of a constant concentration of DFOH at 10 μM with up to 20 μM of luciferase. Fluorescence intensities were similarly determined as above.

Inhibition of Luciferase Activity. The Cu^{I} assay (*31*) was used to determine the activities of luciferase in the absence or presence of DFOH as an inhibitor. Aliquots (10 μL) of each of 0.2% decanal in ethanol and a saturated $CuClO_4$ solution in acetonitrile were sequentially added into 1 mL of 50 mM phosphate containing 50 μM FMN and 20 mM EDTA to reduce the FMN. The solution was injected, by using a syringe, into 1 mL of an air-saturated 10 mM phosphate containing 0.3 μM wild-type or the αC106A variant of luciferase and 0−10 μM DFOH at 23 °C. Light emission was measured using a calibrated photometer.

Other Measurements. UV−visible spectra were recorded with a Milton Roy Spectronic 3000 unit. Mass spectrum was obtained by using a Micromass ZabSpec Hybrid Sector-TOF instrument and the method of positive-mode electrospray ionization.

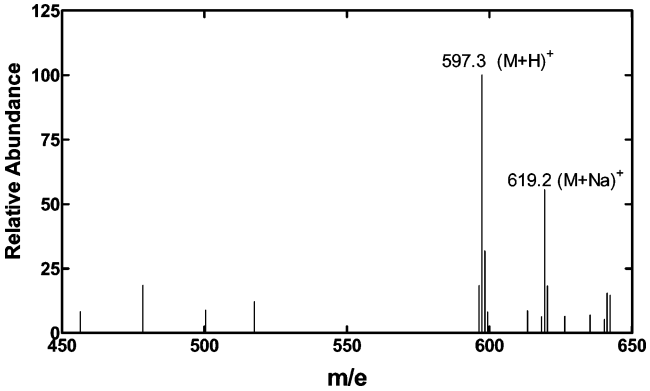


FIGURE 2: Mass spectrum of 5-decylFMN$^+$.

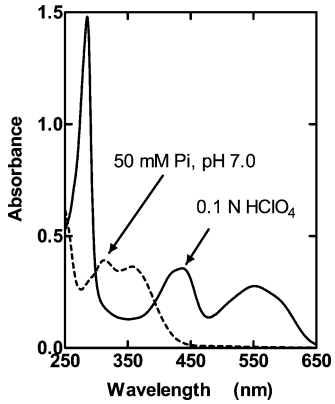


FIGURE 3: UV−visible spectra of 31 μM 5-decylFMN$^+$ in 10 mM phosphate at pH 7.0 (- - -) and in 0.1 N $HClO_4$.

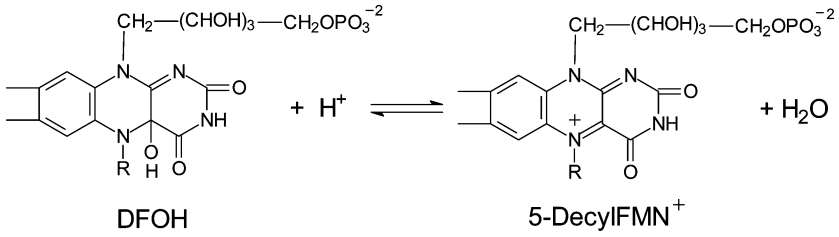
RESULTS

DFOH. The immediate precursor for DFOH is 5-decylFMN$^+$. Figure 2 shows the mass spectrum of 5-decylFMN$^+$, which displayed the M + 1 peak of *m*/*e* 597.3. This peak matches well with the calculated *m*/*e* of 597.2689 for the protonated product $^{12}C_{27}{}^{1}H_{42}{}^{14}N_4{}^{16}O_9{}^{30}P^+$. When this flavin was solubilized in a pH 7.0 aqueous medium, absorption peaks at 313 and 357 nm (- - -, Figure 3) were observed. Acidification yielded a totally different spectrum with peaks at 286, 437, and 551 nm (—, Figure 3). Neutralization of the solution reversed the spectrum change. Such spectra and their reversible changes with pH shift were identical to those of 5-ethyl-3-methyllumiflavin cation under similar conditions shown to be associated with the interconversion between this oxidized flavin cation and the corresponding 4a-hydroxyflavin (*26*, *27*). Accordingly, these spectrum alternations shown in Figure 3 are attributed to the reversible conversion of DFOH pseudobase in neutral solution (•••, Figure 3) to the 5-decylFMN$^+$ cation (—, Figure 3) when the solution was acidified as shown in Scheme 2.

