THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 1, Issue of January 5, pp. 104–110, 1996

Printed in U.S.A.

Interaction of Bacterial Luciferase with 8-Substituted Flavin Mononucleotide Derivatives*

(Received for publication, August 21, 1995)

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Bacterial luciferase catalyzes the emission of visible light from the reaction of reduced flavin, molecular oxygen, and an *n*-alkyl aldehyde. The mechanism of the reaction was probed by measuring the electronic effects of various substituents at the 8-position of the flavin ring system. Substituent effects were obtained for CH_3-, Cl-, CH_3O-, CH_3S-, F-, and H- on the rate of formation and decay of the hydroperoxyflavin intermediate and the time courses for the emission of visible light. The rate constant for the decay of light emission increases for the series Cl < F < H < CH_3S < CH_3 < CH_3O. These results are not compatible with a standard Baeyer-Villiger type mechanism for the chemical transformation, but they are consistent with a decrease in the electron density at the reaction center of the flavin moiety during the rate-limiting step of the reaction.

Bacterial luciferase catalyzes the reaction of $FMNH_2$,[1] O_2, and a long chain aliphatic aldehyde to yield riboflavin-5′-monophosphate (FMN), the carboxylic acid, and blue-green light (Baldwin and Ziegler, 1992). The mechanism proceeds through the formation of a stable C4a-hydroperoxyflavin intermediate by the reaction of $FMNH_2$ with molecular oxygen, presumably by way of a one-electron transfer to yield a radical pair consisting of a flavin radical and superoxide and subsequent recombination to form the C4a-hydroperoxyflavin intermediate (Bruice, 1984). This intermediate has been isolated using low temperature chromatography (Hastings *et al.*, 1973) and characterized by ^{13}C-NMR spectroscopy (Vervoort *et al.*, 1986). In the absence of aldehyde, this intermediate slowly decomposes to FMN and H_2O_2. In the presence of aldehyde, the C4a-hydroperoxyflavin intermediate is presumed to react with the aldehyde to form a C4a-hydroperoxyhemiacetal intermediate. The formation of the hydroperoxyhemiacetal intermediate from $FMNH_2$, O_2 and aldehyde (RCHO) is shown in Scheme I. This intermediate subsequently rearranges to form a 4a-hydroxyflavin intermediate and the carboxylic acid. Finally, the C4a-hydroxyflavin intermediate dehydrates to form FMN and H_2O.

This enzyme system has been studied for over 30 years, and although most of the intermediates involved in the reaction have been isolated and identified, the mechanism by which the excited state is formed is still a subject of much debate. At least seven mechanisms have been proposed for the light-emitting reaction, but some of these can now be eliminated because they are contrary to experimental observations. From a mechanistic point of view, one very important result was obtained by Suzuki *et al.* (1983). Utilizing $^{18}O_2$, they demonstrated the incorporation of one atom of labeled oxygen into the carboxylic acid product. The three most likely mechanisms that are consistent with this result are the Baeyer-Villiger mechanism, the dioxirane mechanism and the chemically initiated electron exchange luminescence (CIEEL) mechanism. These three mechanisms all assume the formation of a C4a-hydroperoxyhemiacetal intermediate, but they describe different ways for the rearrangement of this complex and formation of the excited state. The first chemical mechanism for the luciferase reaction was proposed by Eberhard and Hastings (1972) and consists of a Baeyer-Villiger rearrangement of the C4a-hydroperoxy-hemiacetal intermediate with migration of hydrogen to yield the carboxylic acid and the luciferase-bound flavin in the singlet excited state. This mechanism was later modified to include the formation of the C4a-hydroxyflavin intermediate as the excited state, since it is currently believed that this latter species is the primary emitter in the luciferase reaction (Kurfurst *et al.*, 1984). The Baeyer-Villiger mechanism is shown in Scheme II. Cyclohexanone monooxygenase, an enzyme that catalyzes the oxidation of a variety of substrates including aldehydes, has been shown to proceed through a Baeyer-Villiger mechanism (Schwab *et al.*, 1983; Branchaud and Walsh, 1985). In this enzyme, a flavin C4a-hydroperoxide intermediate is also the active oxygen transfer agent.

The dioxirane mechanism provides an alternative to the Baeyer-Villiger mechanism. This mechanism involves the decomposition of the C4a-hydroperoxyhemiacetal intermediate to form a dioxirane and the C4a-hydroxyflavin (Raushel and Baldwin, 1989). The decay of the dioxirane by one of several mechanisms could lead to the formation of the light-emitting species. The dioxirane mechanism is depicted in Scheme III. There are at least two potentially chemiluminescent pathways for the breakdown of the dioxirane, both sufficiently energetic to populate the first singlet excited state of the flavin. In one of these pathways, the dioxirane could undergo an homolytic cleavage of the oxygen–oxygen bond to yield a biradical, which could subsequently rearrange to form the carboxylic acid in either the triplet or singlet state. The singlet or triplet state of the carboxylic acid could interact with and excite the C4a-hydroxyflavin intermediate prior to light emission.

The CIEEL mechanism describes the decomposition of the C4a-hydroperoxyhemiacetal intermediate via a series of one-

* This work was supported in part by National Institutes of Health Grant GM33894. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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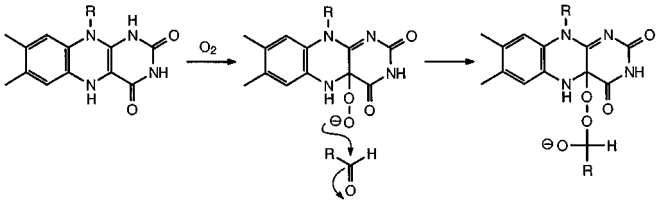
[1] The abbreviations used are: $FMNH_2$ and FMN, reduced and oxidized riboflavin 5′-phosphate; FMNOOH, flavin C4a-hydroperoxide; FMNOOR, flavin C4a-hydroperoxyhemiacetal; FMNOH, flavin C4a-hydroxide; RCHO, *n*-alkylaldehyde; bis-Tris, 2-[bis-(hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; CIEEL, chemically initiated electron exchange luminescence.



