

Interaction of Bacterial Luciferase with Aldehyde Substrates and Inhibitors*

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Bacterial luciferase catalyzes the reaction of FMNH₂, O₂, and an aliphatic aldehyde to yield the carboxylic acid, FMN, water and blue-green light. The kinetics of the bacterial luciferase reaction were measured by stopped-flow spectrophotometry at pH 7 and 25 °C for the series of aldehydes from *n*-heptanal to *n*-undecanal. The rate of formation of the 4a-hydroperoxyflavin intermediate was dependent on the aldehyde concentration when mixtures of enzyme, FMNH₂, and aldehyde were rapidly mixed with O₂. At saturating aldehyde, the rate of formation of this intermediate was 100-fold slower than in the absence of aldehyde, demonstrating that an enzyme·FMNH₂·aldehyde complex can be formed. Numerical simulation of the time courses for these experiments supported the formation of this intermediate and its direct reaction with O₂. The kinetics of the light emitting reaction were dependent upon the chain length of the aldehyde substrate. Although the initial light intensity and the light emission decay rate were different for each aldehyde, the quantum yield for the reaction was independent of the aldehyde used. Luciferase was inhibited by high levels of the aldehyde substrate when the enzyme was assayed by mixing FMNH₂ with an aerobic mixture of enzyme and aldehyde. The extent of inhibition was dependent on the particular aldehyde used, and the binding affinity of the aldehyde for the free enzyme increased in parallel with the aldehyde chain length. The kinetics of the formation and decay of the various intermediates were also studied in the presence of *n*-alkylaldehyde analogs. These compounds decreased the rate of formation of the 4a-hydroperoxyflavin intermediate in much the same way as the aldehyde substrate, presumably by the formation of the enzyme·FMNH₂·analogue ternary complex.

Bacterial luciferase is an $\alpha\beta$ heterodimeric enzyme with a single active center residing on the α subunit. The enzyme catalyzes the reaction of FMNH₂,¹ O₂, and an aliphatic aldehyde to yield FMN, the carboxylic acid and a photon of blue-green light (for a review of the system see Baldwin and Ziegler, 1992). Previous investigations of the kinetic mechanism of the luciferase-catalyzed reaction have led to an understanding of the light producing pathway and have suggested the existence of several off-pathway complexes (Abu-Soud *et al.*, 1992, 1993). These investigations have demonstrated that the enzyme binds FMNH₂ to form a binary complex that isomerizes to an *E'*·FMNH₂ complex. This complex reacts rapidly with O₂ to yield the C4a-hydroperoxyflavin intermediate (Hastings and Gibson, 1963; Vervoort *et al.*, 1986). In the light producing pathway, this intermediate binds the aldehyde substrate to form, ultimately, the excited state of the flavin and the carboxylic acid product. Several chemical mechanisms have been proposed for the light emitting reaction (Ziegler and Baldwin, 1981; Raushel and Baldwin, 1989).

The luciferase reaction has been shown to be sensitive to inhibition by high concentrations of the aldehyde substrate in certain assay formats. There are basically two procedures for assay of bacterial luciferase which are in common use. In the first, the enzyme is incubated in a buffered solution with aldehyde and dissolved O₂. The reaction is initiated by injection of FMNH₂ that is reduced by H₂ and a platinum catalyst or by blue light (Hastings *et al.*, 1978). The second assay format involves incubation of the enzyme with FMNH₂ prepared by the addition of a small amount of dithionite. The reaction is initiated by injection of an aerobic solution of the aldehyde substrate. Aldehyde substrate inhibition is not observed with the latter assay format, but it is with the former (Hastings *et al.*, 1978). Abu-Soud *et al.* (1993) have shown that the aldehyde-induced substrate inhibition is caused by the formation of a dead-end enzyme-aldehyde complex. This complex appears not to bind FMNH₂ to form the ternary complex. Rather, the aldehyde must first dissociate, and during the time required for dissociation of the aldehyde and binding of the flavin, the free flavin reacts nonenzymatically with O₂ to form FMN and H₂O₂ (Gibson and Hastings, 1962; Massey *et al.*, 1971).

Abu-Soud *et al.* (1992) have shown that the addition of aldehyde to the incubation mixture dramatically reduces the rate with which O₂ reacts with the enzyme-bound FMNH₂ to yield the flavin hydroperoxide intermediate. This observation demonstrated the existence of an off-pathway ternary complex of enzyme·FMNH₂·aldehyde, but the issue of the direct reaction of O₂ with the ternary complex was left unanswered. Alternatively, the reduced rate of reaction could have been caused by dissociation of the aldehyde from the ternary complex followed by the reaction of O₂.

The kinetics of the light emitting reaction, as determined by the decay rate of bioluminescence, are dependent, among other factors, upon the carbon chain length of the aldehyde substrate. Aldehydes of any chain length longer than 6 or 8

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¹ The abbreviations used are: FMNH₂ 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.

carbons are substrates for the enzyme (Hastings *et al.*, 1966; Watanabe and Nakamura, 1972), and although similar quantum yields are obtained with the different chain length aldehydes, the catalytic turnover rates are different (Baumstark *et al.*, 1979). The exact nature of the differences observed with the various aldehydes on the kinetic mechanism of the bacterial luciferase is unknown.

Bacterial luciferase can be inhibited by numerous hydrophobic compounds, many of which have been shown to bind competitively with the aldehyde substrate (Curry *et al.*, 1990; Hastings *et al.*, 1966). Some of these compounds have been shown to stabilize the 4a-hydroperoxyflavin intermediate (Ahrens *et al.*, 1991; Makemson *et al.*, 1992; Tu, 1979) and are commonly used to isolate and study the flavin hydroperoxide (Tu, 1986). None of these compounds has been shown to be substrates for the luciferase reaction.

Stopped-flow methods have now been used to study the effect of aliphatic *n*-alkylaldehydes (heptanal to undecanal) and *n*-alkylaldehyde analogs (*i.e.* trifluoromethyl ketones, boronic acids, amines, alcohols, and carboxylic acids) on the kinetic mechanism of the bacterial luciferase reaction, with special attention to their effect on the formation and stability of the 4a-hydroperoxyflavin intermediate. The experiments presented in this publication address the general questions of the effect of the different chain length aldehydes on the kinetic mechanism of bioluminescence and the off-pathway and alternative pathway processes in the luciferase-catalyzed reaction. We have found that the binding affinities of the different aldehydes to the various reaction intermediates are similar for all aldehydes, but the rate of formation and breakdown of the peroxyhemiacetal intermediate can account for the differences observed in the catalytic turnover with the various aldehydes. The inhibitors, *n*-alkylalcohols, acids, amines, trifluoromethyl ketones, and boronic acids all seem to interact with the enzyme in much the same way as the aldehyde substrate. This paper presents a unified kinetic mechanism that allows interpretation of a large number of studies of substrate interaction and inhibition of the luciferase enzyme.