Therefore, DFOH can be easily obtained simply by solubilizing the 5-decylFMN$^+$ in a neutral aqueous medium.

Luciferase:DFOH Complex. Sephadex G-75 chromatography was used to assess whether luciferase can bind DFOH to form a stable complex. Excess DFOH was mixed with luciferase and then passed through a Sephadex G-75 column. The A_{280} profile of the elution showed two peaks (—, Figure 4A). The first peak corresponded to the void volume for luciferase and luciferase-bound species, and the second peak was for the free DFOH on the basis of the elution profile of a control run of DFOH without luciferase (- - -, Figure 4A).

Scheme 2



The absorption spectrum of the first peak was shown in the main panel of Figure 4B (—), exhibiting additional absorption over the 250−450 nm range when compared with the spectrum of luciferase itself (- - -). The difference spectrum (inset of Figure 4B) correlated well with that of DFOH. These results indicate that a luciferase:DFOH complex was formed and isolatable by molecular sieve chromatography. Special care was taken for this experiment. DFOH has polar as well as nonpolar long chain alkyl groups and thus readily forms micelles at high concentrations. Luciferase and micelles of DFOH had about the same elution volume from the Sephadex G-75 column. To prevent this complication, DFOH used for the detection of the luciferase:DFOH complex formation was prepared in the form of a true solution as described in the Experimental Procedures.

The change in spectrum of DFOH with pH was used to further confirm the identity of the bound species by luciferase. If the bound flavin was DFOH, acidification of the complex should convert it into 5-decylFMN$^+$ and give rise to the absorption peaks at 437 and 551 nm. Indeed, these two absorption peaks were observed immediately after the acidification of the luciferase complex sample by mixing with an equal volume of 1 N $HClO_4$ containing 8 M guanidine hydrochloride to avoid protein precipitation (Figure 5).

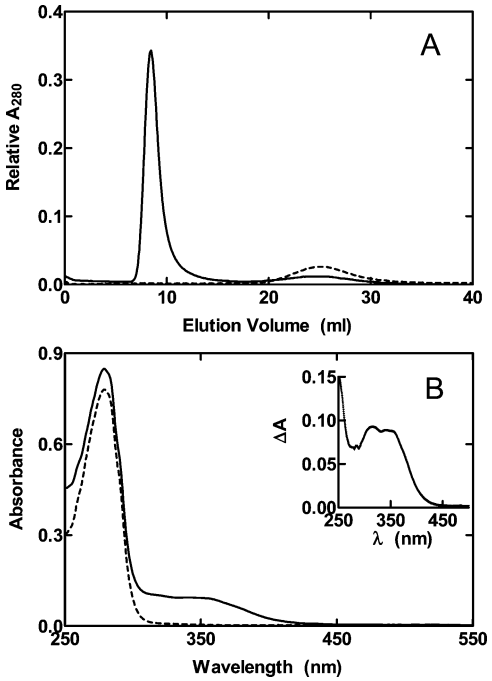


FIGURE 4: Detection of the luciferase:DFOH complex. (A) Sephadex G-75 column (1 × 40 cm) elution profiles for a luciferase and DFOH mixture (—) and a control containing only DFOH (- - -). Samples of 30 μM DFOH itself and a mixture of 20 μM αC106A luciferase and 30 μM DFOH in 1 mL of 5.0 mM phosphate were each loaded on the column and eluted with the same buffer. (B) UV−visible spectra of luciferase alone (- - -) and the peak fraction at the elution volume of 9 mL in A. Both samples had 10 μM luciferase. The difference spectrum of ($A_{complex}$ minus $A_{luciferase}$) is shown in the inset.

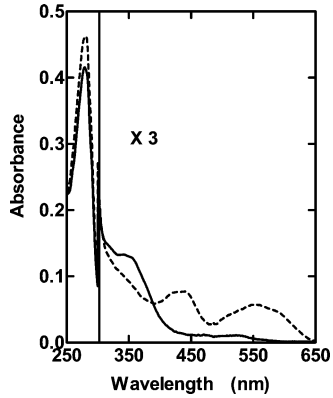


FIGURE 5: Spectral evidence for the identity of DFOH bound to luciferase. UV−visible spectra were recorded for the isolated luciferase:DFOH complex after dilution with an equal volume of either 5 mM phosphate (—) or 8 M guanidine hydrochloride in 1 N $HClO_4$ (- - -).