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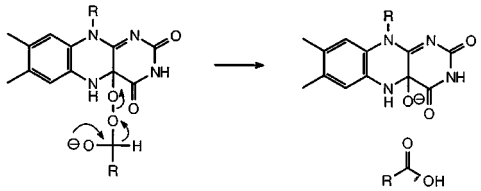

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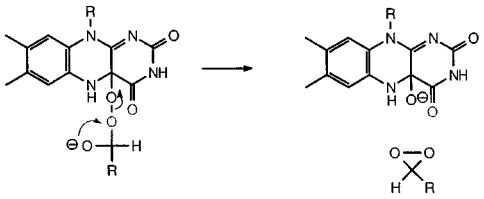

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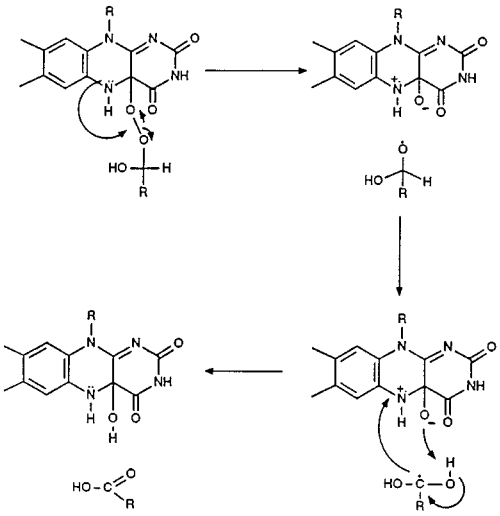
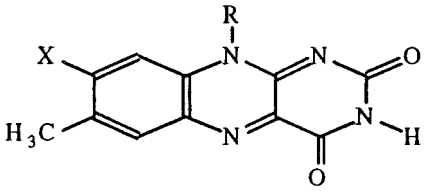


SCHEME IV.



STRUCTURE I.

electron transfer reactions. Schuster (1979) has demonstrated that a CIEEL mechanism can account for certain chemiluminescent and bioluminescent reactions. In this process, a caged radical anion/cation pair is generated, and charge annihilation then populates the excited singlet state of the activator. For the reaction catalyzed by luciferase, it is possible to describe the production of either an excited flavin hydroxide or an excited acid or acylium cation. The CIEEL mechanism for the luciferase reaction proposed by Eckstein *et al.* (1993) is shown in Scheme IV. A CIEEL mechanism can also be proposed for the decomposition of the dioxirane species. In such a mechanism, the dioxirane would receive an electron from the flavin donor to form a radical ion pair. The radical anion of the dioxirane could then yield the radical anion of the carboxylic acid. Back transfer of the electron to the donor would then yield the excited state of the fluorophore.

It has been shown that 8-substituted flavin derivatives are substrates for the luciferase reaction (Watanabe *et al.*, 1978). The structure of the FMN derivatives is shown in Structure I. The position at C-8 is believed to be exposed to the solvent (Schopfer *et al.*, 1981; Chen and Baldwin, 1984) so that modifications at this position should not alter the protein-flavin interactions to any significant extent. It has also been shown that substitutions at this position alter significantly the electronic properties (*i.e.* redox potential) of the flavin. Previous studies directed at determining the effect of these flavin analogs have been limited to simple measurements of bioluminescence intensity and rate of decay of light emission. It is clear that these substitutions should also affect the rates by which the various flavin intermediates react with oxygen and aldehyde substrates. For example, the nucleophilicity of the C4a-hydroperoxyflavin anion should vary with the different substitutions, and as a consequence, the rate of reaction of this intermediate with the aldehyde substrate and the rate of decomposition of the formed C4a-flavin peroxyhemiacetal intermediate should differ from one flavin analog to the other. In this investigation the kinetics of the luciferase-catalyzed reaction with various 8-substituted flavin derivatives have been studied in detail. The rates of formation and decay of the reaction intermediates have been measured. In addition the rate of formation of visible light and the effect of these substitutions on the individual steps of the reaction mechanism have been determined.

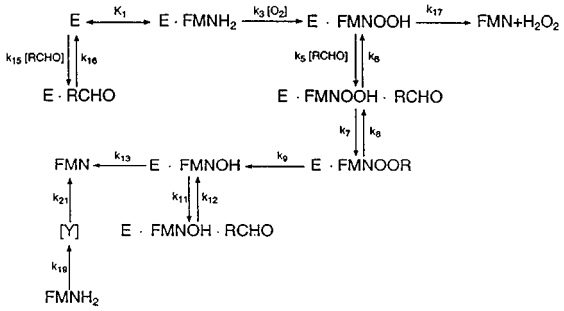
MATERIALS AND METHODS

General—FMN was purchased from Fluka (97%) and used without further purification. 8-Chlororiboflavin was prepared by modification of the method of Lambooy (1971), 8-methoxyriboflavin by the method of Kasai *et al.* (1979), and 8-thiomethylriboflavin by modification of the method of Moore *et al.* (1979). 8-Fluoroflavin was synthesized by coupling of the ribityl derivative of 6-amino-3-fluoro-4-methylaniline with alloxan.[2] The riboflavin derivatives were phosphorylated according to the method of Scola-Nagelschneider and Hemmerich (1976). The 8-H-FMNH$_2$ derivative was a generous gift from Professor Franz Müller (Sandoz Agro, Ltd.). All the FMN analogs were further purified by column chromatography. All purified flavins moved as single spots on thin-layer chromatography and were characterized by ^{1}H, ^{13}C, and ^{31}P NMR and mass spectrometry. All other reagents and solvents were of the highest purity grade available and obtained from either Sigma or Aldrich.

