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Kinetic Destabilization of the Hydroperoxy Flavin Intermediate by Site-directed Modification of the Reactive Thiol in Bacterial Luciferase*

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Bacterial luciferase catalyzes the formation of visible light, FMN, and a carboxylic acid from $FMNH_2$, O_2, and the corresponding aldehyde. The reactive cysteinyl residue at position 106 of the α subunit has been replaced by serine, alanine, and valine by site-directed mutagenesis (Baldwin, T. O., Chen, L. H., Chlumsky, L. J., Devine, J. H., and Ziegler, M. M. (1989) *J. Biolumin. Chemilumin.* 4, 40–48) and the kinetics of the reaction catalyzed by each mutant protein measured by stopped-flow spectrophotometry at pH 7 and 25 °C. The time courses for the formation and decay of the various intermediates for the three αC106 mutants have been followed by monitoring the absorbance at 380 and 445 nm and the emission of visible light using *n*-decanal as the aldehyde substrate. The time courses for these events have been incorporated into a comprehensive kinetic model; 16 individual rate constants have been obtained for this model by numeric simulations of the time courses for the wild-type enzyme and for the three αC106 mutants. The mutants catalyzed the production of visible light demonstrating that the reactive thiol is not involved in the bioluminescence reaction. All three mutants have been found to catalyze the formation of the C4a-hydroperoxy flavin intermediate with rate constants equal to that of the wild-type enzyme. These results are incompatible with those reported by Xi *et al.* who have suggested that the major pathway for the oxidation of αC106V-bound $FMNH_2$ does not involve the C4a-hydroperoxy flavin as an intermediate (Xi, L., Cho, K.-W., Herndon, M. E., and Tu, S.-C. (1990) *J. Biol. Chem.* 265, 4200–4203). The rates of decay of the C4a-hydroperoxy flavin intermediate with the mutant enzymes were found to be two orders of magnitude faster than that of the wild-type enzyme. Luciferase has been shown to be inhibited at high levels of aldehyde substrate when the enzyme is assayed by injecting $FMNH_2$ into an aerobic mixture of enzyme and aldehyde. This aldehyde inhibition has been shown to occur by the formation of a dead-end enzyme-aldehyde complex which blocks the binding of $FMNH_2$ to the enzyme; loss of activity is due to the rapid nonenzymatic decomposition of the reduced flavin with molecular oxygen.

Bacterial luciferase is a heterodimeric enzyme consisting of homologous but nonidentical subunits α and β (Friedland and Hastings, 1967). The enzyme catalyzes the bioluminescent reaction of $FMNH_2$,[1] O_2, and an aliphatic aldehyde to yield FMN, the corresponding carboxylic acid, H_20, and blue-green light (Ziegler and Baldwin, 1981; Baldwin and Ziegler, 1992). The reaction mechanism (see Scheme I) is thought to proceed via the binding of the reduced flavin ($FMNH_2$) to the protein followed by reaction with molecular oxygen to yield the hydroperoxy flavin (FMNOOH) intermediate (Hastings *et al.*, 1973; Vervoort *et al.*, 1986a, 1986b). It has been generally assumed that the aldehyde substrate reacts with the hydroperoxy flavin intermediate to form a tetrahedral adduct (FMNOOR) which eventually rearranges to the ultimate products of FMN, H_2O, and the carboxylic acid. We have previously proposed that a dioxirane intermediate is formed from this tetrahedral complex (Raushel and Baldwin, 1989) but as yet, there is no direct experimental evidence for the formation of any intermediates subsequent to the production of the hydroperoxy flavin. However, rearrangement of the dioxirane to the carboxylic acid product could result in formation of an excited state flavin hydroxide (FMNOH), and ultimately, visible light (Raushel and Baldwin, 1989).

The enzyme is usually assayed in a single turnover format by rapid (manual) injection of $FMNH_2$ into a vial containing enzyme, aldehyde, and dissolved O_2 (Hastings *et al.*, 1978). Any reduced flavin that does not bind to the enzyme is rapidly removed by the nonenzymatic reaction of $FMNH_2$ and O_2 to yield FMN and H_2O_2, such that multiple turnovers are generally not possible. In this assay format, the light production by the luciferase from *Vibrio harveyi* has been shown to be inhibited by elevated levels of the aldehyde substrate (>10 μM). No inhibition is observed if an enzyme-$FMNH_2$ complex is mixed with air-saturated solutions of aldehyde. Holzman and Baldwin (1983) have proposed that the enzyme can bind two molecules of aldehyde leading to inhibition. Baldwin *et al.* (1987) have shown that replacement of the reactive cysteinyl residue of bacterial luciferase at position 106 of the α subunit by a seryl residue resulted in an enzyme that was essentially free of aldehyde substrate inhibition up to 100 μM *n*-decanal. In terms of the model of inhibition proposed by Holzman and Baldwin (1983), the lack of aldehyde inhibition observed with the αC106S mutant suggested that the thiol might be involved in binding the inhibitory aldehyde. Consistent with this model was the finding that the luciferase