EXPERIMENTAL PROCEDURES

Riboflavin 5'-monophosphate (FMN) was purchased from Fluka (97%) and used without further purification. The *n*-aliphatic aldehydes, alcohols, and amines were of the highest grade available and obtained from either Sigma or Aldrich. Trifluoromethyl ketones were prepared from the corresponding bromides (Hammock *et al.*, 1982), purified by distillation under reduced pressure and characterized by ^1H , ^{13}C , and ^{19}F NMR spectroscopy and mass spectrometry. Boronic acids were prepared from the corresponding bromides (Torsell and Larsson, 1957), purified by recrystallization and characterized by ^1H , ^{13}C , and ^{11}B NMR spectroscopy and mass spectrometry. All spectroscopic data were consistent with the corresponding structures and did not show the presence of any impurities.

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 absorbance at 280 nm using a molecular weight of 79,000 and a specific absorption coefficient of $0.94 \text{ cm}^{-1} \text{ mg}^{-1}$.

Stopped-flow Spectrophotometry—All experiments were performed under a nitrogen atmosphere in 50 mM Bis-Tris-HCl buffer, pH 7.0, at $25^\circ\text{C} \pm 0.2^\circ\text{C}$. Care was taken not to expose the flavin solutions to light during the experimental procedures. The flavin was reduced by bubbling hydrogen in the presence of a few crystals of palladium on activated carbon. The FMN concentration was determined spectrophotometrically on the basis of a molar absorption coefficient at 450 nm of $12,200 \text{ M}^{-1} \text{ cm}^{-1}$ (Whitby, 1953). 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-FMN H_2 solutions were made by mixing the anaerobic enzyme solutions with reduced flavin under a nitrogen atmosphere and then transferring the mixture 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 experiments were carried out under the same conditions as described previously (Abu-Soud *et al.*, 1992). Stock solutions of 0.1 M aldehyde and aldehyde analogs were 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.

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

$$y = Ae^{-k_1t} + C \quad (\text{Eq. 2})$$

$$y = Ae^{-k_1t} + Be^{-k_2t} + C \quad (\text{Eq. 3})$$

$$y = A(k_1/(k_2 - k_1))(e^{-k_1t} - e^{-k_2t}) + C \quad (\text{Eq. 4})$$

$$y = A(1 + (1/(k_1 - k_2))(k_2e^{-k_1t} - k_1e^{-k_2t})) + C \quad (\text{Eq. 5})$$

Equation 3 represents the sum of two independent exponentials for a parallel process, and 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 I or II. 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 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).

RESULTS

Effect of *n*-Aldehydes on the Formation of 4a-Hydroperoxyflavin Intermediate—The time courses, monitored at 380 nm, for the reaction of luciferase, FMN H_2 , and decanal with air-equilibrated buffer are shown in Fig. 1. The reaction was initiated by mixing a solution of enzyme and FMN H_2 in the presence of various amounts of decanal (0–400 μM) with a fixed concentration of oxygen (120 μM). At low levels of decanal, the time courses for the reaction were accurately fit to the sum of two exponentials (Equation 3) with values for k_1 and k_2 of 350 s^{-1} and 23 s^{-1} , respectively. The fast phase was similar in magnitude to the reaction of $E' \cdot \text{FMN}\text{H}_2$ with O_2 in the absence of aldehyde (Abu-Soud *et al.*, 1992). The amplitude of the slow phase increased relative to the amplitude of the fast phase as the decanal concentration was increased (see *inset* to Fig. 1). The amplitudes of the two phases were used to obtain an estimate of the equilibrium constant for the formation of the $E' \cdot \text{FMN}\text{H}_2$ -decanal complex from $E' \cdot \text{FMN}\text{H}_2$ and decanal. Similar experiments were performed with the aldehydes heptanal, octanal, nonanal and undecanal, yielding similar results (data not shown).