Enzyme Purification—Wild-type bacterial luciferase from *Vibrio harveyi* was purified by the method of Baldwin *et al.* (1989). The enzyme was judged to be greater than 95% pure based on gel electrophoresis. The enzyme concentration was determined spectrophotometrically by measuring the absorbance at 280 nm using a molecular weight of 76,000 and a specific absorption coefficient of 1.13 cm^{-1} mg^{-1} (Sinclair *et al.*, 1993).

Stopped-flow Spectrophotometry—All experiments were performed under a nitrogen atmosphere in 50 mM bis-Tris-HCl buffer, pH 7.0, at 25° ± 0.2 °C. Care was taken not to expose the flavin solutions to light during the experimental procedures. The flavin substrates were reduced by bubbling hydrogen in the presence of a few crystals of palladium on activated carbon. The concentration of the FMN derivatives was determined spectrophotometrically. The molar absorption coefficients used were 12,200 M^{-1} cm^{-1} at 450 nm for FMN (Whitby, 1953), 10,100 M^{-1} cm^{-1} at 445 nm for 8-Cl-FMN (Fritz *et al.*, 1987), 8,500 M^{-1} cm^{-1} at 448 nm for 8-H-FMN (Fritz *et al.*, 1987), 24,500 M^{-1} cm^{-1} at 445 nm for 8-CH$_3$O-FMN (Fritz *et al.*, 1987), 28,500 M^{-1} cm^{-1} at 474 nm for 8-CH$_3$S-FMN (Moore *et al.*, 1979), and 12,500 M^{-1} cm^{-1} at 435 nm for

[2] R. Topgi, unpublished results.



SCHEME V.

8-F-FMN (Macheroux *et al.*, 1990).

The anaerobic enzyme solutions were prepared using an all glass vacuum system (Williams *et al.*, 1979) by several cycles of evacuation and equilibration with nitrogen gas. Purification of the nitrogen gas was performed by passing the gas over a heated column of BASF catalyst R3–11 (Chemical Dynamics Corp./Kontes Glass Co.). The anaerobic luciferase-$FMNH_2$ solutions were made by mixing the anaerobic enzyme solutions with reduced flavin under a nitrogen atmosphere, and the mixture was then transferred to the stopped-flow instrument using an air-tight Hamilton syringe. The kinetic experiments were carried out using a stopped-flow apparatus from Hi-Tech Ltd. (model SF-51) connected to an HP-300 series computer. A glass cut-off filter (type GU 380) was used for absorbance measurements at 380 nm to avoid interference by the bioluminescence during the course of the reaction. The stopped-flow instrument was equipped with a rapid scanning device (MG-3000) designed to collect a complete spectrum (200–800 nm) every 90 ms. The stopped-flow experiments were carried out under the same conditions as described previously (Abu-Soud *et al.*, 1992, 1993). A stock solution of 0.1 M decanal was freshly prepared before use. The time courses for the various kinetic experiments were fit to one or more of the following rate equations using a nonlinear least-square procedure contained in the software supplied by Hi-Tech Ltd.

$$y = Ax + C \quad \text{(Eq. 1)}$$

$$y = Ae^{-k_1 t} + C \quad \text{(Eq. 2)}$$

$$y = Ae^{-k_1 t} + Be^{-k_2 t} + C \quad \text{(Eq. 3)}$$

$$y = A(k_1/(k_2 - k_1))(e^{-k_1 t} - e^{-k_2 t}) + C \quad \text{(Eq. 4)}$$

$$y = A(1 + (1/(k_1 - k_2))(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})) + C \quad \text{(Eq. 5)}$$

Equation 3 represents the sum of two independent exponentials for a parallel process. Equations 4 and 5 describe the time courses for a sequential process ($X \rightarrow Y \rightarrow Z$) monitoring the formation of Y and Z, respectively. In these equations, k_1 and k_2 are the first-order rate constants, t is time, A and B are amplitude factors, and e is 2.718. Ten individual traces were collected and averaged to improve the signal-to-noise ratio.

All of the kinetic data collected from the stopped-flow studies were transferred to a Silicon Graphics workstation and subsequently analyzed with extensively modified forms of the KINSIM (Barshop *et al.*, 1983) and FITSIM (Zimmerle and Frieden, 1989) programs, using the comprehensive kinetic model that appears in Scheme V. The microscopic rate constants that appear in these kinetic models were estimated by comparison of the experimental time courses for product formation with the calculated time courses derived by numerical integration of the appropriate differential equations with the KINSIM program. The rate constants were first estimated graphically until the simulated time courses matched the experimental data as closely as possible. The final values were then adjusted, and the error limits were obtained using the automated FITSIM routine that calculates the best values by minimization of the difference between the experimental and simulated data using an iterative nonlinear least squares procedure. The standard error for each individual rate constant has been estimated to be less than 15% using the FITSIM program.

Oxygen Measurements—The molecular oxygen concentrations were determined using an Orion pH meter (model 601) equipped with an oxygen electrode (model 97-08).

