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Stopped-Flow Kinetic Analysis of the Bacterial Luciferase Reaction†

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ABSTRACT: The kinetics of the reaction catalyzed by bacterial luciferase have been measured by stopped-flow spectrophotometry at pH 7 and 25 °C. Luciferase catalyzes the formation of visible light, FMN, and a carboxylic acid from $FMNH_2$, O_2, and the corresponding aldehyde. The time courses for the formation and decay of the various intermediates have been followed by monitoring the absorbance changes at 380 and 445 nm along with the emission of visible light using *n*-decanal as the alkyl aldehyde. The synthesis of the 4a-hydroperoxyflavin intermediate (FMNOOH) was monitored at 380 nm after various concentrations of luciferase, O_2, and $FMNH_2$ were mixed. The second-order rate constant for the formation of FMNOOH from the luciferase–$FMNH_2$ complex was found to be 2.4×10^6 M^{-1} s^{-1}. In the absence of *n*-decanal, this complex decays to FMN and H_2O_2 with a rate constant of 0.10 s^{-1}. The enzyme–$FMNH_2$ complex was found to isomerize prior to reaction with oxygen. The production of visible light reaches a maximum intensity within 1 s and then decays exponentially over the next 10 s. The formation of FMN from the intermediate pseudobase (FMNOH) was monitored at 445 nm. This step of the reaction mechanism was inhibited by high levels of *n*-decanal which indicated that a dead-end luciferase–FMNOH–decanal could form. The time courses for the these optical changes have been incorporated into a comprehensive kinetic model. Estimates for 15 individual rate constants have been obtained for this model by numeric simulations of the various time courses.

Bacterial luciferase is a flavin hydroxylase which catalyzes the reaction of $FMNH_2$, O_2, and an aliphatic aldehyde to yield the carboxylic acid, FMN, and blue-green light (λ_{max} = 490 nm). The enzyme is a heterodimer consisting of homologous subunits, α and β, with molecular weights of 40 108 (Cohn et al., 1985) and 36 349 (Johnston et al., 1986), respectively. The active center resides primarily, if not exclusively, on the α subunit; the role of the β subunit remains unclear, but it is required for the efficient generation of light (Baldwin & Ziegler, 1991). The enzyme is commonly assayed by monitoring light emission following injection of $FMNH_2$ into a vial containing enzyme, an aliphatic aldehyde (e.g., *n*-decanal), and O_2 dissolved in a buffered solution (Hastings et al., 1978). The light intensity rises to a maximum, which is proportional to the amount of enzyme, and then decays exponentially with a rate constant characteristic of the enzyme and the alkyl chain length of the aldehyde (Hastings et al., 1966). This format comprises a single-turnover assay since free (excess) $FMNH_2$ is quickly depleted by the nonenzymatic autoxidation pathway

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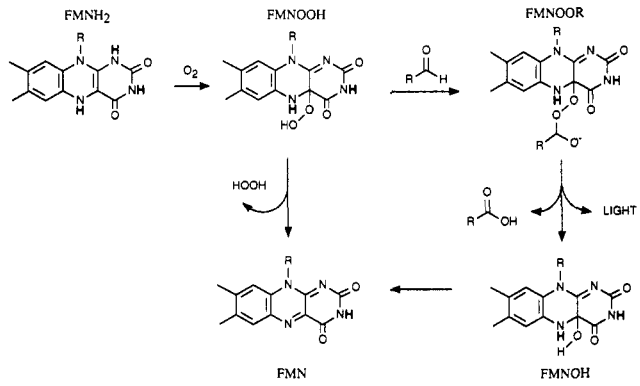
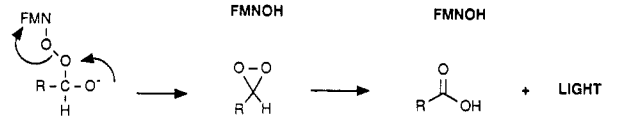
Scheme I



Scheme II



(Gibson & Hastings, 1962; Massey et al., 1971), while light emission continues for several seconds.

Detailed investigations from several laboratories have generated a clear picture of the stoichiometry and identity of the substrates and products, as well as several intermediates in the luciferase-catalyzed reaction (see Scheme I). The enzyme–$FMNH_2$ complex reacts with molecular oxygen to form the 4a-hydroxyperoxyflavin intermediate (FMNOOH) (Hastings et al., 1973; Vervoort et al., 1986a,b). In the absence of the aliphatic aldehyde substrate, this intermediate decays in a nonluminescent reaction to yield FMN and H_2O_2 (Hastings & Balny, 1975). In the presence of an aliphatic aldehyde, the 4a-hydroperoxyflavin intermediate reacts to form, through intermediates that have not been definitely identified, the final products of FMN and the carboxylic acid. Studies using $^{18}O_2$ incorporation have demonstrated that one oxygen of the carboxylic acid is derived from molecular oxygen through the intermediacy of the 4a-hydroperoxyflavin intermediate (Suzuki et al., 1983). The reaction is formally a flavin-mediated monooxygenation in which the enzyme-bound reduced flavin activates molecular oxygen, splitting it into water and a hydroxylated organic compound (Eberhard & Hastings, 1972). It has been postulated that the 4a-hydroperoxyflavin intermediate reacts with the aldehyde to form a tetrahedral adduct (FMNOOR) but this intermediate has not been demonstrated.