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[1] The abbreviations used are: $FMNH_2$, reduced flavin; FMNOOH, hydroperoxy flavin; FMNOOR, tetrahedral adduct; FMNOH, excited state flavin hydroxide; bis-Tris, 2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

from *V. fischeri* has a valyl reside at position α106, and the enzyme is not sensitive to aldehyde substrate inhibition (Baldwin *et al.*, 1987). However, based on the detailed kinetic analyses reported here and elsewhere (Abu-Soud *et al.*, 1992), it appears much more likely that the mechanism of inhibition by the aldehyde involves formation of a dead-end enzyme-aldehyde complex. If binding of $FMNH_2$ to the enzyme were to be blocked or inhibited by aldehyde binding, then apparent inhibition would result due to the rapid nonenzymatic decomposition of the reduced flavin by reaction with molecular oxygen. The earlier experiments did, however, conclusively demonstrate that the reactive thiol at α106 is not required for the bioluminescence reaction (Baldwin *et al.*, 1987).

We have constructed three mutants of the luciferase protein in which the highly reactive cysteinyl residue (Nicoli *et al.*, 1974) at position α106 has been replaced by serine, alanine, and valine (Baldwin *et al.*, 1989). These mutants were originally constructed to determine if the reactive thiol was in any way involved in the aldehyde substrate inhibition of the bioluminescence reaction, as described above. A recent reinvestigation by Xi *et al.* (1990), using the same mutant enzymes, confirmed the report that the chemically reactive thiol is not involved in the bioluminescent reaction, but, in addition, these authors have made the intriguing suggestion that the mutant αC106V actually resulted in a mechanistic shift from a flavin hydroxylase to a flavin oxidase. They have proposed that the hydroperoxy flavin (FMNOOH) intermediate is not formed in the enzymatic oxidation of $FMNH_2$ to FMN in the absence of aldehyde. This interpretation was based, in part, on the fact that $FMNH_2$ is converted to FMN in the presence of the α106V mutant faster than in the nonenzymatic reaction, and faster than in the presence of the wild type luciferase.

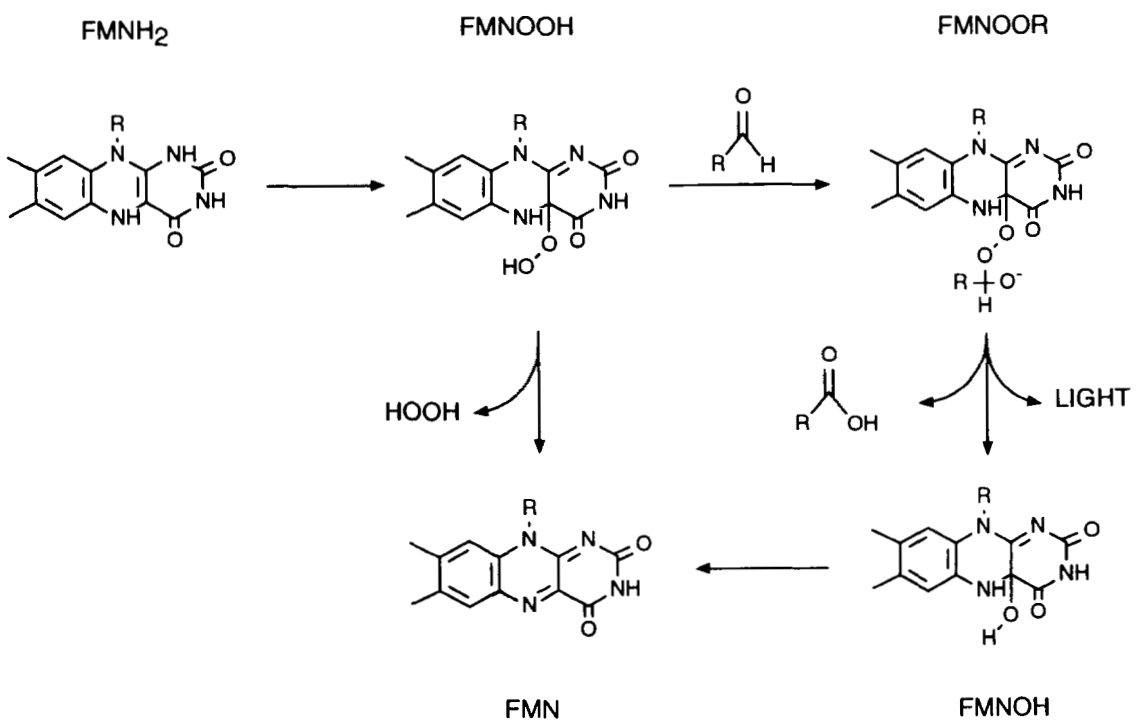
We report here a detailed kinetic analysis of the reactions catalyzed by the three luciferase mutants, αC106S, αC106A, and αC106V. These studies were undertaken initially to determine the mechanism for aldehyde substrate inhibition as outlined above. However, the intriguing suggestion that the αC106V mutant luciferase was a flavin oxidase, rather than a flavin hydroxylase, constituted an additional reason to conduct a more thorough and detailed kinetic investigation of these mutant luciferases.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Construction of the α106 mutants was performed as described (Baldwin *et al.*, 1989) using uridinylated single-strand DNA template prepared from *Escherichia coli* strains RZ1032 and CJ236 according to the method of Kunkel *et al.* (1987) with the addition of 1 mg of T4 gene 32 protein to the extension/ligation reaction. The mutations were confirmed by dideoxy chain termination sequencing (Sager *et al.*, 1977). A small region of the *lux*A coding region for each mutant was transferred into another plasmid (pLAV11; Baldwin *et al.* (1989)) to be certain that the remainder of the coding region had not been altered in the mutagenesis reactions; the sequence of the transferred DNA for each mutant was again determined by dideoxy sequencing.