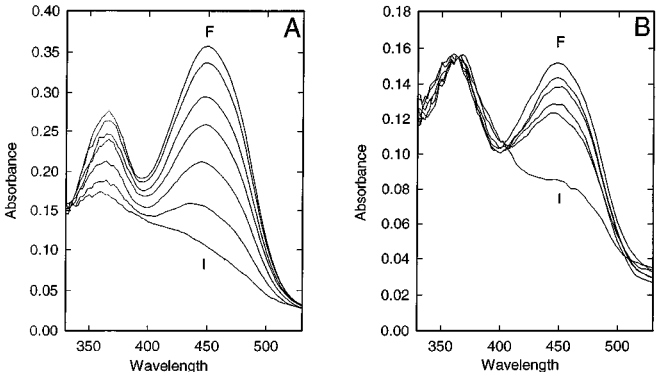


FIG. 1. **Rapid scanning absorption spectra for the autooxidation of 8-Cl-$FMNH_2$ and for the formation and decomposition of the *E*·8-Cl-FMNOOH intermediate.** *A*, absorption spectra following the reaction of 8-Cl-$FMNH_2$ (30 μM) with air-equilibrated buffer (120 μM O_2) at 25 °C. *Spectrum I* was taken 7 ms after mixing. Additional spectra were taken at 90-ms intervals for 900 ms. Not all spectra are shown. *B*, absorption spectra following the reaction of luciferase (75 μM) and 8-Cl-$FMNH_2$ (15 μM) with air-equilibrated buffer (120 μM O_2) at 25 °C. *Spectrum I* was taken 23 ms after mixing. Additional spectra were taken at 0.9-s intervals for 8.5 s. Not all spectra are shown.

RESULTS

Decomposition of 8-X-$FMNH_2$—The nonenzymatic decomposition of the reduced 8-substituted FMN derivatives was studied by measuring the absorbance changes that followed the mixing of 8-*X*-$FMNH_2$ with O_2. Fig. 1*A* shows the absorption spectra for the reaction of 8-Cl-$FMNH_2$ with O_2. *Spectrum I* corresponds to that of the reduced form, and spectrum F corresponds to the fully oxidized form. Similar spectra were collected for all of the 8-substituted flavin derivatives studied. Single-wavelength experiments were done to measure the kinetics of the nonenzymatic decomposition of the 8-substituted $FMNH_2$ derivatives. The wavelengths at which the absorbance changes were measured were the following: 380 and 445 nm for $FMNH_2$, 365 and 445 nm for 8-Cl-$FMNH_2$, 370 and 472 nm for 8-CH_3S-$FMNH_2$, 330 and 445 nm for 8-CH_3O-$FMNH_2$, 350 and 448 nm for 8-H-$FMNH_2$, and 356 and 435 nm for 8-F-$FMNH_2$. In general, these wavelengths correspond to the absorbance maxima of the corresponding 8-*X*-FMN. The time courses for the appearance of 8-*X*-FMN measured at both wavelengths were very similar, and each had a distinctive lag followed by a single exponential. Fig. 2 shows the time course for the reaction of 15 μM 8-Cl-$FMNH_2$ with 120 μM O_2 measured at 365 and 445 nm. The time courses for this reaction were fit to Equation 5 with values for k_1 and k_2 of 3.6 s^{-1} and 7.5 s^{-1}, respectively. In the same way, the time courses for the autooxidation of the other 8-*X*-$FMNH_2$ derivatives were also fit to Equation 5 with values for k_1 and k_2 of 4.6 s^{-1} and 11.2 s^{-1} for $FMNH_2$, 3.8 s^{-1} and 14.1 s^{-1} for 8-CH_3S-$FMNH_2$, 3.4 s^{-1} and 11.1 s^{-1} for 8-CH_3O-$FMNH_2$, 5.0 s^{-1} and 9.3 s^{-1} for 8-H-$FMNH_2$, and 2.2 s^{-1} and 10.4 s^{-1} for 8-F-$FMNH_2$.

Formation and Decomposition of the 4a-Hydroperoxyflavin Intermediate—The formation of the enzyme-bound hydroperoxyflavin intermediates was studied for each of the 8-substituted flavin derivatives. The absorption spectrum, obtained as a function of time, when a mixture of enzyme and 8-Cl-$FMNH_2$ was rapidly mixed with oxygen is shown in Fig. 1*B*. The formation of the 8-Cl-FMNOOH intermediate was essentially complete prior to the collection of the first spectrum (*spectrum I*). This intermediate has an absorption maximum at approximately 365 nm. This also corresponds to an isosbestic point for the hydroperoxyflavin intermediate and oxidized flavin. The subsequent change in absorbance at 445 nm was due to the decomposition of this intermediate to 8-Cl-FMN and H_2O_2. The time course for the formation of the *E*·8-CH_3O-FMNOOH com-

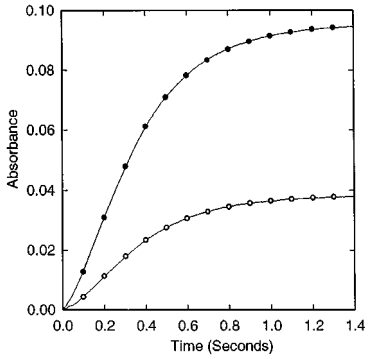


FIG. 2. **Time courses for the reoxidation of 8-Cl-FMNH$_2$.** The reaction was initiated by mixing 8-Cl-FMNH$_2$ (15 μM) with O_2 (120 μM), and the reaction was monitored at 365 (●) and 445 nm (○). The *solid lines* represent the experimental data, and the *symbols* represent the fit of the data to Equation 5 with values of k_1 and k_2 of 3.6 and 7.5 s^{-1}, respectively.