The nature of the primary excited state in the luciferase-catalyzed reaction and the identity of the emitter remain subjects to much conjecture. Recently, we have proposed an alternative to the often-quoted suggestion that the tetrahedral intermediate reacts by the Baeyer–Villiger pathway to directly yield the carboxylic acid and the excited state of the flavin pseudobase (FMNOH) (Eberhart & Hastings, 1972). We have proposed formation of a dioxirane and the flavin pseudobase from the tetrahedral intermediate with subsequent breakdown of the dioxirane to yield the carboxylic acid as the primary excited state (as shown in Scheme II) (Raushel & Baldwin, 1989; Cho & Lee 1984).

Compared with the effort that has been applied to characterization of intermediates on the reaction pathway, there has been little effort applied to precisely defining the kinetic mechanism of the enzyme in terms of a consistent set of rate constants and binding equilibria. The difficulties caused by the lack of such fundamental data are readily apparent. We have now undertaken a detailed evaluation of the kinetic mechanism of the luciferase-catalyzed reaction using stopped-flow methods. This is possible using rapid reaction techniques because the transformation of enzyme-bound $FMNH_2$ into the various oxygenated intermediates can be monitored at 380 nm because there is an isosbestic point at this wavelength for FMNOOH and FMN (Hasting & Presswood, 1980). The final conversion of these intermediates to FMN can be measured at 445 nm (Hastings & Presswood, 1980). Moreover, the time course for light emission provides an additional spectroscopic signal. The time courses and the associated rate constants obtained from these experiments have been incorporated into a comprehensive kinetic model for the luciferase-catalyzed reaction. This model comprises an internally consistent set of rate constants from which progress curves for the various portions of the complex bioluminescence reaction can be calculated. In all cases, the calculated curves fit the experimental data very well.

Materials and Methods

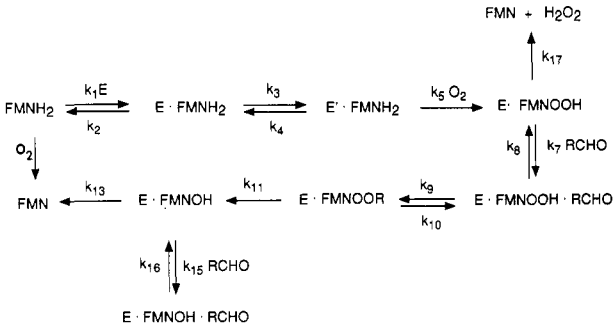
Materials. Riboflavin 5′-monophosphate (FMN) was obtained from Fluka (97%) and used without further purification. All other reagents and solvents were of the highest purity grade available and obtained from either Sigma or Aldrich.

Purification of the Bacterial Luciferase. The bacterial luciferase from *Vibrio harveyi* was purified from recombinant *Escherichia coli* according to literature methods (Baldwin et al., 1989). The enzyme purity was estimated by gel electrophoresis to be >95%. Luciferase concentrations were determined spectrophotometrically on the basis of a specific absorption coefficient of 0.94 (0.1%, 1 cm) at 280 nm and a molecular weight of 76457. Anaerobic enzyme solutions were prepared in an all-glass apparatus (Williams et al., 1979) by several cycles of evacuation and equilibration with nitrogen. The nitrogen gas was purified to remove trace contamination of oxygen by passing the gas over a heated column of BASF catalyst R3-11 (Chemical Dynamics Corp./Kontes Glass Co.). Buffer solutions were made anaerobic by bubbling nitrogen gas for at least 1 h through a septum-tipped flask.

Rapid Reaction Measurements. All kinetic studies were performed with a temperature-controlled stopped-flow apparatus obtained from Hi-Tech Ltd. (Model SF-51 or PQ/SF-53) interfaced with a Hewlett-Packard Series 3000 computer. These instruments are designed for efficient anaerobic work and equipped with a rapid-scanning device (Model MG-3000 from Hi-Tech Ltd.). A glass cutoff filter (type GU 380) was used for absorbance measurements at 380 nm to avoid interference from bioluminescence during the course of the reaction. All the kinetic measurements were carried out in the dark under nitrogen atmosphere in Bis-Tris-HCl buffer (0.05 M), pH 7.0 at 25 ± 0.2 °C. The flavin solutions were reduced by hydrogen gas in the presence of a few crystals of palladium on activated carbon. The final FMN concentrations were determined spectrophotometrically on the basis of a molar absorption coefficient of 12200 M^{-1} cm^{-1} at 445 nm (Whitby, 1953). A stock solution of 0.1 M *n*-decanal in ethanol was freshly prepared before use. The time courses for light emission and/or absorption were obtained after mixing equal volumes of the various solutions. The signal-to-noise ratios were improved by averaging 10 individual experiments.