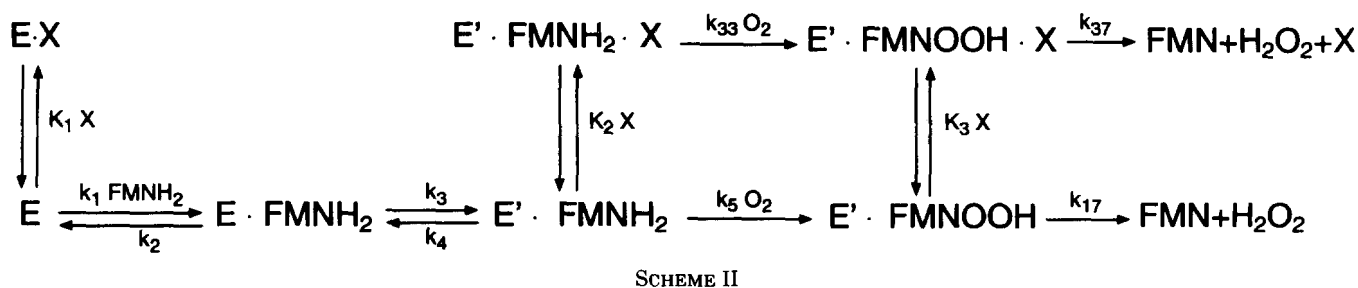
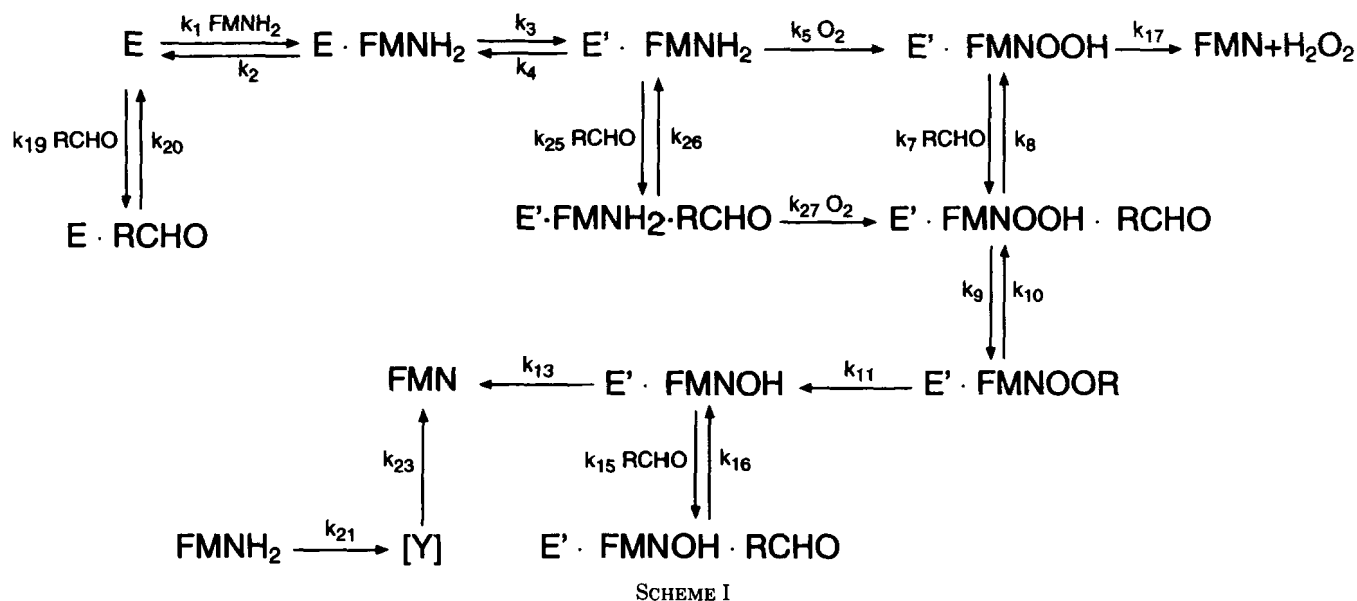
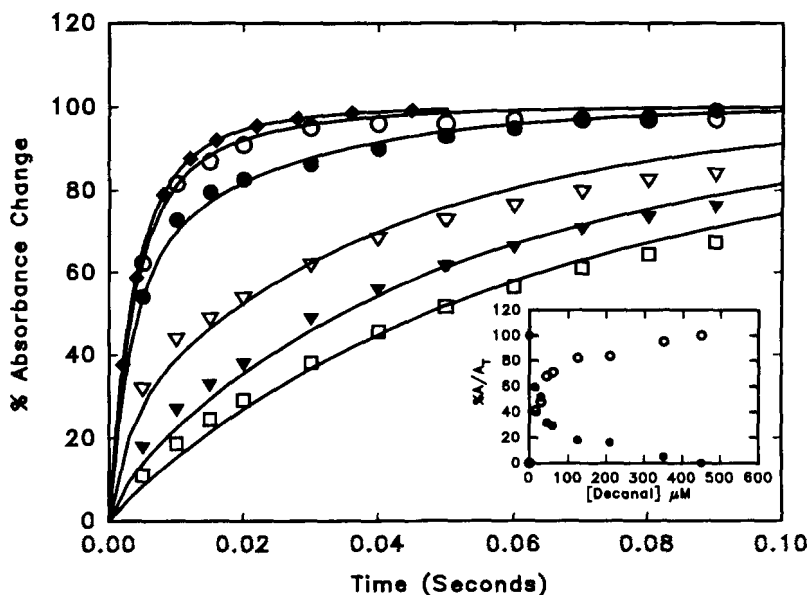


FIG. 1. Time courses for the reaction of E-FMNH₂-decanal with O₂ when the reaction was monitored at 380 nm. The experiments were conducted at a fixed concentration of enzyme (75 μ M) and FMNH₂ (15 μ M) in the presence of variable amounts of decanal (0 (\blacklozenge), 10 (\circ), 38 (\bullet), 100 (∇), 200 (\blacktriangledown), and 400 (\square) μ M), and the samples were mixed with an equal volume of air-equilibrated buffer (120 μ M O₂). The *symbols* represent portions of the experimental data; the *solid lines* represent the simulated time courses using the rate constants that appear in Table II and the model in Scheme I. The *inset* shows a plot of the percent absorbance change that is caused by the fast phase (\circ) and the slow phase (\bullet) for the formation of the 380 nm absorbing species for each concentration of decanal used. The amplitudes were determined by fitting the time courses to Equation 3.



Since high concentrations of decanal caused a decrease in the rate of formation of the 4a-hydroperoxyflavin intermediate (Fig. 1), we concluded that the aldehyde can bind to the E' -FMNH₂ complex to yield the ternary complex of E' -FMNH₂·RCHO. To determine the fate of this ternary complex in the reaction with O₂, we established experimental conditions in which the equilibria strongly favored the formation of the E' -FMNH₂·RCHO complex. Enzyme, FMNH₂, and decanal (75, 15, and 500 μM final concentrations) were mixed with equal volumes of O₂-equilibrated buffer (final O₂

concentrations of 60–600 μM), and the formation of the 4a-hydroperoxyflavin intermediate was monitored at 380 nm (Fig. 2, not all data shown). These kinetic traces were fit to a single exponential (Equation 2) to determine the apparent first-order rate constants which were then plotted *versus* the O_2 concentration to obtain an apparent second-order rate constant of $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (data not shown).

Formation and Decomposition of Light-emitting Species— Plots of the relative maximum light intensity (I_{\max}) as well as the decay rate of the light produced when a mixture of enzyme

FIG. 2. Time courses for the reaction of $E \cdot FMNH_2$ ·decanal with O_2 when the reaction was monitored at 380 nm. The experiments were conducted at a fixed concentration of enzyme (75 μM), $FMNH_2$ (15 μM), and decanal (500 μM), and the samples were mixed with various amounts of O_2 (120 (Δ), 200 (\blacksquare), 400 (∇), and 600 (\bullet) μM) in 50 mM Bis-Tris buffer. The symbols represent portions of the experimental data; the solid lines represent the simulated time courses using the rate constants that appear in Table II and the model in Scheme I.