Enzyme Purification—Wild-type bacterial luciferase from *V. harveyi*, as well as mutant enzymes, were purified by the method of Baldwin *et al.* (1989). The enzymes were 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 cm^{-1} 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 ± 0.2 °C. Care was taken not to expose the flavin solutions to light during the experimental procedures. The flavin (Fluka, 97%) 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 M^{-1} 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-$FMNH_2$ solutions were made by mixing the anaerobic enzyme solutions with reduced flavin under a nitrogen atmosphere and the mixture then transferred to the stopped-flow



SCHEME I.

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 wavelength was calibrated relative to the four principal peak absorption wavelengths of a didymium filter (BG 20) at 236, 358, 584, and 738 nm. The stopped-flow experiments for the three mutant enzymes were carried out under the same conditions as were used previously for the wild type enzyme (Abu-Soud *et al.*, 1992). 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, while 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 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 processed with modified forms of the KINSIM (Barshop *et al.*, 1983) and FITSIM (Zimmerle & Frieden, 1989) programs, using the comprehensive kinetic model that appears in Scheme II. Quantitation of the microscopic rate constants that appear in this kinetic model was made 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.

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

RESULTS

Mutations at Position 106 of α Subunit—Mutant proteins with the substitutions αC106S, αC106A, and αC106V were purified to homogeneity, and their catalytic properties were determined. All of the mutant proteins catalyzed the emission of visible light. When an anaerobic solution of enzyme (75 μM) and $FMNH_2$ (15 μM) was rapidly mixed with an equal volume of *n*-decanal (100 μM) containing 120 μM oxygen at pH 7.0, the emission of visible light rapidly reached a maximum and then decayed exponentially. The maximum light intensities for the mutant proteins relative to the wild protein were found to be 0.31, 0.56, and 0.022 for the serine, alanine, and valine mutants, respectively.

Formation of the E·FMNOOH Intermediate—The formation of the hydroperoxy flavin intermediate has been previously shown to be required for the emission of visible light by bacterial luciferase. This intermediate has an absorption maximum at approximately 380 nm. The absorption spectrum, obtained as a function of time, when a mixture of the wild-type luciferase and $FMNH_2$ was rapidly mixed with oxygen is shown in Fig. 1*A*. The formation of the FMNOOH intermediate was essentially complete prior to the collection of the first spectrum. The subsequent change in absorbance at 445 nm was due to the decomposition of this intermediate to FMN and H_2O_2. The three mutant proteins also catalyzed the production of the FMNOOH intermediate with the same stoichiometry as the wild type protein. The absorption spectra upon mixing the αC106V enzyme and $FMNH_2$ with O_2 is shown in Fig. 1*B*. The intermediate was completely formed within the dead-time for collection of the first spectrum, as for the wild-type enzyme, but the decay of this intermediate was significantly faster than for the wild-type enzyme as indicated by the more rapid changes in the spectra at 445 nm. Similar spectra (data not shown) were obtained with the αC106A and αC106S mutants.

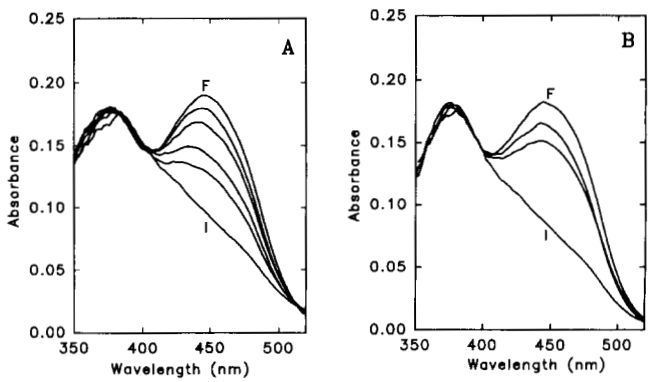


FIG. 1. **Detection of hydroperoxyflavin intermediate.** *A*, absorption spectra following the reaction of wild-type luciferase (75 μM) and $FMNH_2$ (15 μM) with air-equilibrated buffer (120 μM O_2) at 25 °C. Spectrum I was taken 7 ms after mixing. Additional spectra were taken at 2.8-s intervals for 25.8 s. Not all spectra are shown. *B*, absorption spectra following the reaction of αC106V luciferase (75 μM) and $FMNH_2$ (15 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 0.09-s intervals for 0.8 s. Not all spectra are shown.