Fluorescence of the Luciferase:DFOH Complex. Both the wild-type and αC106A luciferases were used to isolate the enzyme:DFOH complex as described above, and their fluorescence excitation (with emission monitored at 430 nm) and emission (with excitation monitored at 360 nm) spectra were recorded. Similar results were obtained for both enzyme samples, and those for the αC106A:DFOH complex are shown in Figure 6. In contrast to the weak background signals associated with luciferase alone or free DFOH, the αC106A:DFOH complex exhibited intense fluorescence. The excitation spectrum showed two peaks similar to the two absorption peaks of DFOH. The corrected fluorescence emission showed a peak at 440 nm. The fluorescence quantum yield of αC106A-bound DFOH was determined to

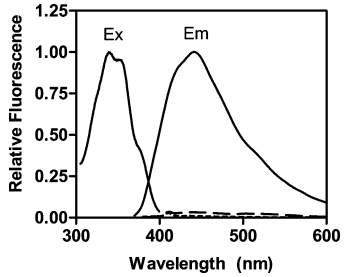


FIGURE 6: Fluorescence spectra of the luciferase:DFOH complex. The isolated complex was obtained as described in Figure 4 and had A_{280} of 0.81 and A_{360} of 0.068. The excitation (Ex) and corrected emission (Em) spectra of the sample were obtained by monitoring emission at 430 nm and using excitation at 360 nm, respectively. Weak emission signals of controls of αC106A luciferase (- - -) and DFOH (— — —) are also shown.

be 0.05 using FMN (quantum yield = 0.24) as a standard, with both samples adjusted to the same 0.02 absorbance at 360 nm used for excitation. In a parallel experiment using the wild-type luciferase, intense fluorescence emission was also observed for the luciferase:DFOH complex showing a 440 nm peak for the corrected emission spectrum and a quantum yield of 0.08.

Binding Stoichiometry. When fluorescence signals associated with the luciferase:DFOH complexes were plotted against the mole fractions of DFOH in a series of samples that contained a constant 10 μM total concentration of luciferase plus DFOH following the Job's method of continuous variation analysis (Figure 7A), the linear portions of the plot can be extrapolated to reach a point of interception corresponding to 0.48 mole fraction of DFOH. Hence, a binding stoichiometry of 0.92 (=0.48/0.52) DFOH per luciferase was obtained. Using a second method, the DFOH concentration was kept at 10 μM and [luciferase]/[DFOH] molar ratios were varied from 0 to 2. As shown in Figure 7B, fluorescence associated with the enzyme:DFOH complex increased linearly at lower ratios of [luciferase]/[DFOH] and reached a constant plateau at higher ratios of [luciferase]/[DFOH]. These two linear portions of the plot intercepted at a point corresponding to a binding of 1.0 DFOH per luciferase.

Inhibition of Luciferase Activity by DFOH. We found that DFOH up to 10 μM caused inhibition of luciferase in a dose-dependent manner (Figure 8A). At 10 μM of DFOH, 90 and 80% of the activities were inhibited for αC106A and wild-type luciferase, respectively. In the assay, 1 mL of 50 μM $FMNH_2$ with 0.002% decanal was injected into 1 mL of air-saturated phosphate buffer containing luciferase and various concentrations of DFOH. The excess free $FMNH_2$ was rapidly autooxidized after the injection (*32*), much faster than the rate of the luciferase-catalyzed bioluminescence. Therefore, the assay was a single turnover reaction for luciferase. If the dissociation of the luciferase:DFOH inactive complex upon dilution is much slower than both the binding of $FMNH_2$ and the autoxidation of free $FMNH_2$ in the solution, only the original inhibitor-free enzyme can participate in the bioluminescent reaction. The observed bioluminescent activity is governed by the equilibrium of the inactive complex with free luciferase and DFOH before the $FMNH_2$ injection and can be described by eq 1 and the rearranged eq 2 where

$$v = \frac{v_0 K_d}{K_d + [\mathrm{DFOH}]} \quad (1)$$

$$\frac{1}{v} = \frac{1}{v_0} + \frac{[\mathrm{DFOH}]}{v_o K_d} \quad (2)$$

v_0 and v are observed light intensities without and with DFOH, respectively, and K_d is the dissociation constant of the complex. The plots of $1/v$ versus the DFOH concentration were linear for both the wild-type and αC106A luciferases (Figure 8B), consistent with eq 2. Accordingly, K_d values of 1.2 and 2.4 μM for αC106A and wild-type luciferase, respectively, were obtained.