Oxygen Measurements. The molecular oxygen concentration was determined using an Orion pH meter (Model 601) equipped with an oxygen electrode (Model 97-08). The various solutions were made by mixing different volumes of O_2-saturated buffer with air-equilibrated or anaerobic buffer solutions. The O_2-saturated buffer was made by bubbling O_2 gas through the solution for 6 h in a septum-tipped flask.

Scheme III



Data Analysis. The time courses for the various kinetic experiments were fitted to one or more of the following rate equations (eq 1–5) by a nonlinear least-squares procedure using

$$y = 1 - e^{-k_1 t} \quad (1)$$

$$y = Ae^{-k_1 t} + Be^{-k_2 t} \quad (2)$$

$$y = [k_1/(k_2 - k_1)](e^{-k_1 t} - e^{-k_2 t}) \quad (3)$$

$$y = 1 + [1/(k_1 - k_2)](k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \quad (4)$$

$$y = ax + b \quad (5)$$

the software supplied by Hi-Tech Ltd. Equation 2 represents the sum of two independent exponentials for a parallel process while eq 3 and 4 describe the time courses for a sequential process (X → Y → Z) monitoring the formation of Y and Z, respectively. In these equations, k_1 and k_2 are first-order rate constants, t is time, and A and B are amplitude factors, and e is 2.718. The KINSIM program (Barshop et al., 1983) was used to simulate the time courses using the comprehensive kinetic model which appears in Scheme III by numerical integration of the equations describing this mechanism. The best fit was achieved by sequential adjustment of the values for the various rate constants by graphical visualization until the simulated time courses matched the experimental data sets as closely as possible.

Results

Stopped-flow kinetic techniques were used to study the mechanism of the luciferase-catalyzed reaction in the absence and presence of various concentrations of $FMNH_2$, enzyme, *n*-decanal, and oxygen. Changes in the flavin absorption at 380 and 445 nm, as well as the time course of emission of visible light with time, were used to detect the formation and decay of the various intermediates. Progress curves show that changes in these optical probes occur on time scales ranging from <0.01 to 100 s during single-turnover experiments. In the absence of *n*-decanal, the studies focused on the formation of the 4a-hydroperoxyflavin intermediate and its decay; the formation of the intermediate was measured at 380 nm, while its decay was monitored at 445 nm. In the presence of *n*-decanal, production of visible light provided an additional optical signal.

Decomposition of $FMNH_2$. Previous reports have demonstrated that the decomposition of $FMNH_2$ in the presence of O_2 is not a simple process (Gibson & Hastings, 1962; Massey et al., 1971). Figure 1 shows the time course for the reaction of 15 μM $FMNH_2$ with 120 μM O_2 when the absorbance is measured at 380 and 445 nm. The time courses for the appearance of FMN measured at both wavelengths are very similar, and each has a distinctive lag phase followed by a single exponential. The time courses for this reaction at a fixed oxygen concentration of 120 μM were fit to eq 4 with values for k_1 and k_2 of 4.7 and 11.5 s^{-1}, respectively.

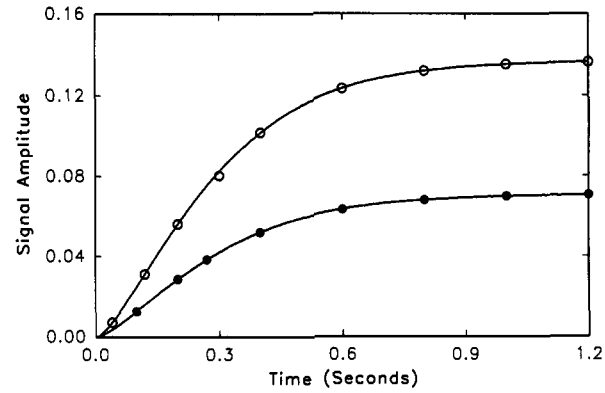


FIGURE 1: Time course for the reoxidation of $FMNH_2$ (15 μM) with O_2 (120 μM) monitored at 380 (●) and 445 nm (○). The solid lines represent the experimental data, and the symbols represent the fit of the data to eq 4 with values for k_1 and k_2 of 4.7 and 11.5 s^{-1}, respectively.