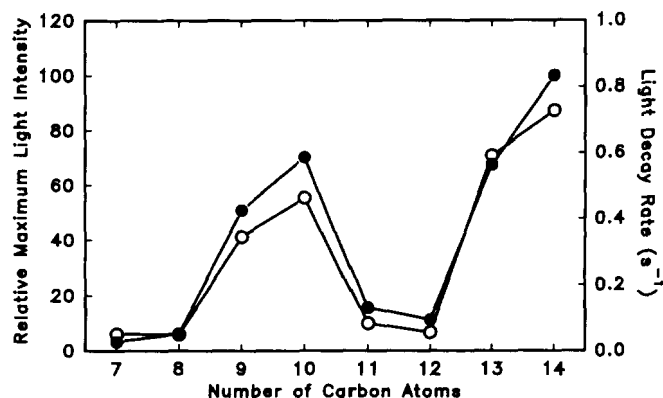
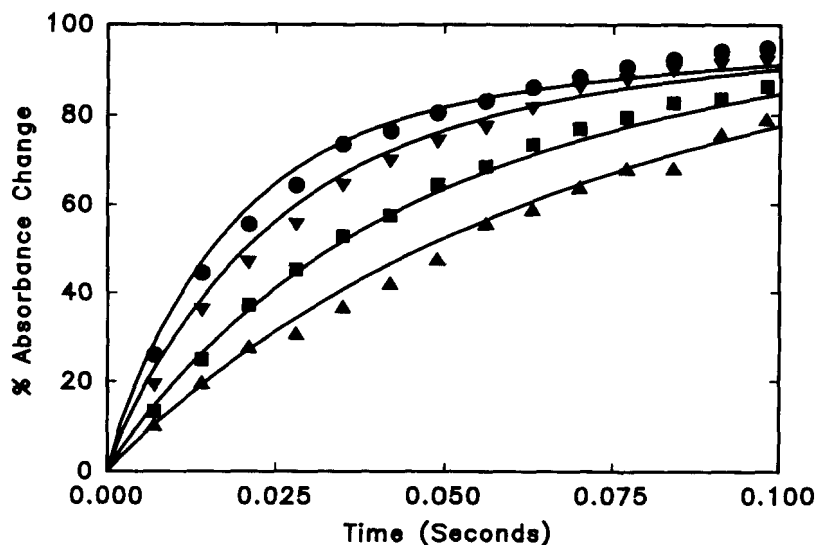


FIG. 3. Relative maximum light intensity (\bullet) and bioluminescence decay rate (\circ) for the reaction of enzyme (75 μM) and $FMNH_2$ (15 μM) with an equal volume of n -alkylaldehyde (100 μM) in air-equilibrated buffer (120 $\mu M O_2$).

and $FMNH_2$ was mixed with aldehyde (100 μM) in air-equilibrated buffer (120 $\mu M O_2$) for the aldehydes in the series from heptanal to tetradecanal are shown in Fig. 3. For all aldehydes, the rate of light emission reaches a maximum in less than 5 s and then decays exponentially with rate constants that vary from one aldehyde to another (Fig. 3). Both the initial light intensity and the light decay rate varied from one aldehyde to another. However, in all cases, if the initial intensity was higher, the light decay rate was also faster, and thus the quantum yield was essentially the same for all of the aldehydes used.

The time courses for the production of visible light when the $E' \cdot FMNH_2$ complex was mixed with increasing amounts of n -undecanal in air equilibrated buffer are shown in Fig. 4. The rate of light emission reaches a maximum in less than 3 s and then decays exponentially over a period of approximately 25 s. The time required for maximum light emission decreases as the concentration of n -undecanal is increased. Similar experiments were performed with heptanal, octanal, nonanal, and decanal (data not shown).

Formation of the Enzyme-Aldehyde Complex—The inhibition of luciferase by the aldehyde substrate was evaluated by mixing enzyme and aldehyde in air-equilibrated buffer with $FMNH_2$. Plots of the maximum light intensity produced *ver-*

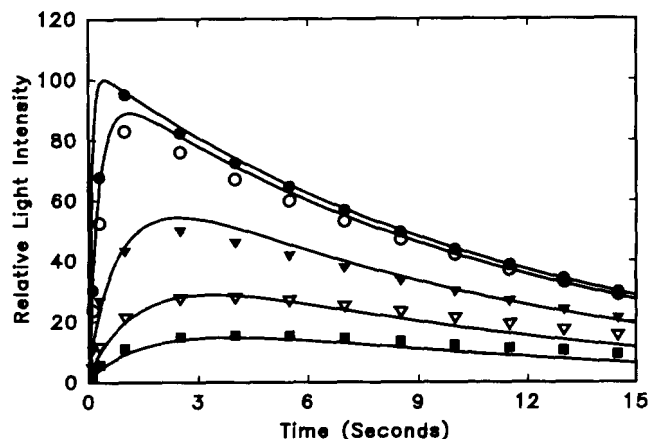


FIG. 4. Time courses for light emission after mixing a solution of luciferase (75 μM) and $FMNH_2$ (15 μM) with various amounts of n -undecanal (10 (\blacksquare), 20 (∇), 40 (Δ), 80 (\circ), and 200 (\bullet) μM) in air-equilibrated buffer. The symbols represent portions of the experimental data; the solid lines represent the simulated time courses using the rate constants that appear in Table II and the model in Scheme I.

sus the aldehyde concentration when the reaction was initiated by mixing a fixed amount of enzyme (75 μM) and O_2 (120 μM) in the presence of increasing amounts of aldehyde (heptanal, nonanal, and undecanal; 10–500 μM) with a fixed amount of $FMNH_2$ (15 μM) are shown in Fig. 5, panels A–C. At high aldehyde concentrations, a reduced enzyme activity was observed. The extent of inhibition at a specific aldehyde concentration was greater for the longer chain length aldehydes. With heptanal, virtually no inhibition was observed below 600 μM .

No aldehyde-induced substrate inhibition was observed when the $E' \cdot FMNH_2$ complex was mixed with air-equilibrated solutions (120 $\mu M O_2$) containing varying concentrations of aldehyde (Fig. 5, panels D–F). Similar results were also obtained when octanal and decanal were used as the aldehyde substrate (data not shown).

When a solution of enzyme and aldehyde in air-equilibrated buffer was mixed with $FMNH_2$ and the reaction monitored at 380 nm and 445 nm, the time courses (in the presence of low concentrations of any of the aldehydes studied here) were