The rate of formation of the FMNOOH complex with the wild-type protein and the three mutants was measured by mixing a complex of enzyme (75 μM) and $FMNH_2$ (15 μM) with O_2 and monitoring the change in absorbance at 380 nm. The pseudo-first-order rate constants were obtained by a fit of the data to Equation 2. The second-order rate constants for the reaction of $E \cdot FMNH_2$ with O_2 were obtained by a fit of the pseudo-first-order rates, determined as a function of the oxygen concentration (70–250 μM), to Equation 1. These second order rate constants (k_5, summarized in Table I) were essentially the same for all four proteins.

Decomposition of FMNOOH—In the absence of an aldehyde substrate, the FMNOOH intermediate has been previously shown to decay to FMN and H_2O_2 (Hastings and Balny, 1975). The rate of decomposition is variable and dependent on pH, temperature, and the presence of aldehyde analogs (Tu, 1979). The decay of the FMNOOH complex in the absence of aldehyde was monitored at 445 nm. For the wild-type, C106S, and C106V proteins, the change in absorbance at 445 nm upon mixing of enzyme and $FMNH_2$ with O_2 was fit to Equation 2. The rate constants (k_{17}) were found to be 0.10, 13, and 15 s^{-1} for the wild type, serine, and valine mutants, respectively. For the alanine mutant the data were successfully fit to Equation 3 with rate constants of 10.5 and 1.0 s^{-1}. For all three mutant proteins the rate of formation of the $E \cdot FMNOOH$ complex is the same as the wild-type protein, but the rate of decay of this complex is two orders of magnitude faster.

Formation of Luciferase-$FMNH_2$—The affinity of the three mutant enzymes for the reduced flavin substrate was determined by two complementary experiments, both involving

TABLE I

Rate constants for the kinetic model of Scheme II

pH 7.0, 25 °C. Note that k_{21} and k_{23} were determined by a fit of the time courses for the reaction of $FMNH_2$ with O_2 monitored at 380 and 445 nm to a sequential mechanism (Equation 5; Abu-Soud *et al.*, 1992). These values are 4.7 and 11.5 s^{-1}, respectively.

	Wild-type	αC106V	αC106A	αC106S
k_1	1.7×10^7 M^{-1} s^{-1}	1.0×10^7 M^{-1} s^{-1}	1.8×10^7 M^{-1} s^{-1}	1.2×10^7 M^{-1} s^{-1}
k_2	1200 s^{-1}	1400 s^{-1}	800 s^{-1}	1000 s^{-1}
k_3	200 s^{-1}	120 s^{-1}	200 s^{-1}	350 s^{-1}
k_4	14 s^{-1}	11 s^{-1}	35 s^{-1}	70 s^{-1}
k_5	2.4×10^6 M^{-1} s^{-1}	2.7×10^6 M^{-1} s^{-1}	2.3×10^6 M^{-1} s^{-1}	1.3×10^6 M^{-1} s^{-1}
k_7	1.9×10^7 M^{-1} s^{-1}	1.2×10^7 M^{-1} s^{-1}	1.0×10^7 M^{-1} s^{-1}	1.2×10^7 M^{-1} s^{-1}
k_8	120 s^{-1}	2200 s^{-1}	270 s^{-1}	480 s^{-1}
k_9	1.6 s^{-1}	13 s^{-1}	4.7 s^{-1}	3.2 s^{-1}
k_{10}	1.2 s^{-1}	1.4 s^{-1}	8.8×10^{-3} s^{-1}	0.01 s^{-1}
k_{11}	1.1 s^{-1}	0.02 s^{-1}	0.35 s^{-1}	0.68 s^{-1}
k_{13}	0.60 s^{-1}		0.48 s^{-1}	1.8 s^{-1}
k_{15}	3.0×10^3 M^{-1} s^{-1}		3.4×10^3 M^{-1} s^{-1}	9.4×10^3 M^{-1} s^{-1}
k_{16}	0.06 s^{-1}		0.18 s^{-1}	3.3 s^{-1}
k_{17}	0.10 s^{-1}	15 s^{-1}	4.7 s^{-1}[a]	13.0 s^{-1}
k_{19}	9.1×10^5 M^{-1} s^{-1}	1.0×10^6 M^{-1} s^{-1}	2.4×10^6 M^{-1} s^{-1}	1.8×10^6 M^{-1} s^{-1}
k_{20}	5.8 s^{-1}	6.6 s^{-1}	24 s^{-1}	61 s^{-1}

[a] Decomposition of the αC106A hydroperoxy flavin intermediate occurred by a two exponential process with rate constants of 1.0 and 10.5 s^{-1}. The single exponential value given here was determined by simulation.