The slow dissociation of bound DFOH was confirmed by first mixing 3 μM of αC106A with 15 μM DFOH and then diluting the sample 10-fold by the standard buffer. $FMNH_2$ was then injected at 3−15 s after the dilution to initiate bioluminescence. The observed light intensities gradually increased to a maximal level at about 10 s after the sample dilution, indicating a slow release of bound DFOH and, hence, a slow recovery of additional active αC106A. Because the dissociation of bound DFOH was much slower than the autoxidation of free $FMNH_2$, the conventional competitive inhibition test could not be performed by injecting an $FMNH_2$ solution into a solution containing luciferase and DFOH. Preincubation of luciferase with DFOH and $FMNH_2$ together followed by an aldehyde solution injection was also prob-

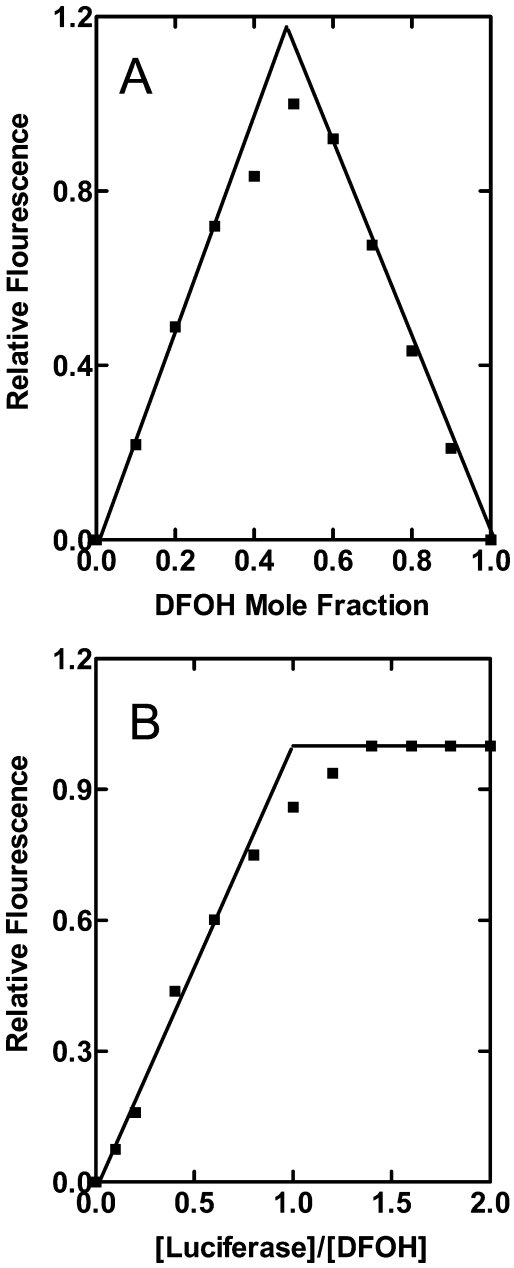


FIGURE 7: Determination of the binding stoichiometry of the luciferase:DFOH complex. One set of samples kept a constant total concentration of luciferase and DFOH constant at 10 μM (A), and another series of samples had a constant concentration of DFOH at 10 μM with various levels of luciferase (B). The emission of each sample at 430 nm was measured using excitation at 360 nm. Weak background signals from luciferase alone and from free DFOH, if significant, were subtracted from the observed fluorescence to determine the fluorescence signals associated with the luciferase:DFOH complexes, which are presented for both A and B.