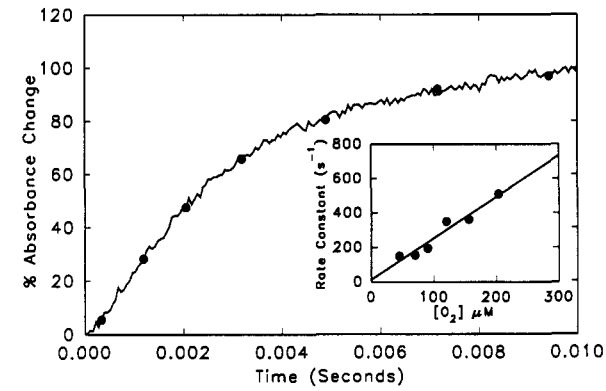


FIGURE 2: Time course for the formation of the E–FMNOOH intermediate. The reaction was monitored at 380 nm after mixing a solution of luciferase (75 μM) and $FMNH_2$ (15 μM) with O_2 (120 μM). The experimental data (solid line) were fit to eq 1 with a first-order rate constant of 350 s^{-1} as indicated 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–FMNOOH. A second-order rate constant of 2.4×10^6 M^{-1} s^{-1} was obtained from a fit of these data to eq 5.

Formation and Decomposition of the 4a-Hydroperoxyflavin Intermediate. The time course for the formation of the E–FMNOOH complex is presented in Figure 2. This reaction was initiated by the mixing of the E–$FMNH_2$ complex with O_2, and the progress of the reaction was monitored at 380 nm. The enzyme concentration was 75 μM, 5 times the flavin concentration and 10–100 times the apparent K_m of the E–$FMNH_2$ complex (Meighen & Hastings, 1971) in order to ensure that all of the subsequent reactions of the flavin with O_2 occurred while the cofactor was associated with the protein. At a final O_2 concentration of 120 μM, the pseudo-first-order rate constant, obtained from a fit of the data to eq 1, is 350 s^{-1}. A fit of these data to eq 2 gave rate constants of 346 and 319 s^{-1} for k_1 and k_2, respectively, and thus the time course for this reaction is adequately described by a single-exponential equation. As illustrated in the inset to Figure 2, the apparent rate constant for the pseudo-first-order formation of E–FMNOOH is directly proportional to the concentration of O_2. A fit of these data to eq 5 gives a second-order rate constant of 2.4×10^6 M^{-1} s^{-1}. In the absence of aldehyde, the decomposition of E–FMNOOH to FMN and H_2O_2 was monitored at 445 nm. The time course for the absorbance change at 445 nm after mixing E–$FMNH_2$ with O_2 is shown in Figure 3. The data were fit to eq 1 with a first-order rate constant of

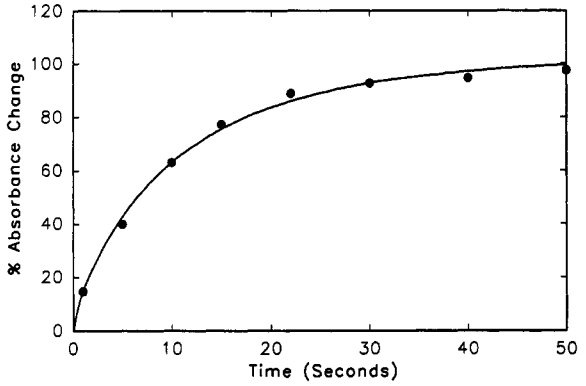


FIGURE 3: Time course for the decomposition of E–FMNOOH to FMN and H_2O_2 when monitored at 445 nm. The reaction was initiated by mixing a solution of luciferase (75 μM) and $FMNH_2$ (15 μM) with O_2 (120 μM). The experimental data (solid line) were fit to eq 1 with a first-order rate constant of 0.10 s^{-1} as indicated by the filled circles. Additional details are given in the text.

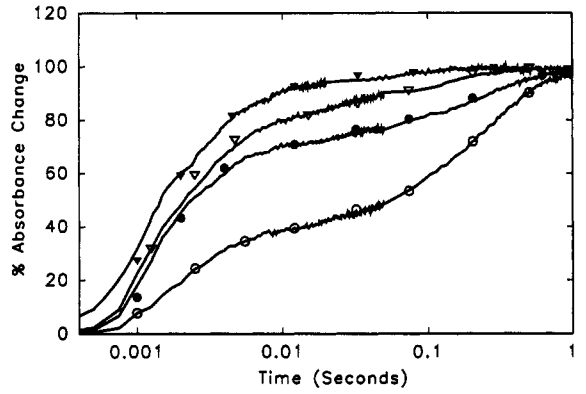


FIGURE 4: Time courses for the reaction of E–$FMNH_2$ with O_2 when the formation of E–FMNOOH was monitored at 380 nm. The experiments were conducted at a fixed concentration of $FMNH_2$ (15 μM) in the presence of variable amounts of enzyme [10 (○), 15 (●), 20 (▽), and 80 μM (▼)] and mixed with an equal volume of air-equilibrated buffer. The solid lines represent the actual experimental data while the symbols represent the simulated time courses using the rate constants which appear in Table I with the model in Scheme III.

0.10 s^{-1}. No significant difference in the decomposition rates was observed upon variation of O_2 concentration.