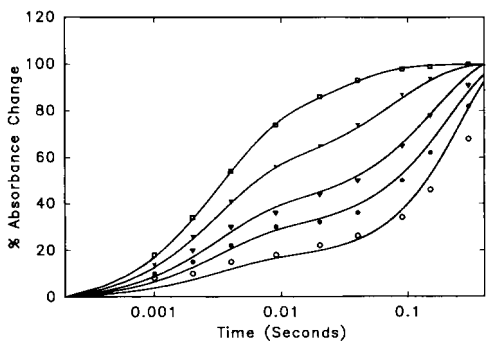
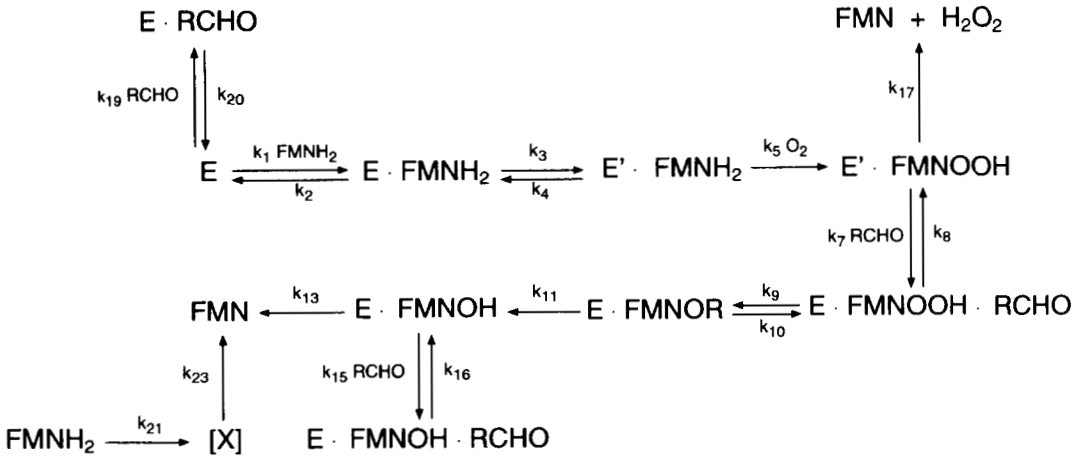


FIG. 2. **Time courses for the reaction of $E \cdot FMNH_2$ with O_2 for the αC106V mutant when the formation of $E \cdot FMNOOH$ was monitored at 380 nm.** The experiments were conducted at a fixed concentration of $FMNH_2$ (15 μM) in the presence of variable amounts of enzyme (5 (○), 10 (●), 15 (▽), 30 (▼), and 80 μM (□)) and the samples mixed with an equal volume of air-equilibrated buffer (120 μM O_2). 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 with the model in Scheme II.

monitoring the reaction of O_2 with enzyme-bound and free $FMNH_2$. In the first experiment, $E \cdot FMNH_2$ was mixed with O_2 under conditions of increasing enzyme concentration (0–80 μM) with a fixed concentration of $FMNH_2$ (15 μM) and O_2 (120 μM). The absorbance of the flavin was monitored at 380 nm as a function of time; the results obtained with the αC106V luciferase are shown in Fig. 2. In the presence of low levels of enzyme, the time courses for the reaction were accurately fit to the sum of two exponentials (Equation 3), suggesting the existence of two flavin species, both reacting with O_2 to yield 380-nm-absorbing species (Scheme II). The fast step was similar in magnitude to that assigned to the formation of the $E \cdot FMNOOH$ intermediate, while the second phase was similar to that for the nonenzymatic autoxidation of $FMNH_2$. In the presence of higher levels of enzyme, only the fast phase was observed, and the changes in the flavin absorption were fit to a single exponential (Equation 2). For the second experiment, variable amounts of luciferase in air-equilibrated buffer were mixed with 15 μM $FMNH_2$. At low levels of enzyme, the time courses could be fit to the sum of two exponentials (Equation 3). At higher concentrations of enzyme, the amplitude of the first phase increased relative to the amplitude of the slower phase. For all proteins the fast phase occurred with a limiting rate constant of 80–90 s^{-1} at approximately 80 μM enzyme, suggesting the existence of a rate-determining isomerization (Abu-Soud *et al.*, 1992). The time courses for the reaction of air-equilibrated enzyme with $FMNH_2$, using the αC106V mutant enzyme, are shown in Fig. 3. Similar data (not shown) were obtained for the other mutants. The rate constants, k_1, k_2, k_3, and k_4 for the three mutant enzymes were estimated by the simultaneous fit of the complete set of time courses for the two experiments with the numerical simulations derived using the model which appears in Scheme II. The *solid lines* in these two figures represent the simulated time courses using the constants that appear in Table I.