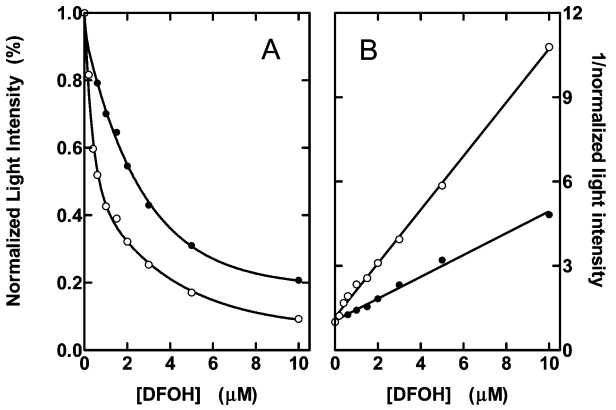


FIGURE 8: Inhibition of luciferase activity by DFOH and the determination of the dissociation constants. (A) Effect of DFOH on luciferase activity. Bioluminescence activities were initiated by injecting 1 mL of 0.002% decanal and 50 mM $FMNH_2$ (reduced by Cu^I-EDTA) into 1 mL of 5 mM phosphate containing 0.3 μM wild-type or αC106A luciferase and 0−10 mM DFOH. The intensities of the emission peaks were normalized by setting the intensity of the reaction without DFOH at 1. (B) Plot of the reciprocal of the normalized light intensity versus the DFOH concentration using the data in A. For both A and B, wild-type and αC106A luciferases are shown as ● and ○, respectively.

lematic for the conventional competitive inhibition test because of instability of DFOH under conditions for FMN (photochemical or chemical) reduction.

DISCUSSION

The main goal of this work is to test whether a 4a-hydroxyflavin type of flavin model whose fluorescence quantum yield can be elevated, upon binding to bacterial luciferase, from the reported $\leq 10^{-5}$ for the free flavin (*19*) to near the bioluminescence quantum yield of the luciferase emitter (*6*). To this end, DFOH was developed for the intended study. The chemical identity of DFOH was supported by the excellent fit of the mass spectrum of the immediate precursor, 5-decylFMN$^+$ (Figure 2), the pH-dependent reversible conversion of DFOH and 5-decylFMN$^+$, and the matching of absorption characteristics of these two flavins (Figure 3) with those of 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin and 5-ethyl-3-methyllumiflavin cation under identical pH-shift treatments (*26*, *27*).

After mixing luciferase with DFOH, the complex can be isolated by molecular sieve chromatography (Figure 4A). The identity of the bound DFOH was confirmed by the absorption spectrum of the flavin in the complex (Figure 4B) and its conversion to 5-ethylFMN$^+$ upon acidification (Figure 5). From two fluorescent titration measurements (parts A and B of Figure 7), DFOH bound to luciferase in a 1:1 molar ratio. DFOH inhibited the activity of both the wild-type and αC106A luciferases (Figure 8A), enabling the determination of K_d values of 2.4 and 1.2 μM, respectively (Figure 8B) and suggesting that DFOH bound to the luciferase active center. From all tests, DFOH appears to be an excellent model for the proposed intermediate **IV**.

Consistent with the reported $\leq 10^{-5}$ fluorescence quantum yield of EtFOH (*19*), the fluorescence of free DFOH was barely detectable by our fluorometer. Upon binding to either wild-type of αC106A luciferase, intense fluorescence was observed with an emission spectrum peaking at 440 nm (Figure 6). Most importantly, the fluorescence quantum yields of the wild-type luciferase- and αC106A-bound DFOH were found to be 0.08 and 0.05, respectively. In both cases, the fluorescence quantum yield of bound DFOH was enhanced several orders of magnitude to a level of 50% of the corresponding bioluminescence quantum yield of 0.16 for the wild-type luciferase emitter and 0.10 for the αC106A emitter. These tremendous levels of fluorescence enhancements of DFOH upon binding to two species of luciferase provide a strong support to the proposed identity of an excited state 4a-hydroxy-4a,5-dihydroFMN as the luciferase emitter. Notably, the emission spectrum of the bound DFOH is at a shorter wavelength range than that of luciferase bioluminescence ($\lambda_{max} \sim 490$ nm). From earlier (*33*) and recent (*34*) mutational studies, it has been shown that the color of bioluminescence is sensitive to perturbations of the emitter:luciferase active-site interactions. Hence, the blue shift of the bound DFOH is not surprising, in view of the tagging of a hydrophobic decyl group at the flavin N5.