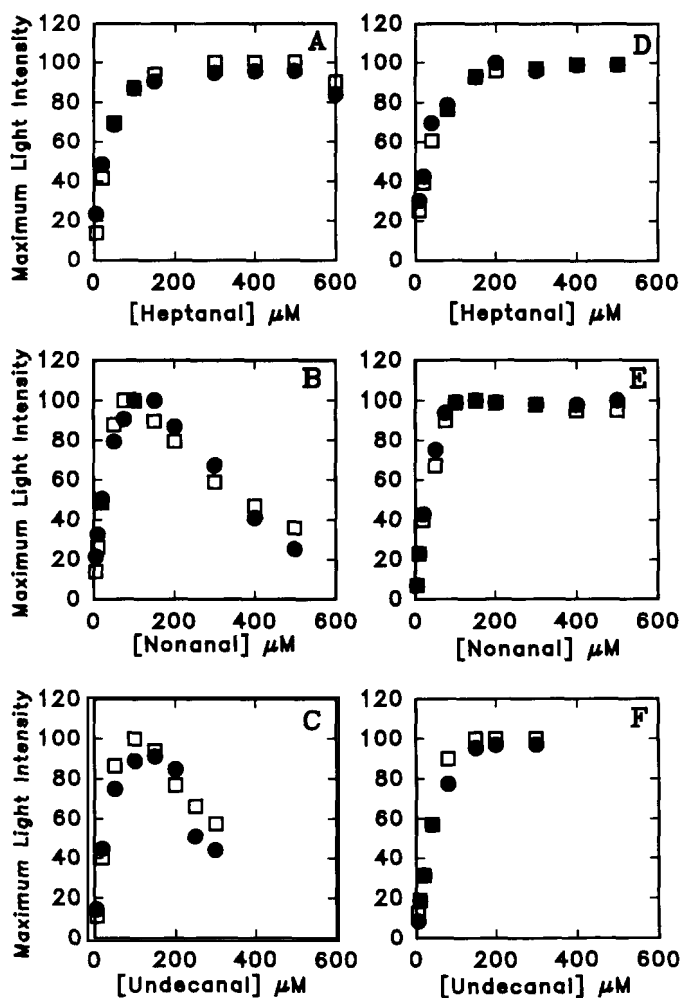


FIG. 5. Effect of the chain length of aldehyde and order of substrate addition on the aldehyde-induced inhibition of bioluminescence intensity. Panels A–C, actual (●) and simulated (□) maximum light intensities when enzyme and various amounts of *n*-heptanal (panel A), *n*-nonanal (panel B), and *n*-undecanal (panel C) in air-equilibrated buffer ($120 \mu\text{M O}_2$) were mixed with FMNH₂. The final concentrations of enzyme and FMNH₂ were 75 and 15 μM , respectively. Panels D–F, actual (●) and simulated (□) maximum light intensities when enzyme (75 μM) and FMNH₂ (15 μM) were mixed with various amounts of *n*-heptanal (panel D), *n*-nonanal (panel E), and *n*-undecanal (panel F) in air-equilibrated buffer.

virtually identical to the time course of the reaction of aerobic enzyme with FMNH₂ without any added aldehyde. However, in the presence of high concentrations of aldehyde, the time courses were similar to those observed for the nonenzymatic autooxidation of FMNH₂ by O₂ to produce FMN and HOOH (data not shown).

Formation of FMN—The ultimate formation of FMN was determined by monitoring the flavin absorbance at 445 nm for the reaction of E'·FMNH₂ with various amounts of aldehyde in air-equilibrated buffer. The time courses for the reaction of the E'·FMNH₂ complex with increasing amounts of *n*-nonanal are shown in Fig. 6. The kinetic traces were successfully fit to the sum of two exponentials (Equation 3). As the concentration of *n*-nonanal increases, the slower phase steadily decreases in rate. Similar results were obtained with heptanal, octanal, decanal, and undecanal (data not shown).

Effect of *n*-Alkyl Compounds on the Formation and Decay of the 4a-Hydroperoxyflavin Intermediate—Other investigators have shown that *n*-alkylcarboxylic acids, alcohols, amines, and boronic acids are inhibitors of bacterial luciferase,

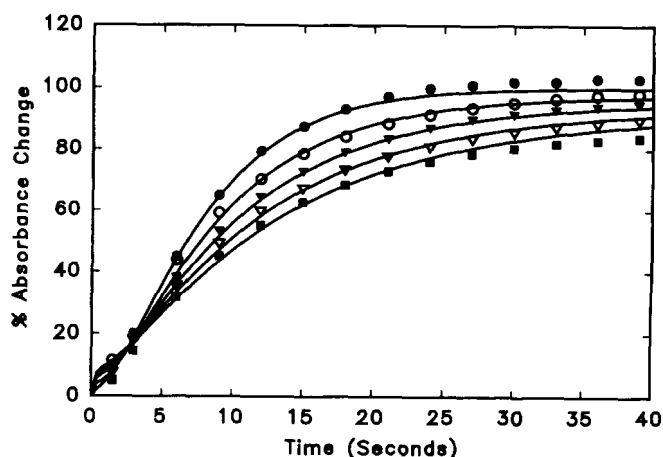


FIG. 6. Time courses for the formation of FMN when E'·FMNH₂ is mixed with air-equilibrated *n*-nonanal and the reaction monitored at 445 nm. The experiments were conducted at fixed concentrations of luciferase (75 μM) and FMNH₂ (15 μM) with various amounts of *n*-nonanal (50 (●), 200 (○), 300 (▼), 400 (▽), and 500 (■) μM) in air-equilibrated buffer. The symbols represent portions of the experimental data; the solid lines represent the simulated time courses using the rate constants that appear in Table II and the model in Scheme I.

and they are competitive with the aldehyde substrate (Ahrens *et al.*, 1991; Hastings *et al.*, 1966; Makemson *et al.*, 1992). We have determined the direct effect of these compounds on the rate of formation of the 4a-hydroperoxyflavin (absorbance at 380 nm) and on the formation of FMN from the hydroperoxyflavin intermediate (absorbance at 445 nm). The data presented in panel A of Fig. 7 demonstrate that these *n*-alkyl inhibitors decrease the rate with which O₂ reacts with the enzyme-bound reduced flavin to yield the 4a-hydroperoxyflavin intermediate, in much the same way as with the aldehyde substrates (see Fig. 1 for the example of *n*-decanal). The data presented in panel B of Fig. 7 demonstrate that these compounds also decrease the rate in which the hydroperoxyflavin intermediate is ultimately converted to FMN.

The effect of several *n*-alkylaldehyde analogs (*i.e.* alcohols, carboxylic acids, trifluoromethyl ketones, amines, and boronic acids) on the rate of formation and decomposition of the 4a-hydroperoxyflavin intermediate was studied. A mixture of enzyme, FMNH₂, and aliphatic compound was mixed with O₂, and the change in absorbance at 380 and 445 nm was measured. All compounds tested were found to slow down the formation of the peroxyflavin intermediate. The rate of formation of this intermediate was two to four times slower in the presence of these compounds (Table I). The magnitude of this effect was found to vary from one class of compound to another, alcohols being the most effective and carboxylic acids being the least effective. The effect on the rate of formation was also found to be dependent on the chain length of the alkyl compound. The magnitude of the stabilization generally increased as the alkyl chain length increased. The effect of these alkyl compounds is similar to that observed with the aldehyde substrate. The *n*-alkyl aliphatic compounds were also found to stabilize the decay of the 4a-hydroperoxyflavin intermediate. Amines were found to be the most effective for stabilization of the hydroperoxyflavin intermediate.