plex is presented in Fig. 3. This reaction was initiated by the mixing of the *E*·8-CH_3O-FMNH$_2$ complex with O_2. The final enzyme and flavin concentrations were 75 and 15 μM, respectively. At a final O_2 concentration of 120 μM, the pseudo-first-order rate constant, obtained from a fit of the data to Equation 2, is 190 s^{-1}. As illustrated in the *inset* to Fig. 5, the apparent rate constant for the pseudo-first-order formation of *E*·8-CH_3O-FMNOOH is directly proportional to the concentration of O_2. A fit of these data to Equation 1 gives a second-order rate constant of 1.5×10^6 M^{-1} s^{-1}. The same experiments were performed with the other 8-substituted flavin derivatives yielding similar results (data not shown). A second-order rate constant of 2.4×10^6 M^{-1} s^{-1} was obtained for FMNH$_2$, 8.8×10^5 M^{-1} s^{-1} for 8-CH_3S-FMNH$_2$, 5.6×10^5 M^{-1} s^{-1} for 8-Cl-FMNH$_2$, and 1.0×10^6 M^{-1} s^{-1} for 8-H-FMNH$_2$. It was not possible to measure the rate for the 8-F-FMNH$_2$ derivative.

In the absence of aldehyde, the decomposition of the 8-substituted FMNOOH intermediates to 8-*X*-FMN and H_2O_2 was studied. The time course for the absorbance change at 445 nm after mixing *E*·8-CH_3O-FMNH$_2$ with O_2 is shown in Fig. 4. The data were fit to Equation 2 with a first-order rate constant of 12.4 s^{-1}. No significant difference in the decomposition rates was observed upon variation of O_2 concentration. The first-order rate constant for the decomposition of the hydroperoxyflavin intermediates was 0.10 s^{-1} for FMNH$_2$, 0.07 s^{-1} for 8-Cl-FMNH$_2$, 0.14 s^{-1} for 8-CH_3S-FMNH$_2$, 0.2 s^{-1} for 8-H-FMNH$_2$, and 0.85 s^{-1} for 8-F-FMNH$_2$.

Formation and Decomposition of Light-emitting Species—The time courses for the production of visible light when the enzyme·8-CH_3O-FMNH$_2$ complex was mixed with increasing amounts of *n*-decanal in air-equilibrated buffer are shown in Fig. 5. The rate of light emission reaches a maximum in approximately 0.1 s and then decays exponentially over a period of about 0.5 s. Similar experiments were performed with the other 8-substituted flavin analogs. The relative maximum light intensity for the various 8-substituted flavin derivatives at a given concentration of enzyme, aldehyde, and flavin was not determined. The main difference between the various analogs is the rate with which the emission of light decays. For all the flavin analogs, the light decay can be accurately fit to Equation 2 with a rate constant of 4.4 s^{-1} for 8-CH_3O-FMN, 0.50 s^{-1} for FMN, 0.18 s^{-1} for 8-CH_3S-FMN, 0.025 s^{-1} for 8-Cl-FMN, 0.10 s^{-1} for 8-H-FMN, and 0.04 s^{-1} for 8-F-FMN.

Calculation of Microscopic Rate Constants—The microscopic rate constants in Table I for the kinetic model that appears in Scheme V for the various 8-substituted flavin mononucleotide derivatives used in these studies were obtained by direct comparison of the experimental time courses for intermediate and

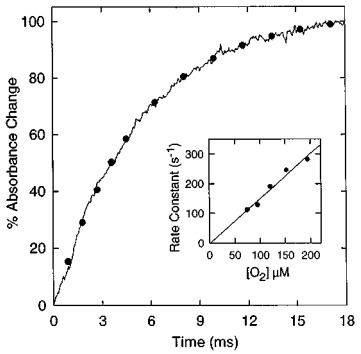


FIG. 3. **Time course for the formation of the *E*·8-CH_3O-FMNOOH intermediate.** The reaction was monitored at 365 nm after mixing a solution of luciferase (75 μM) and 8-CH_3O-FMNH$_2$ (15 μM) with O_2 (120 μM). The experimental data (*solid line*) were fit to Equation 2 with a first-order rate constant of 190 s^{-1} as indicated by the *filled circles*. The *inset* shows the effect of O_2 concentration on the pseudo-first-order rate constant for the formation of *E*·8-CH_3O-FMNOOH. A second-order rate constant of 1.5×10^6 M^{-1} s^{-1} was obtained from a fit of these data to Equation 1.

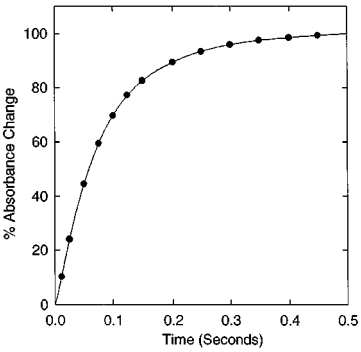


FIG. 4. **Time course for the decomposition of *E*·8-CH_3O-FMNOOH to 8-CH_3O-FMN and H_2O_2 when monitored at 445 nm.** The reaction was initiated by mixing a solution of luciferase (75 μM) and 8-CH_3O-FMNH$_2$ (15 μM) with air-equilibrated buffer (120 μM O_2). The experimental data (*solid line*) were fit to Equation 2 with a first-order rate constant of 12 s^{-1} as indicated by the *filled circles*.