Two additional experiments were conducted in order to obtain more information concerning the rate and equilibrium constants for the formation of the enzyme–$FMNH_2$ complex. The time courses for the absorbance changes at 380 nm when E–$FMNH_2$ was mixed with a fixed amount of O_2 are shown in Figure 4. These experiments were conducted at increasing ratios of enzyme to flavin. At low levels of enzyme, the time courses could be accurately fit to the sum of two exponentials using eq 2. At higher concentrations of enzyme, the amplitude of the fast phase increased relative to the amplitude of the slower phase. The relative magnitudes of the two phases were used to approximate the fraction of $FMNH_2$ oxidized in the unbound and bound complex with luciferase.

The time courses when increasing amounts of air-equilibrated luciferase were mixed with a fixed concentration of $FMNH_2$ are shown in Figure 5. At low levels of enzyme, the time courses are again characterized by the sum of two exponentials. At a concentration of 80 μM enzyme, the fast phase occurs with a rate constant of 85 s^{-1}.

Formation and Decomposition of Light-Emitting Species. The time courses for the production of visible light when the enzyme–$FMNH_2$ complex was mixed with increasing amounts

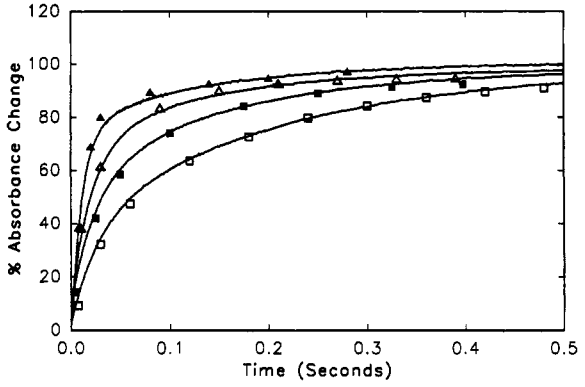


FIGURE 5: Time courses for the reaction of air-equilibrated luciferase with $FMNH_2$ when the reaction was monitored at 380 nm. The experiments were initiated by mixing various amounts of luciferase [10 (□), 15 (■), 40 (△), and 80 μM (▲)] in air-equilibrated buffer (120 μM O_2) with a fixed concentration of $FMNH_2$ (15 μM). The volumes of the two solutions were equal. The solid line represents the experimental time courses, and the symbols represent the simulated time courses using the rate constants which appears in Table I with the model in Scheme III.

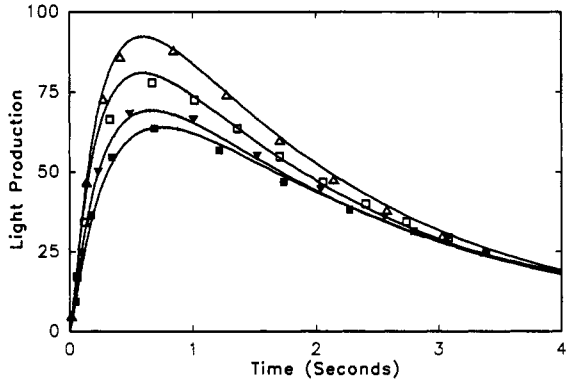


FIGURE 6: Time courses for light emission after mixing a solution of luciferase (75 μM) and $FMNH_2$ (15 μM) with various amounts of *n*-decanal [30 (■), 40 (▼), 100 (□), and 500 μM (△)] in air-equilibrated buffer. The solid lines represent the actual experimental data while the symbols represent the simulations when the constants which appear in Table I are used with the model in Scheme III.

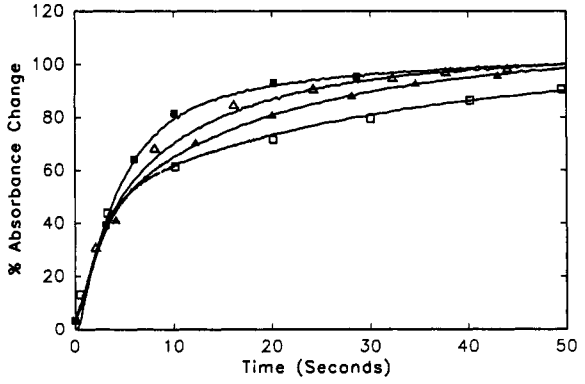


FIGURE 7: Time courses for the formation of FMN when E–$FMNH_2$ is mixed with air-equilibrated *n*-decanal and the reaction monitored at 445 nm. The experiments were conducted at fixed concentrations of luciferase (75 μM) and $FMNH_2$ (15 μM) with various amounts of *n*-decanal [40 (■), 120 (△), 300 (▲), and 500 μM (□)] in air-equilibrated buffer. The solid lines represent the actual experimental data while the symbols represent the simulations using the model which appears in Scheme III with the rate constants in Table I.

of *n*-decanal in air-equilibrated buffer are shown in Figure 6. The rate of light emission reaches a maximum intensity in less than 1 s and then decays exponentially over a period of approximately 10 s.