More extensive kinetic studies were performed with *n*-decanol and *n*-decylamine. The existence of the enzyme-inhibitor complex was demonstrated by the observation that at very high inhibitor concentrations, the rate of formation of FMN upon mixing of enzyme, inhibitor, and O₂ with FMNH₂ was identical to the rate of FMN formation in the

FIG. 7. Effect of *n*-alkyl aliphatic compounds on the formation and decay of the 4a-hydroperoxyflavin intermediate. The experiments were conducted at a fixed concentration of enzyme (75 μ M) and FMNH₂ (15 μ M) in the presence of variable amounts of either *n*-decanol (●), *n*-decanoic acid (○), *n*-decylamine (▽), or *n*-decyltrifluoromethyl ketone (▼) and the reactions initiated by mixing with an equal volume of air-equilibrated buffer (120 μ M O₂). The reaction was monitored at 380 nm (panel A) and 445 nm (panel B), and the pseudo-first-order rate constants were plotted against the *n*-alkyl aliphatic compound concentration.

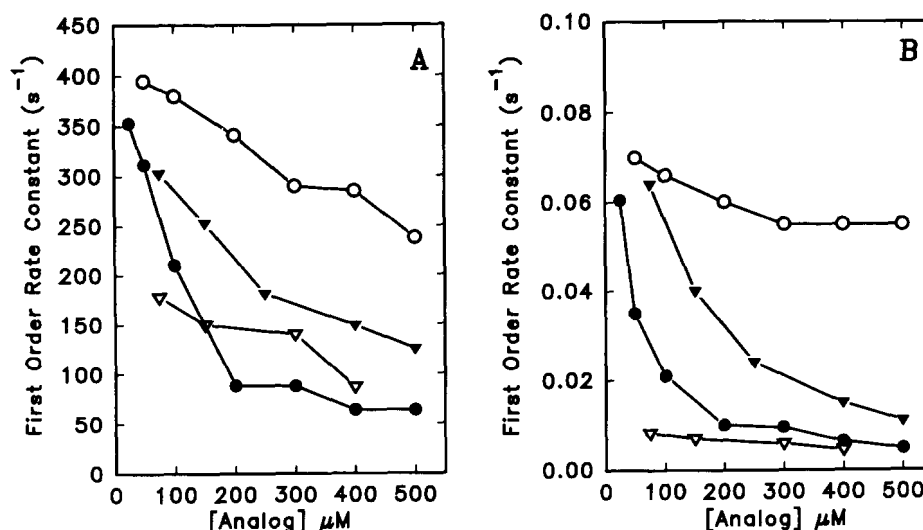


TABLE I

Formation and decay rates of 4a-hydroperoxyflavin-aliphatic compound complexes

Rates were determined in 50 mM Bis-Tris buffer, pH 7, at 25 °C. The rate constants $k_{380\text{ nm}}$ and $k_{445\text{ nm}}$ were determined by fitting the time courses for the reaction of luciferase (75 μ M), FMNH₂ (15 μ M), and aliphatic compound (500 μ M) with air-equilibrated buffer (120 μ M O₂) monitored at 380 and 445 nm to a single exponential function (Equation 2).

Aliphatic compound	$k_{380\text{ nm}}$ s^{-1}	$k_{445\text{ nm}}$ s^{-1}
None ^a		0.10
CH ₃ (CH ₂) ₇ OH	65	0.005
CH ₃ (CH ₂) ₉ OH	50	0.005
CH ₃ (CH ₂) ₁₁ OH ^b	185	0.0008
CH ₃ (CH ₂) ₆ COOH	370	0.05
CH ₃ (CH ₂) ₈ COOH	240	0.055
CH ₃ (CH ₂) ₁₀ COOH ^c	185	0.045
CH ₃ (CH ₂) ₆ COCF ₃	110	0.03
CH ₃ (CH ₂) ₇ COCF ₃	100	0.02
CH ₃ (CH ₂) ₈ COCF ₃	125	0.01
CH ₃ (CH ₂) ₅ NH ₂	255	0.03
CH ₃ (CH ₂) ₆ NH ₂	140	0.005
CH ₃ (CH ₂) ₇ NH ₂	100	0.006
CH ₃ (CH ₂) ₈ NH ₂	90	0.0035
CH ₃ (CH ₂) ₉ NH ₂ ^d	90	0.0045
CH ₃ (CH ₂) ₇ B(OH) ₂ ^e	100	0.02
CH ₃ (CH ₂) ₈ B(OH) ₂ ^e	100	0.02
CH ₃ (CH ₂) ₉ B(OH) ₂ ^e	90	0.01

^a Determined by fitting the time course for the reaction of luciferase (75 μ M) and FMNH₂ (15 μ M) with air-equilibrated buffer (120 μ M O₂) monitored at 380 and 445 nm to a single exponential function (Equation 2).

^b [CH₃(CH₂)₁₁OH] = 100 μ M.

^c [CH₃(CH₂)₁₀COOH] = 300 μ M.

^d [CH₃(CH₂)₉NH₂] = 400 μ M.

^e [CH₃(CH₂)₈B(OH)₂] = 200 μ M.

absence of enzyme (data not shown). When enzyme, FMNH₂, and high concentrations of inhibitor were mixed with various amounts of O₂, the pseudo-first-order rate constants for the formation of the 380 nm absorbing species were directly proportional to the oxygen concentration. Similar results have

been presented in Fig. 2 for *n*-decanol. The second-order rate constant for the reaction of the *E'*·FMNH₂·inhibitor ternary complex (with either saturating *n*-decanol or *n*-decylamine) with O₂ was 100-fold slower than the corresponding reaction of *E'*·FMNH₂ with O₂ in the absence of the inhibitor. These results are comparable to those observed when aldehyde is used as a substrate (Fig. 2).

Calculation of Microscopic Rate Constants—The microscopic rate constants and the equilibrium constants for the kinetic models that appear in Schemes I and II for the aldehydes in the series from heptanal to undecanal (Table II) and for *n*-decanol and *n*-decylamine (Table III) were obtained by direct comparison of the experimental time courses for intermediate and product formation with the calculated time courses using the KINSIM program. The rate constants for the formation of the *E'*·FMNH₂ complex (k_1 to k_4), the decomposition of the *E'*·FMNOOH complex to FMN and H₂O₂ (k_{17}), the final dehydration of *E'*·FMNOH complex to FMN (k_{13}), and the autooxidation of FMNH₂ (k_{21} and k_{23}) have been reported previously by Abu-Soud *et al.* (1992, 1993) and were held constant during the simulation.