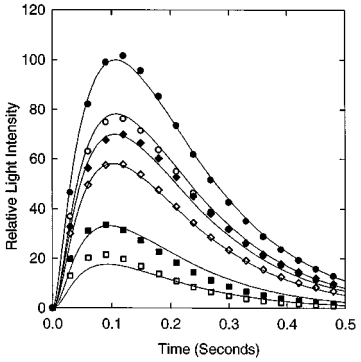


FIG. 5. **Time courses for the bioluminescence with 8-CH_3O-FMNH$_2$.** The light emission was measured after mixing a solution of luciferase (75 μM) and 8-CH_3O-FMNH$_2$ (15 μM) with various amounts of *n*-decanal (15 (□), 30 (■), 60 (◇), 80 (◆), 100 (○), and 400 (●) μM) in air-equilibrated buffer. The *symbols* represent portions of the experimental data, while the *solid lines* represent the simulated time courses using the rate constants that appear in Table I and the model in Scheme V.

product formation with the calculated time courses using the KINSIM program. The dissociation constants K_1 for the 8-substituted flavin derivatives were taken from Eckstein *et al.* (1993). These dissociation constants were obtained by the method of Eadie-Hoftstee using the maximal light intensity in standard assays at different reduced flavin concentrations. The

TABLE I
Rate constants for the kinetic model in Scheme V

pH 7.0, 25 °C. The rate constants k_{15} (9.1×10^5 M^{-1}s^{-1}) and k_{16} (5.8 s^{-1}) have been reported elsewhere (Abu-Soud *et al.*, 1993).

	8-CH_3-$FMNH_2$	8-Cl-$FMNH_2$	8-CH_3O-$FMNH_2$	8-CH_3S-$FMNH_2$	8-H-$FMNH_2$
K_1[a]	0.3×10^{-7} M	9.0×10^{-8} M	4.0×10^{-7} M	2×10^{-6} M	6.5×10^{-7} M
k_3	2.4×10^6 M^{-1} s^{-1}	5.6×10^5 M^{-1} s^{-1}	1.5×10^5 M^{-1} s^{-1}	8.75×10^5 M^{-1} s^{-1}	1.0×10^6 M^{-1} s^{-1}
k_5	1.9×10^7 M^{-1} s^{-1}	1.0×10^7M^{-1} s^{-1}	2.4×10^7 M^{-1} s^{-1}	1.9×10^7 M^{-1} s^{-1}	1.8×10^6 M^{-1} s^{-1}
k_6	120 s^{-1}	150 s^{-1}	25 s^{-1}	82 s^{-1}	200 s^{-1}
k_7	1.6 s^{-1}	1.3 s^{-1}	11 s^{-1}	1.0 s^{-1}	1.6 s^{-1}
k_8	1.15 s^{-1}	1.16 s^{-1}	0.55 s^{-1}	0.92 s^{-1}	1.8 s^{-1}
k_9	1.1 s^{-1}	0.09 s^{-1}	8.8 s^{-1}	0.4 s^{-1}	0.16 s^{-1}
k_{11}	3000 M^{-1} s^{-1}				
k_{12}	0.06 s^{-1}				
k_{13}	0.6 s^{-1}				
k_{17}	0.1 s^{-1}	0.07 s^{-1}	12.4 s^{-1}	0.14 s^{-1}	0.20 s^{-1}
k_{19}	4.6 s^{-1}	3.6 s^{-1}	3.4 s^{-1}	3.8 s^{-1}	5.0 s^{-1}
k_{21}	11.2 s^{-1}	7.5 s^{-1}	11.1 s^{-1}	14.1 s^{-1}	9.3 s^{-1}

[a] Taken from Eckstein *et al.* (1993).

second-order rate constants (k_3) for the formation of *E*·8-*X*-FMNOOH from E·8-*X*-$FMNH_2$ and O_2 were obtained from the time courses that followed the mixing of solutions of luciferase and 8-substituted $FMNH_2$ derivatives with various concentrations of O_2. In all cases, the variation of the pseudo-first-order rate constant with the oxygen concentration was linear, and the plots intersected the axes near the origin. The rate constants for the processes associated with the binding of aldehyde to the *E*·8-*X*-FMNOOH complexes through the chemical formation of *E*·8-*X*-FMNOH (k_5-k_9) were established from the time courses of the light emission that followed the mixing of air-equilibrated decanal with mixtures of enzyme and 8-*X*-$FMNH_2$. The rate constants for the decomposition of the *E*·8-*X*-FMNOOH complexes to FMN and H_2O_2 were obtained by monitoring the formation of 8-*X*-FMN at the corresponding absorption maxima in the absence of decanal. The time course for this decay could be fit to a single-exponential (Equation 2). The rate constants for the dehydration of *E*·8-*X*-FMNOH and the rate constants associated with aldehyde binding were only determined for $FMNH_2$ as described by Abu-Soud *et al.* (1992). The rate constants associated with the binding of aldehyde to free enzyme (k_{15} and k_{16}) have been reported previously by Abu-Soud *et al.* (1992, 1993) and were held constant during the simulation. Finally, the rate constants for the conversion of unbound 8-*X*-$FMNH_2$ to 8-*X*-FMN and H_2O_2 in the presence of O_2 (k_{19} and k_{21}) were obtained from a fit of the time courses of the reaction of 8-*X*-$FMNH_2$ (15 μM) with O_2 (120 μM) to Equation 5.

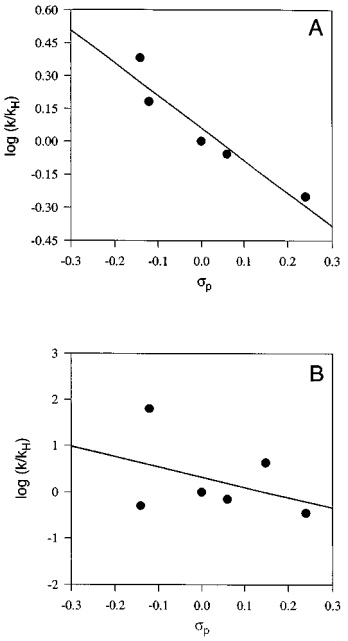


FIG. 6. **Hammett plots for the rates of formation and decomposition of the 4a-hydroperoxyflavin intermediate.** Correlation of the second-order rate constant for the formation (*panel A*) and first-order rate constant for the decomposition (*panel B*) of the 4a-hydroperoxyflavin intermediate with the electronic substituent constant σ_p.