Formation of FMN. The final formation of FMN was monitored at 445 nm. The time courses when a fixed amount of the enzyme–$FMNH_2$ complex was rapidly mixed with increasing amounts of *n*-decanal are shown in Figure 7. At 40 μM *n*-decanal, the rate of formation of FMN can be fit to a single exponential with a first-order rate constant of 0.17 s^{-1}. As the concentration of *n*-decanal increases, the time courses can be fit to the sum of two exponentials, and the slower phase steadily decreases in rate as the *n*-decanal concentration increases.

Discussion

The primary objective for this study was to elucidate the kinetic mechanism of the bacterial luciferase reaction. The chemical instability of $FMNH_2$ in the presence of O_2 made the analysis technically more difficult because O_2 is also required as a substrate for the reaction. Therefore, it was necessary that certain substrate mixtures be made anaerobic while others need to be at a defined oxygen concentration. Moreover, the decomposition of $FMNH_2$ limited the kinetic analysis to essentially single turnovers since the rate of decay of unbound $FMNH_2$ in the presence of O_2 is significantly faster than the overall enzyme-catalyzed reaction.

Three spectroscopic signals were measured in order to obtain the time courses for the formation and decay of the various intermediates. Absorbance measurements at 380 nm were used to monitor the reaction up to the formation of the 4a-hydroperoxyflavin intermediate. In the absence of *n*-decanal, this phase of the overall reaction is over within 10 ms. The emission of visible light was used to follow the events after the addition of *n*-decanal, and the absorbance measurements at 445 nm were used to follow the formation of the FMN from the proposed FMNOH pseudobase (Kurfürst et al., 1987). The time courses for these transformations were collected as a function of the concentration of luciferase, O_2, and *n*-decanal at a fixed $FMNH_2$ concentration of 15 μM over time periods which ranged from 10 ms to 100 s.

The minimal model which can be utilized to describe the kinetics of the reaction catalyzed by the bacterial luciferase is presented in Scheme III. This model is based on the known intermediates in the reaction sequence (FMNOOH and FMNOH) as well as the postulated tetrahedral adduct formed upon the addition of *n*-decanal to FMNOOH (i.e., FMNOOR). We have explicitly assumed that light emission is coupled to the oxidation of the aldehyde to the carboxylic acid. The overall strategy for the assignment of individual rate constants to this model was to subdivide the mechanism into segments with a limited number of steps and then build upon the model as more substrates were added. The kinetic analyses of individual time courses were used to estimate rate constants for the final model. These rate constants were then utilized in the numerical solution to the model to compare the derived time courses with the experimental ones.

The rate constants for the final model were progressively obtained using the following analysis. The conversion of unbound $FMNH_2$ to FMN and H_2O_2 in the presence of O_2 was followed at 380 and 445 nm upon the rapid mixing of 15 μM $FMNH_2$ with 120 μM O_2. Both of these time courses could be fit to the sum of two consecutive first-order reactions (i.e., $A \rightarrow B \rightarrow C$; eq 4) where the two rate constants are 4.7 and 11.5 s^{-1}. Although Massey et al. (1971) have demonstrated that the autoxidation of $FMNH_2$ is a very complicated reaction, we have used this simplified analysis for our model since it adequately describes the loss of $FMNH_2$ at a fixed oxygen concentration when not bound to the enzyme. However, the order of the two rate constants is arbitrary since we have only measured the time course for the formation of the final product, FMN.

The second-order rate constant (k_5) for the formation of E–FMNOOH from E–$FMNH_2$ and O_2 was obtained by mixing the luciferase–$FMNH_2$ complex with various concentrations of O_2. The luciferase concentration was 5 times the flavin concentration and well above the apparent K_d to ensure that all of the absorbance changes were due to the flavin bound to the protein. As shown in Figure 2 at high O_2 concentration, the time course could be fit to a single exponential. The variation of the pseudo-first-order rate constant with the final oxygen concentration was linear, and the plot intersected the axes near the origin. Therefore, there is no indication that O_2 binds to the protein prior to reacting with the flavin and the reaction is essentially irreversible. The second-order rate constant (k_5) for the reaction of E–$FMNH_2$ with O_2 is 2.4×10^6 M^{-1} s^{-1}.