The rate constants associated with the formation of the *E'*·FMNH₂·aldehyde complex (k_{25} and k_{26}) and the rate constant for the reaction of this complex with O₂ (k_{27}) were determined by the simultaneous fit of the complete set of time courses at 380 nm for the experiments in which a mixture of enzyme, FMNH₂, and varying concentrations of aldehyde were mixed with a fixed concentration of O₂ and also in which a mixture of enzyme, FMNH₂, and a fixed concentration of aldehyde was combined with varying amounts of O₂ (Figs. 1 and 2).

The rate constants associated with the binding of aldehyde with free enzyme (k_{19} and k_{20}) and the rate constants for the processes associated with the binding of aldehyde to the *E'*·FMNOOH complex through the formation of *E'*·FMNOH for the different aldehydes (k_7 to k_{11}) were established as described by Abu-Soud *et al.* (1993) by the simultaneous simulation of the time courses for the emission of visible light that followed the mixing of air-equilibrated aldehyde with a mixture of enzyme and FMNH₂ and those obtained when FMNH₂ is mixed with an aerobic mixture of enzyme and aldehyde to the kinetic model that appears in Scheme I (Figs. 4 and 5).

TABLE II
Rate constants for the kinetic model in Scheme I

pH 7.0, 25 °C. The rate constants k_1 ($1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), k_2 ($1,200 \text{ s}^{-1}$), k_3 (200 s^{-1}), k_4 (14 s^{-1}), k_5 ($2.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$), k_{13} (0.6 s^{-1}), and k_{17} (0.1 s^{-1}) have been reported elsewhere (Abu-Soud *et al.*, 1993). Note that k_{21} and k_{23} (4.7 and 11.5 s^{-1} , respectively) were determined by a fit of the time courses for the reaction of FMNH₂ with O₂ monitored at 380 and 445 nm to a sequential mechanism (Equation 5; Abu-Soud *et al.*, 1992).

	Heptanal	Octanal	Nonanal	Decanal	Undecanal
k_7	$1.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$3.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$1.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$	$1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
k_8	$1,000 \text{ s}^{-1}$	$2,400 \text{ s}^{-1}$	73 s^{-1}	120 s^{-1}	$3,400 \text{ s}^{-1}$
k_9	5 s^{-1}	4.3 s^{-1}	1.4 s^{-1}	1.6 s^{-1}	37 s^{-1}
k_{10}	0.9 s^{-1}	0.46 s^{-1}	0.98 s^{-1}	1.15 s^{-1}	0.24 s^{-1}
k_{11}	0.04 s^{-1}	0.08 s^{-1}	0.76	1.11 s^{-1}	0.09 s^{-1}
k_{15}		$3,200 \text{ M}^{-1} \text{ s}^{-1}$	$2,500 \text{ M}^{-1} \text{ s}^{-1}$	$3,000 \text{ M}^{-1} \text{ s}^{-1}$	$3,000 \text{ M}^{-1} \text{ s}^{-1}$
k_{16}		0.28 s^{-1}	0.80 s^{-1}	0.06 s^{-1}	0.30 s^{-1}
k_{19}	$5.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$4.6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	$1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	$9.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$
k_{20}	12 s^{-1}	12 s^{-1}	7.2 s^{-1}	5.8 s^{-1}	5.2 s^{-1}
k_{25}	$1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	$2.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$5.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$	$1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$	$1.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$
k_{26}	39 s^{-1}	37 s^{-1}	21 s^{-1}	37 s^{-1}	40 s^{-1}
k_{27}	$4.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$8.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$7.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$5.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$

TABLE III
Kinetic constants for the model in Scheme II

pH 7.0, 25 °C. The rate constants k_1 ($1.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$), k_2 ($1,200 \text{ s}^{-1}$), k_3 (200 s^{-1}), and k_4 (14 s^{-1}) have been reported elsewhere (Abu-Soud *et al.*, 1993).

	Decanol	Decylamine
K_1	$3.9 \times 10^3 \text{ M}^{-1}$	$6.7 \times 10^3 \text{ M}^{-1}$
K_2	$6.1 \times 10^3 \text{ M}^{-1}$	$3.1 \times 10^5 \text{ M}^{-1}$
K_3	$3.5 \times 10^4 \text{ M}^{-1}$	$7.6 \times 10^6 \text{ M}^{-1}$
k_{33}	$7.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$5.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$
k_{37}	0.004 s^{-1}	0.002 s^{-1}

Estimation of the association and dissociation rate constants of the $E' \cdot \text{FMNOH}$ complex with the different aldehydes (k_{15} and k_{16}) were obtained by the simultaneous simulation of the time courses for the change in absorbance at 445 nm and the production of visible light upon mixing $E \cdot \text{FMNH}_2$ with air-equilibrated aldehyde. The simulated time courses for the change in absorbance at 445 nm using *n*-nonanal as the aldehyde substrate are illustrated as the *solid lines* in Fig. 6.

The equilibrium rate constants K_1 and K_2 for *n*-decanol and *n*-decylamine which appear in Table III for Scheme II were estimated by simultaneous simulation of the time courses for the reaction of enzyme and FMNH₂ with aerobic solutions of inhibitor, the reaction of aerobic enzyme, and inhibitor with FMNH₂ and the reaction of enzyme, FMNH₂, and inhibitor with O₂ monitored at 380 nm. The equilibrium constant K_3 and the rate constant k_{37} were estimated by simultaneous fit of the time courses for the reactions mentioned above when monitored at 445 nm.

DISCUSSION

Formation of the $E \cdot \text{FMNH}_2$ -Aldehyde Complex—When mixtures of enzyme, FMNH₂, and aldehyde are mixed with O₂ and the reactions monitored at 380 nm, the time courses can be fit to the sum of two exponentials. As the concentration of the aldehyde increases, the amplitude of the slow phase also increases. The substantial decrease in the net rate of formation of the 4a-hydroperoxyflavin intermediate at high concentrations of aldehyde clearly indicates the formation of an $E' \cdot \text{FMNH}_2$ -aldehyde ternary complex. As the alkyl chain length of the aldehyde was increased from 7 to 11 carbons, the apparent binding affinity of the aldehyde for the $E' \cdot \text{FMNH}_2$ complex also increased, resulting in a shift from the fast phase to the slow phase at lower concentrations of aldehyde (see *inset* to Fig. 1). This increase in binding affinity as

a function of aldehyde chain length is accounted for as a decrease in the dissociation constant (k_{26}/k_{25} in Table II) for the aldehyde from the $E' \cdot \text{FMNH}_2$ -aldehyde ternary complex as obtained by simulation of the experimental data to the kinetic model that appears on Scheme I.