DISCUSSION

Substitution at the 8-position of the isoalloxazine ring of the flavin mononucleotide markedly alters the kinetics of the bacterial luciferase reaction. A linear free-energy relationship analysis can therefore be conducted to determine the effect of these flavin substitutions on the individual steps of the reaction mechanism. However, not all of the calculated rate constants listed in Table I show linear plots when analyzed by the Hammett equation (6).

$$\log (k_x/k_o) = \sigma \rho \qquad \text{(Eq. 6)}$$

The Hammett plot for the calculated rate constants for the formation of the 4a-hydroperoxyflavin intermediate and for decomposition of this intermediate is shown in Fig. 6. The rate constant for the formation of the 4a-hydroperoxyflavin intermediate exhibits a good fit to the Hammett equation ($r^2 = 0.90$) with a value of ρ of -1.5 (*panel A*). On the other hand, the rate of decomposition of this intermediate shows a very poor fit ($r^2 = 0.15$; *panel B*). This latter result might indicate the more complex nature of this process. The substituent constants used for these plots are the classic σ_p; no special substituent constants were used. It is reasonably well established that substituent effects in biological systems can also reflect a combination of electronic, steric, and hydrophobic contributions to the observed rate constants. The failure to obtain a good correlation with these rate constants, based only on electronic contributions, might therefore be a reflection of further contributions from steric and hydrophobic factors. In some cases it is possible to assess the importance of these other contributions by performing a multiple regression analysis (Shorter, 1978). However, it was not possible to perform this type of analysis with the data obtained in this study, since a three-parameter correlation (electronic, steric, and hydrophobic) would not be statistically valid with the five sets of rate constants determined here for the limited number of available flavin analogs.

The Hammett equation could also be applied with success to the rate of decay of light emission. The semilog plot of k/k_H *versus* σ_p is shown in Fig. 7. The slope of this plot, ρ, has a value of -4. A negative value for this slope is a direct indication that the reaction in question is facilitated by increasing the electron

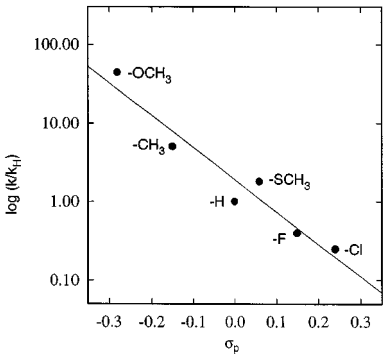


FIG. 7. **Correlation of the rate of decay of bioluminescence with the electronic substituent constant σ_p.**

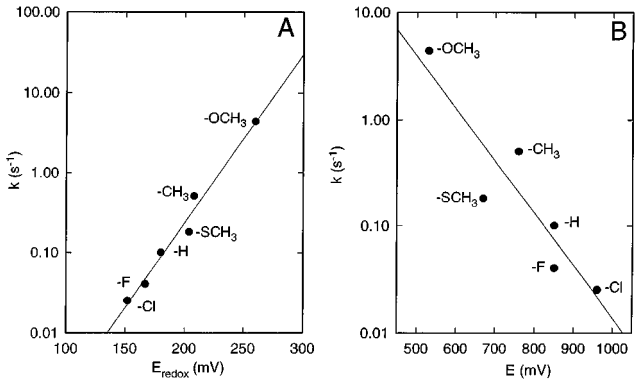
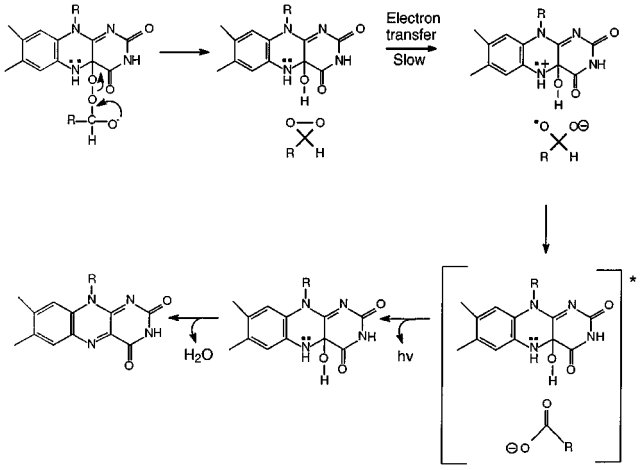


FIG. 8. **Correlation of the rates of decay of light emission with the flavin redox potential.** The redox potentials used are of the 8-substituted FMN/FMNH$_2$ couples (*panel A*) and the one-electron oxidation potentials of the 8-substituted-4a,5-dihydroflavin analogs determined by Eckstein *et al.* (1993) (*panel B*).



SCHEME VI.

density at the reaction center. The value obtained here is a very large value for ρ, typical of processes involving ionic transition states. Baeyer-Villiger reactions of benzaldehydes with substituted perbenzoic acids under conditions where a hydrogen is the migrating group show small positive values of ρ (0.2–0.6) (Ogata and Sawaki, 1972). The results obtained here are therefore inconsistent with a Baeyer-Villiger type mechanism for the bioluminesence reaction catalyzed by the bacterial luciferase.