Formation and Decomposition of the Light-emitting Species—The time courses for light emission were monitored when $E \cdot FMNH_2$ was mixed with increasing amounts of *n*-decanal in air-equilibrated buffer. For the mutants, the rate of light formation (intensity) reached a maximum in less than 0.5 s and then decayed exponentially over a period of time that varied from one mutant to another. The time courses for light emission during the reaction of the αC106S enzyme and $FMNH_2$ with increasing concentrations of *n*-decanal in air-equilibrated buffer are shown in Fig. 4. For the alanine (data not shown) and serine mutant proteins, the rate of decay for light emission was found to be relatively insensitive to the concentration of aldehyde. At low aldehyde concentrations, the luminescence decay rate constants were 0.40 and 0.60 s^{-1} for the alanine and serine mutants, respectively, while at saturating *n*-decanal concentration, the rate constants were 0.29 and 0.65 s^{-1}, respectively. The valine mutation, however, was different; the decay rate for light emission decreased substantially at elevated levels of *n*-decanal. In the presence of low levels of *n*-decanal (10 μM), the rate constant for light decay was 1.9 s^{-1}, while at saturating *n*-decanal (500 μM), the rate constant was 0.6 s^{-1}.

Formation of Luciferase-Decanal Complex—We have previously described the construction of a comprehensive kinetic model for the wild-type bacterial luciferase reaction (Scheme II) with a single self-consistent set of rate constants that accounts for nearly all of the possible combinations of enzyme and substrates except for the substrate inhibition induced by high levels of aldehyde (Abu-Soud *et al.*, 1992). The effect of



SCHEME II.

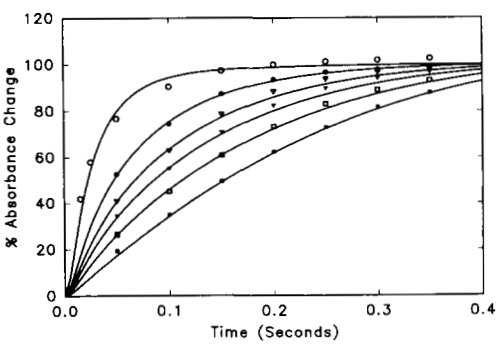


FIG. 3. **Time courses for the reaction of air-equilibrated αC106V luciferase with $FMNH_2$ when the reaction was monitored at 380 nm.** The experiments were initiated by mixing various amounts of enzyme (5 (■), 10 (□), 15 (▼), 20 (▽), 30 (●), and 80 μM (○)) in air-equilibrated buffer (120 μM O_2) with a fixed concentration of $FMNH_2$ (15 μM). The volumes of the two solutions were equal. The *symbols* represent the experimental time courses, and the *solid lines* represent the simulated time courses using the rate constants that appear in Table I with the model in Scheme II.

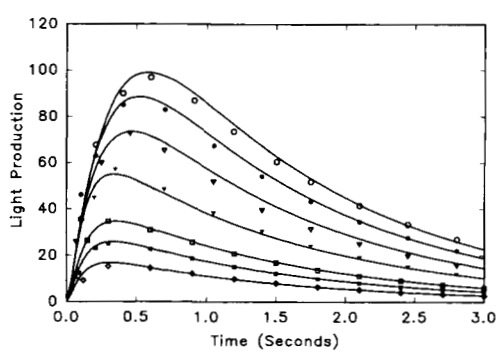


FIG. 4. **Time courses for light emission after mixing a solution of αC106S luciferase (75 μM) and $FMNH_2$ (15 μM) with various amounts of *n*-decanal ((40 (◇), 60 (■), 80 (□) 120 (▼), 200 (▽), 300 (●), and 500 μM (○)) in air-equilibrated buffer.** The *symbols* represent the experimental data, while the *solid lines* represent the simulations when the constants that appear in Table I are used with the model in Scheme II.

aldehyde concentration on the activity of the wild-type luciferase was examined by following the changes in optical density at both 380 and 445 nm. The production of light was also measured for the reaction of air-equilibrated enzyme (75 μM) in the presence of increasing amounts of *n*-decanal ranging from 0 to 500 μM with a fixed concentration of $FMNH_2$ (15

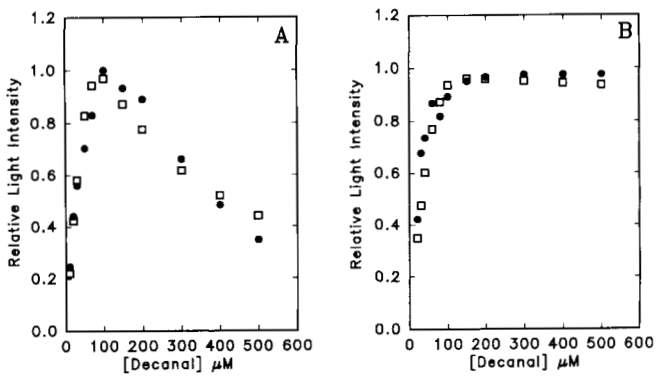


FIG. 5. **Activity profiles for wild-type luciferase.** *A*, actual (●) and simulated (□) maximum light intensities for the reaction of wild-type enzyme and various amounts of *n*-decanal in air-equilibrated buffer with $FMNH_2$. The final concentrations of enzyme and $FMNH_2$ were 75 and 15 μM, respectively. *B*, actual (●) and simulated (□) maximum light intensities for the reaction of the wild-type enzyme (75 μM) and $FMNH_2$ (15 μM) with various amounts of *n*-decanal in air-equilibrated buffer.