A second-order rate constant of $2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ was obtained for the oxidation of the $E' \cdot \text{FMNH}_2$ -aldehyde complex by O₂, using *n*-decanal as the aldehyde substrate. This rate constant is 100-fold smaller than the rate constant for the reaction of the $E' \cdot \text{FMNH}_2$ complex with O₂ (Abu-Soud *et al.*, 1992). A similar decrease in the second-order rate constant for the reaction of the ternary complex with O₂ was observed for all of the aldehydes used in this study. Two possible pathways can be written for the reaction of O₂ with the flavin in the ternary $E' \cdot \text{FMNH}_2 \cdot \text{RCHO}$ complex (Scheme I). In the first, the aldehyde must dissociate to yield $E' \cdot \text{FMNH}_2$ which then reacts with O₂ to form the $E' \cdot \text{FMNOOH}$ complex. However, with this restricted mechanism we were unable to simulate all of the time courses using this pathway alone. We have therefore incorporated into the model a second pathway for the reaction of O₂ directly with the flavin in the ternary complex (Scheme I). By inclusion of this step, together with the other steps depicted in Scheme I, we have been able to simulate accurately the time courses for the reaction of O₂ with the $E' \cdot \text{FMNH}_2 \cdot \text{RCHO}$ complex (Fig. 2). The *solid lines* in Figs. 2 and 3 were determined by fitting the data to Scheme I and allowing the first- and second-order rate constants (k_{25} , k_{26} , and k_{27}) to vary. The second-order rate constant that gave the best fit to the data was $5.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, in close agreement with the second-order rate constant obtained from the plot of the apparent first-order rate constants at 380 nm *versus* the O₂ concentrations as described above.

Substrate Inhibition by the Aldehyde Substrate—The maximum rate of light emission from luciferase is reduced at high concentrations of the aldehyde substrate when the assay is performed by mixing FMNH₂ with an aerobic mixture of enzyme and aldehyde. This inhibition is a consequence of the formation of an E -aldehyde complex that cannot bind FMNH₂ until the aldehyde dissociates from the enzyme. The nonenzymatic aerobic decomposition of FMNH₂ to FMN competes with the slow dissociation of decanal from the E -aldehyde complex, thereby limiting the amount of FMNH₂ available for the light producing pathway (Abu-Soud *et al.*, 1993). The extent of inhibition at a specific aldehyde concentration was greater for the longer chain aldehydes (Fig. 5). This is a direct result of the increased binding affinity ($k_{19}/$

k_{20} in Table II) of the aldehyde for the free enzyme, which increases as the aldehyde chain length increases.

Formation and Decomposition of Light-emitting Species—At a given aldehyde concentration, the maximum light intensity and the light decay rate vary from one aldehyde to the other (Fig. 3). In all cases, if the maximum light intensity is high, then the light decay is also fast, and thus the quantum yield remains nearly constant. We have found that the overall equilibrium constant for the formation of the peroxyhemiacetal intermediate (k_7k_9/k_8k_{10}) from the peroxyflavin intermediate and aldehyde slightly increases as the aldehyde chain length increases (i.e. $5.6 \times 10^4 \text{ M}^{-1}$ for heptanal to $6.3 \times 10^5 \text{ M}^{-1}$ for undecanal). The main difference among the various aldehydes in the kinetic mechanism is the rate of breakdown of the peroxyhemiacetal intermediate (k_{11}). The observed trend for this rate constant is identical to that of the initial light intensity and light decay rate (Fig. 3).

Interaction of Luciferase with Aliphatic Compounds—When mixtures of enzyme, FMNH₂, and inhibitor are combined with O₂ and the reactions monitored at 380 nm, the time courses can be fit to a the sum of two exponentials, as observed previously with the aldehyde substrates. As the concentration of the inhibitor increases, the amplitude of the slow phase also increases. This decrease in the rate of formation of the 4a-hydroperoxyflavin intermediate at high concentrations of these inhibitors is indicative of the formation of an *E'*-FMNH₂-inhibitor ternary complex. The observed rate reduction for the oxidation of the bound flavin of approximately 100-fold is similar to that observed for the binding of aldehyde. Not all of the inhibitors decreased the rate of formation of the 4a-hydroperoxyflavin intermediate by the same magnitude. From the inhibitors used in these studies, carboxylic acids were found to be less effective, whereas alcohols were found to be the most effective (Fig. 7A). When enzyme in the presence of high concentrations of these inhibitors was mixed with FMNH₂ and the reaction monitored at 380 nm the time courses were similar to that observed for the autooxidation of FMNH₂. This observation suggests the formation of an *E*-inhibitor dead-end complex, as observed previously with the aldehyde. The primary difference is that the affinity of these inhibitors for the free enzyme is much weaker than observed for the aldehyde. The equilibrium constant for the formation of the *E*-X complex was $1.7 \times 10^5 \text{ M}^{-1}$ for *n*-decanal, while it was only $3.9 \times 10^3 \text{ M}^{-1}$ for *n*-decanol and $6.7 \times 10^3 \text{ M}^{-1}$ for *n*-decylamine.

Several aldehyde analogs (amines, alcohols, carboxylic acids, boronic acids, and trifluoromethyl ketones) were found

to decrease the rate in which the 4a-hydroperoxyflavin intermediate decomposes to FMN and H₂O₂ as indicated by the change in absorbance at 445 nm. This observation is a result of the formation of *E*-FMNOOH-inhibitor ternary complexes, which decay to FMN and H₂O₂ very slowly, with rate constants depending on the specific inhibitor used. This phenomenon has been studied and described in detail by others (Ahrens *et al.*, 1991; Makemson *et al.*, 1992; Tu, 1979). The equilibrium constant for the formation of the *E*-FMNH₂-inhibitor complex was $6.1 \times 10^3 \text{ M}^{-1}$ for *n*-decanol and $3.1 \times 10^3 \text{ M}^{-1}$ for *n*-decylamine (Table III). The rate of decomposition of the ternary complex to FMN was 0.004 s^{-1} for *n*-decanol and 0.002 s^{-1} for *n*-decylamine, a rate reduction of approximately 25–50 relative to the rate observed for the *E*-FMNOOH complex.

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