The results obtained from the linear free-energy relationship analysis described above are, however, in accordance with the formation of a C4a-hydroperoxyflavin radical cation intermediate, as suggested by the CIEEL mechanism (Scheme IV). The CIEEL mechanism also predicts that the oxidation potential of the flavin should affect the rate of decay for the bioluminescence reaction. Semilog plots of the light emission decay rate against the two-electron redox potential of the FMN/1,5-FMNH$_2$ couples and the one-electron redox potential for the oxidation of 4a,5-dihydroflavin model compounds to the radical cation as determined by Eckstein *et al.* (1993) are shown in Fig. 8, *A* and *B*. There is a linear relationship between the two redox potential values and the light emission decay rate. The decay of light emission is fastest with FMN analogs of the lowest oxidation potential. This result is an indication that in the rate-limiting step there is a decrease in the electron density at the reaction center of the flavin moiety. Substituents that can donate electron density favor this process. This is in agreement with the results obtained with the Hammett analysis.

It is not possible with the information gained through these studies to differentiate between the CIEEL mechanism depicted in Scheme IV and that involving the dioxirane species. It has been recently shown that dioxiranes can participate in one-electron transfer reactions. Nelsen *et al.* (1993) have demonstrated that unsaturated hydrazines react with dimethyldioxirane to form *N*-methylhydrazinium acetate. The mechanism of the reaction involves a single-electron transfer from the hydrazine to the dioxirane to initially form an ion pair; methyl transfer then generates the final product.

A similar mechanism can be drawn for the luciferase reaction and is shown in Scheme VI. In this mechanism, the 4a-hydroperoxyhemiacetal intermediate decomposes to form a dioxirane and the 4a-hydroxyflavin intermediate. This process is identical to that of the oxidation of ketones by monopersulfuric acid. Dioxiranes have been successfully isolated and characterized in nonbiological model systems. In these oxidations, it is important to notice that the reaction proceeding through a Baeyer-Villiger type mechanism competes with the reaction pathway forming the dioxirane species, which prevails at neutral pH. After formation of the dioxirane and the 4a-hydroxyflavin intermediate, electron transfer from the flavin to the dioxirane produces an ion pair, consisting of the 4a-hydroxyflavin radical cation and the dioxirane radical anion. The production of this flavin hydroxide radical cation should be more favorable with substituents that can donate electron density to the newly formed positively charged center. This is in agreement with the results of the Hammett analysis. The formation of the radical pair would be the slow step in the reaction mechanism. In the next step, rearrangement of the dioxirane radical anion with transfer of the hydrogen atom to the N5 of the flavin would then result in the production of the excited state of the flavin hydroxide and the carboxylic acid. The excited hydroxyflavin then decays to ground state with the production of a photon of light. The flavin hydroxide then dehydrates and deprotonates to yield the final FMN product.

This mechanism is also in agreement with the results obtained for the deuterium kinetic isotope effect experiments. The deuterium isotope effect for light formation and decay of light emission is approximately 1.6.[3] This value is somewhat larger than expected for a secondary isotope effect but might indicate that the breakage of the carbon–hydrogen bond does not happen in the rate-determining step. In the mechanism depicted here, the rate-determining step is the formation of the radical pair, but actual breakage of the carbon–hydrogen bond is involved in the formation of the excited state intermediates.

Although the mechanism presented in Scheme VI provides a reasonable pathway for the decomposition of the C4a-hydroperoxyhemiacetal intermediate and the generation of an excited state, there are potentially two problems with the dioxirane mechanism. One is that dioxiranes of aldehydes have not yet

[3] W. Francisco, unpublished results.

been identified or isolated, and second, attempts to generate light in the presence of oxidizable electron donor activators have not been successful. On the other hand, it has been established that the rearrangement of a dioxirane into a carboxylic acid is a highly exothermic process. The energy difference between a dioxirane and the corresponding carboxylic acid is approximately 80 kcal/mol (Adam *et al.*, 1989). A CIEEL type mechanism for the generation of light from the decomposition of dioxiranes has been proposed (Adam *et al.*, 1989), but it awaits experimental support.

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Additions and Corrections

Vol. 271 (1996) 104–110

Interaction of bacterial luciferase with 8-substituted flavin mononucleotide derivatives.

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In response to concerns expressed by Drs. Eckstein, Ghisla, and Hastings, we wish to rescind parts of the above-referenced paper, which duplicates material published in several earlier papers by them and their colleagues of which we were aware (Eckstein *et al.*, 1993; Eckstein and Ghisla, 1990; Macheroux *et al.*, 1987). We should have indicated in our Introduction and elsewhere that the purpose of our work had been, in part, to repeat, using stopped-flow methods and a more detailed kinetic analysis of the data, their earlier studies on the effects of 8-substituted flavins on the bioluminescence reaction. We should have stated that our results confirmed their experimental observations and supported their conclusions, namely that a CIEEL-type mechanism is consistent with the data, whereas a Baeyer-Villiger mechanism is not. We should also have cited a previous paper (Ahrens *et al.*, 1991), which had addressed the same question via a different approach. We should have made it clear that our conclusions are based on the dependence of the rate constant of light intensity decay on the oxidation potential of the substituted flavins, exactly as in the work of Eckstein *et al.* (1993). Finally, we should have stated more explicitly under "Discussion" that even though the 8-substituents caused multiple changes in individual rate constants throughout the entire reaction profile, none of those changes resulted in a significant modulation of the observed rate of decay of bioluminescence from the microscopic rate constant of the chemical step leading to light emission.

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Enzymology:

Interaction of Bacterial Luciferase with 8-Substituted Flavin Mononucleotide Derivatives

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J. Biol. Chem. 1996, 271:104-110.
doi: 10.1074/jbc.271.1.104