μM). The plot of the maximum light intensity (I_{max}) produced during the course of the reaction *versus* the initial *n*-decanal concentration is shown in Fig. 5*A*. At high concentrations of aldehyde a reduction in the level of luciferase activity was observed, similar to the behavior observed by others (Holzman and Baldwin, 1983). No inhibition of the luciferase catalyzed activity was observed when an enzyme-$FMNH_2$ complex was mixed with an air-saturated solution of aldehyde, consistent with the earlier observations. The maximum light intensity obtained for the reaction of $E \cdot FMNH_2$ with *n*-decanal in air-equilibrated buffer *versus* the *n*-decanal concentration is shown in Fig. 5*B*. At 380 and 445 nm, in the presence of low levels of *n*-decanal, the time courses were very similar to those observed upon mixing of air-equilibrated luciferase with $FMNH_2$. In the presence of high levels of *n*-decanal the reaction time courses were very similar to those observed for the nonenzymatic autoxidation of $FMNH_2$ in the presence of O_2 (Fig. 6, *A* and *B*). That is, the effect of increasing concentrations of *n*-decanal appeared to be to decrease the formation of the hydroperoxy flavin intermediate and to accelerate the rate of formation of FMN. These results are consistent with the formation of a dead-end luciferase-decanal complex that must dissociate before the productive $E \cdot FMNH_2$ complex can form. The inhibition thus results from the rapid nonenzymatic decomposition of the unbound $FMNH_2$ in the presence of O_2.

The affinity of the three mutant proteins for the aldehyde

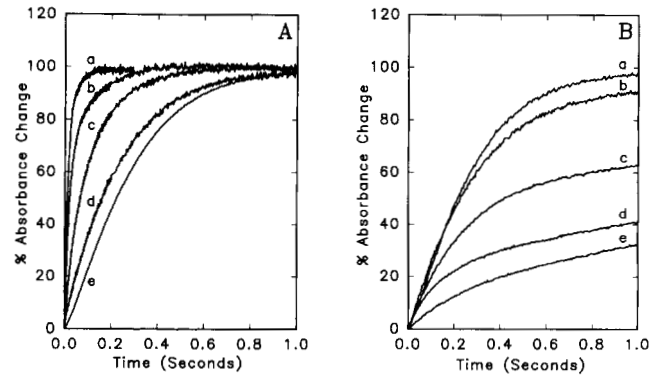


FIG. 6. **Time courses for the reaction of air equilibrated wild-type luciferase and *n*-decanal with $FMNH_2$.** *A*, reaction monitored at 380 nm. The experiments were initiated by mixing wild-type luciferase (75 μM) and various amounts of *n*-decanal ((*a*) 20, (*b*) 150, (*c*) 300, and (*d*) 400 μM) in air-equilibrated buffer (120 μM O_2) with a fixed concentration of $FMNH_2$ (15 μM). Curve *e* is the time course for the reaction of air-equilibrated buffer with $FMNH_2$ (15 μM). *B*, reaction monitored at 445 nm. The experiments were initiated by mixing luciferase (75 μM) and various amounts of *n*-decanal ((*b*) 400, (*c*) 300, (*d*) 200, and (*e*) 150 μM) in air-equilibrated buffer (120 μM O_2) with a fixed concentration of $FMNH_2$ (15 μM). Curve *a* is the time course for the reaction of air-equilibrated buffer with $FMNH_2$ (15 μM) in the absence of enzyme.

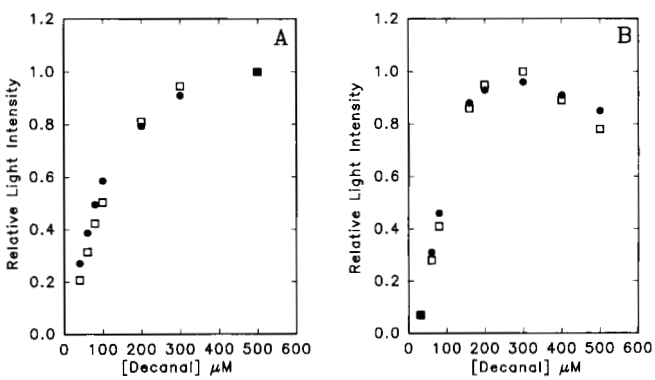


FIG. 7. **Activity profiles for the αC106S and αC106V mutants.** *A*, actual (●) and simulated (□) maximum light intensity for the reaction of αC106S enzyme and various amounts of *n*-decanal in air-equilibrated buffer with $FMNH_2$. *B*, actual (●) and simulated (□) light peak for the reaction of C106A enzyme and various amounts of *n*-decanal in air-equilibrated buffer with $FMNH_2$. The final concentration of enzyme and $FMNH_2$ for both experiments was 75 and 15 μM, respectively.

substrate was also studied by following light emission upon mixing of air-equilibrated luciferase-decanal with $FMNH_2$. The experiments were performed as described above for the wild-type enzyme and were conducted over an *n*-decanal concentration range of 0–500 μM. At high levels of aldehyde both the αC106A and αC106V enzymes were inhibited, but the concentrations required for inhibition were higher than for the wild-type enzyme. The αC106S enzyme showed very low aldehyde inhibition over an *n*-decanal concentration range of 0–500 μM (Fig. 7*A*; Baldwin *et al.*, 1987, 1989). For αC106A (Fig. 7*B*) and αC106V (data not shown), the I_{max} levels were maximal at 200 μM *n*-decanal.