The equilibrium constants and associated rate constants for the formation of the E–$FMNH_2$ complex were determined from data obtained by the mixing of either enzyme and O_2 with $FMNH_2$ or enzyme and $FMNH_2$ with oxygen. The productive complexes were then monitored by following the rate at which E–$FMNH_2$ was converted to E–FMNOOH since the rate constant for this transformation was known from the previous experiments. When increasing amounts of air-equilibrated luciferase were mixed with $FMNH_2$, the rate constant for the formation of E–FMNOOH increased to a limiting value of about 85 s^{-1}. This value is approximately 4-fold smaller than the rate constant for the formation of this same complex when E–$FMNH_2$ is mixed directly with O_2. If the initially formed E–$FMNH_2$ complex were to react directly with O_2 to form FMNOOH in a single step, then the rate of formation of this intermediate at the highest levels of enzyme would match the rate observed when E–$FMNH_2$ is mixed with O_2. This situation is not observed experimentally, and thus these results suggest that the initial complex of E–$FMNH_2$ does not react with O_2 until a unimolecular reaction to E′–$FMNH_2$ occurs. A two-step mechanism has previously been observed for the binding of $FMNH_2$ to the luciferase from *Photobacterium phosphoreum* (Watanabe et al., 1976), and the enzyme from *Vibrio harveyi* has been shown to undergo a conformational change during the catalytic cycle (AbouKhair et al., 1985). The transformation that is apparent from our kinetic analyses may represent a protein conformational change or perhaps the ionization of the bound $FMNH_2$ (Vervoort et al., 1986a,b). It is this reaction which partially limits the rate of formation of E–FMNOOH when the reaction is initiated by the mixing of luciferase and $FMNH_2$. The rate constants k_1, k_2, k_3, and k_4 were obtained by comparison of the time courses which appear in Figures 4 and 5 with the numerical simulations derived using the model which appears in Scheme III. The solid lines drawn in Figures 4 and 5 are the actual experimental time courses while the symbols represent individual points taken from the numerical simulations when k_1, k_2, k_3, and k_4 are 1.7×10^7 M^{-1} s^{-1}, 1.2×10^3 s^{-1}, 200 s^{-1}, and 14 s^{-1}, respectively. The comparison of the experimental data to the simulated time courses is very good.

The rate constant (k_{17}) for the decomposition of the E–FMNOOH complex to FMN and H_2O_2 was obtained by monitoring the formation of FMN at 445 nm in the absence of *n*-decanal. As shown in Figure 4, the time course for this decay could be fit to eq 1. The solid line represents the experimental time course while the symbols indicate the numerical fit to Scheme III in the absence of decanal with the constants that appear in Table I.

Table I: Rate Constants for the Kinetic Model of Scheme III[a]

$k_1 = 1.7 \times 10^7$ M^{-1} s^{-1}	$k_2 = 1200$ s^{-1}
$k_3 = 200$ s^{-1}	$k_4 = 14$ s^{-1}
$k_5 = 2.4 \times 10^6$ M^{-1} s^{-1}	
$k_7 = 1.0 \times 10^7$ M^{-1} s^{-1}	$k_8 = 300$ s^{-1}
$k_9 = 3.8$ s^{-1}	$k_{10} = 1.3$ s^{-1}
$k_{11} = 0.77$ s^{-1}	
$k_{13} = 0.60$ s^{-1}	
$k_{15} = 3.0 \times 10^3$ M^{-1} s^{-1}	$k_{16} = 0.06$ s^{-1}
$k_{17} = 0.10$ s^{-1}	

[a] pH 7.0, 25 °C.

The rate constants for the processes associated with binding of aldehyde through the formation of E–FMNOH (k_7 through k_{11} in Scheme III) were established by monitoring the emission of visible light that followed mixing of variable amounts of air-equilibrated *n*-decanal with E–FMNH$_2$ (E′–FMNH$_2$). The formation of light is assumed to be concomitant with the formation of E–FMNOH. As observed in the time courses which appear in Figure 6, the rate of formation of visible light reaches a maximum within 1 s and then decays exponentially over the next 10 s. These time courses can be simulated using the model which appears in Scheme III with the rate constants for k_7 through k_{11} of 1.0×10^7 M^{-1} s^{-1}, 300 s^{-1}, 3.8 s^{-1}, 1.3 s^{-1}, and 0.77 s^{-1}, respectively.

The final conversion to FMN in the presence of various amounts of *n*-decanal was monitored at 445 nm. As indicated in Figure 7, at 40 μM *n*-decanal the time course can be fit to a single exponential. When increasing amounts of *n*-decanal were used, the decay rate decreased. This indicates that the binding of *n*-decanal to the E–FMNOH complex inhibits the final dehydration to FMN and thus an E–FMNOH–aldehyde dead-end complex has been added to the model. More complicated schemes are possible, but this is the simplest model to which we could fit the available data using values for k_{13}, k_{15}, and k_{16} of 0.60 s^{-1}, 3.0×10^3 M^{-1} s^{-1}, and 0.06 s^{-1}, respectively. The effect of bound *n*-decanal on the dehydration of FMNOH is consistent with the stabilization of E–FMNOOH upon binding of aldehyde analogues (e.g., decanol; Tu, 1979 and dodecanol; Lee et al., 1991).

The time courses for the reactions catalyzed by bacterial luciferase can be simulated quite well using the model shown in Scheme III. The kinetic mechanism is consistent with the ordered addition of FMNH$_2$, O$_2$, and *n*-decanal to the enzyme active site. The intermediate reactions progressively become slower as the overall reaction proceeds. The formation of the E–FMNOOH complex is complete within 10 ms while light production takes approximately 1 s to reach the maximum rate and an additional 10 s to decay. Finally, the formation of FMN from FMNOH is not complete until at least 1 min has elapsed. This is a significant time range for a single enzyme turnover.