A number of technical obstacles were encountered during the course of this study. Initially, EtFOEt and EtFOH were found not to bind to luciferase appreciably, and moreover, HFOH cannot be obtained in a stable form for studies in aqueous media. More recently, we discovered that the naturally occurring P-flavin, reported to be identical in structure to the 6-(3"-myristic acid)-FMN Q-flavin (*35*, *36*), in *Photobacterium phosporeum* can bind to the *V. harveyi* luciferase active site tightly ($K_d = 0.16$ μM) in a 1:1 molar ratio (*37*). In comparison with the FMN binding, the attachment of a long-chain aliphatic group to the C6 position of the flavin ring enhances the binding affinity of P-flavin by 3 orders of magnitude. Consistent with this finding, we found that the attachment of a decyl group to N5, together with the restoration of the ribityl-phosphate chain at N1 and the removal of methyl from N3, enabled wild-type and αC106A luciferases to bind DFOH, in contrast to EtFOEt and EtFOH, in a 1:1 molar ration with K_d values at micromolar levels.

A second technical concern was the stability of the flavin model in aqueous media. It has been shown that 4a-substituted flavins are labile in aqueous media and their stabilities can be significantly increased in a more hydrophobic medium or environment (*8*, *18*, *38*). Moreover, the luciferase 4a-hydroperoxy-4a,5-dihydroFMN intermediate **II** can be markedly stabilized by the cobinding of nonaldehyde long-chain aliphatics, particularly alcohols, to keep the bound flavin at the luciferase active site less accessible to the aqueous medium (*5*, *6*). Hence, it was hoped that the attachment of a hydrophobic decyl group to N5, adjacent to the C4a position, would stabilize the 4a-hydroxyflavin. Indeed, free DFOH was obtainable in neutral aqueous media at room temperature in a form sufficiently stable for the intended studies. Fresh solutions of DFOH were always used in this work because, as with earlier findings with 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin (*18*), prolonged storage in aqueous solutions led to conversion of DFOH to oxidized 5-decylFMN$^+$ cation and subsequently flavin radicals because of comproportionantion of the DFOH pseudobase and 5-decylFMN$^+$.

The binding of DFOH to wild-type luciferase encountered additional technical obstacles, necessitating even more stringent precautions. *V. harveyi* luciferase has a particularly reactive αCys106 residue (*39*). This residue, per se, is not

essential to substrate binding (*40*) or catalysis (*24*). However, because of its close proximity to the luciferase active site (*31*, *41*, *42*), its modification with functional groups bulkier than a methyl results in the loss of luciferase catalytic activity (*39*, *40*). To preserve the wild-type *V. harveyi* luciferase in an active form, its working and/or stock solutions usually contain β-mercaptoethanol or dithiothreitol to prevent modifications of the αCys106. These sulfhydryl-containing agents must be thoroughly removed before luciferase is mixed with DFOH. Otherwise, modifications of DFOH would and did occur following, we believe, reactions similar to that shown previously between nucleophilic sulfur-containing agents and 5-ethyl-3-methyllumiflavin cation in equilibrium with 5-ethyl-4a-hydroxy-3-methyl-4a,5-dihydrolumiflavin (*38*). However, such a precaution was found essential to but not sufficient for a satisfactory stabilization of DFOH in the presence of wild-type luciferase. Changes of the DFOH absorption spectrum began several minutes after the DFOH:luciferase complex was isolated. Although useful data can still be collected when measurements were carried out immediately after the mixing of luciferase and DFOH, routine use of the wild-type luciferase became impractical for most of the intended studies. Fortunately, the αC106A luciferase was catalytically active and capable of binding DFOH (even slightly tighter than the wild-type luciferase). More importantly, the replacement of the highly reactive αCys106 at the active center of luciferase with an alanine enhanced the stability of the luciferase-bound DFOH. Such a finding further suggests that DFOH was bound to the luciferase active site. In the case of wild-type luciferase, the bound DFOH could conceivably be attacked by the reactive αCys106 residue leading to instability. Consequently, most of the work reported herein involved the use of αC106A, with the wild-type luciferase used only for a limited number of critical measurements. Even when αC106A was used, the luciferase:DFOH complex was used as soon as possible after isolation because the bound DFOH, similar to free 4a-hydroxyflavins (*18*), still gradually decayed to generate oxidized flavin and flavin radicals upon standing.

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