An apparent enzyme isomerization following binding of FMNH$_2$ was observed in these studies. Reaction of O$_2$ with enzyme-bound FMNH$_2$ was second-order when initiated by mixing of E–FMNH$_2$ with O$_2$, but when FMNH$_2$ was mixed with enzyme and O$_2$, the rate of formation of E–FMNOOH appeared to be limited by a first-order process, even at very high concentrations of enzyme (see Figure 5). We have therefore proposed an enzyme isomerization following binding of FMNH$_2$. The apparent increase in oxygen reactivity of the luciferase-bound flavin as a consequence of a conformational change is of some interest. The facility of O$_2$ diffusion through globular proteins, measured by quenching of tryptophan fluorescence (Lakowitz & Weber, 1973), suggests that the availability of O$_2$ for reaction prior to the conformational change should not limit the rate of reaction. Reaction of FMNH$_2$ with oxygen is generally believed to proceed via a one-electron transfer, leading to formation of superoxide and the flavin radical, followed by formation of the 4a-hydroperoxyflavin (Ballou et al., 1969; Bruice, 1984). Wang and Thorpe (1991) have proposed that suppression of oxygen reactivity of reduced flavoproteins might reflect desolvation of the active site and destabilization of the superoxide anion intermediate. It is possible that the apparent increase in O$_2$ reactivity of luciferase-bound FMNH$_2$ following binding could reflect such an effect in addition to the control of the FMNH$_2$ reactivity by enforced planarity through binding site interactions.

Previous kinetic studies with bacterial luciferase have been limited in scope by focusing primarily on partial reactions. However, Hastings and Gibson (1963) reported the results of experiments that utilized stopped-flow mixing to elucidate the kinetic mechanism of bacterial luciferase. Their experiments clearly demonstrated the complex nature of the bioluminescence reaction. They showed the existence of both oxygen-dependent and oxygen-independent aspects of the overall reaction, demonstrating that O$_2$ enters the reaction scheme prior to aldehyde. In more recent experiments, Hastings and Presswood (1980) monitored the effect of O$_2$ concentration on the increase in absorbance at 380 nm that occurs upon mixing of E–FMNH$_2$ with O$_2$-equilibrated buffer and determined the second-order rate constant for the reaction to be 8.7×10^5 M^{-1} s^{-1} at 2 °C, in good agreement with the rate constant reported here (2.4×10^6 M^{-1} s^{-1} at 25 °C). Matheson and Lee (1983) have reported measurements of the rates of formation and decay of a fluorescent intermediate and of the bioluminescence of mixtures of enzyme, FMNH$_2$, O$_2$, and tetradecanal at 2 °C. Kurfürst et al. (1984) have suggested that the fluorescent transient might be the 4a-hydroperoxyflavin, consistent with the report that the formation of the fluorescent transient requires the aldehyde substrate (Matheson & Lee, 1983). The 4a-hydroperoxyflavin is also fluorescent (Balny & Hastings, 1975), but its formation does not require aldehyde. Matheson and Lee (1983) found that in air-equilibrated 50 mM phosphate, with 50 μM tetradecanal, pH 7.0, 2 °C, the rate of formation of the fluorescent transient was 6.6×10^{-2} s^{-1} and its decay rate was 3.3×10^{-4} s^{-1}. Under the same conditions, the decay of bioluminescence was fit to a three-exponential equation with rate constants of 9.8×10^{-2}, 6.9×10^{-3}, and 6.8×10^{-4} s^{-1}.

Although the model presented in Scheme III is reasonably complete, there are other complexes of enzyme and substrates that are known to exist. For example, when complexes of E–decanal are mixed with FMNH$_2$, there is an inhibition of maximum light production at elevated aldehyde concentrations. This inhibition is not observed when these same aldehyde concentrations are mixed with E–FMNH$_2$. Preliminary modeling of these results suggests that FMNH$_2$ cannot bind to the E–decanal complex and thus significant FMNH$_2$ decay occurs during the time it takes for the *n*-decanal to dissociate from the E–decanal complex. Previous models for this inhibition involved the binding of two *n*-decanal molecules per mole of enzyme (Holzman & Baldwin, 1983). It also appears that oxygen cannot react (or does so very slowly) with the E–FMNH$_2$–decanal complex. Again, it appears that *n*-decanal must dissociate from this complex prior to the reaction with O$_2$ to form the 4a-hydroperoxyflavin intermediate. Even though the data presented here comprise a reasonably complete kinetic evaluation of the reactions catalyzed by bacterial luciferase which result in light emission, a thorough description of the off-pathway complexes (E–aldehyde and E–

$FMNH_2$–aldehyde) will require further studies.

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