The rate constants associated with the binding of aldehyde with luciferase (k_{19} and k_{20}) and the rate constants for the process associated with binding of aldehyde to $E \cdot FMNOOH$ through the formation of $E \cdot FMNOH$ (k_7–k_{11}) were estimated by monitoring the emission of visible light that followed mixing of either various amounts of air-equilibrated *n*-decanal with $E \cdot FMNH_2$ or air-equilibrated *E*-decanal with $FMNH_2$. The rate constants k_{19}, k_{20}, and k_7 through k_{11} were estimated by the simultaneous simulation of the time courses for the emission of visible light for both experiments to the model which appears in Scheme II. The *solid lines* displayed in Fig. 4 are the simulated time courses and indicate the general agreement between the calculated and experimental time courses using the rate constants that appear in Table I. The *solid circles* in Figs. 5 and 7 are the calculated light intensities for the wild-type and mutant proteins.

Formation of FMN—The formation of FMN was determined by monitoring the flavin absorbance at 445 nm for the reaction of $E \cdot FMNH_2$ with various levels of *n*-decanal in air-equilibrated buffer. Shown in Fig. 8 are the time courses for the reaction of αC106S-$FMNH_2$ with various amounts of air-equilibrated *n*-decanal. For the alanine and serine mutations, the rate of formation of FMN was fit to the sum of two exponentials (data not shown). The amplitude of the second phase increased relative to the amplitude of the first phase at higher levels of *n*-decanal. For the valine mutation at low levels of *n*-decanal (<100 μM), the rate of formation of FMN was fit to a single exponential with first-order rate constant of 10 s^{-1}. At higher concentrations of decanal, the time courses were fit to the sum of two exponentials with rate constants of 6.83 and 0.40 s^{-1}, respectively. The ratio of the amplitudes for the first phase relative to the second phase is 1:2 at 500 μM decanal.

Estimation for the dissociation and association rate constants of the $E \cdot FMNOH$ complex with decanal (k_{15}, k_{16}) and the rate constant for the dehydration of $E \cdot FMNOH$ to FMN (k_{17}) 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 of $E \cdot FMNH_2$ and air-equilibrated *n*-decanal. The simulated time courses for the change in absorbance at 445 nm using the serine mutant are illustrated as the *solid lines* in Fig. 8.

DISCUSSION

Mutation of Reactive Cysteinyl Residue—The experiments presented in this paper provide direct evidence that substitution of the cysteinyl residue at position 106 of the α-subunit with a serine, alanine, or valine resulted in proteins that have significant catalytic activity, indicating that the chemically reactive thiol at position 106 of the α-subunit is not directly involved in the bioluminescence reaction. The valine mutant

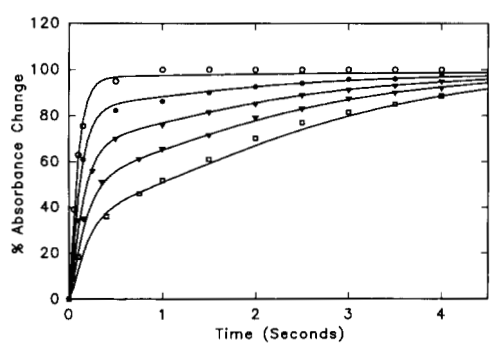


FIG. 8. **Time courses for the formation of FMN for the αC106S mutant when $E \cdot FMNH_2$ is mixed with air-equilibrated *n*-decanal and the reaction monitored at 445 nm.** The experiments were conducted at a fixed concentrations of enzyme (75 μM) and $FMNH_2$ (15 μM) with various amounts of *n*-decanal (10 (○), 60 (●), 120 (▽), 200 (▼), 400 μM (□)) in air-equilibrated buffer (120 μM O_2). The *symbols* represent the experimental data, while the *solid lines* represent the simulations using the model that appears in Scheme II with rate constants presented in Table I.

enzyme has suffered the most significant reduction in bioluminescence activity, but the other two mutant proteins emit one-third to one-half of the maximum light intensity produced by the wild-type protein. It is interesting to note that position α106 in the luciferases from *V. fischeri*, *P. leiognathi*, and *P. phosphoreum* is occupied by valine, and that the amino acid sequence in this region of the α subunit is one of the most highly conserved regions of the luciferase subunits (Baldwin and Ziegler (1992)).

Formation of Hydroperoxyflavin Intermediate—A recent investigation by Xi *et al.* (1990) using the mutant αC106V concluded that the hydroperoxy flavin (FMNOOH) intermediate is not involved in the enzymatic oxidation of $FMNH_2$ to FMN that is known to occur with the wild-type protein in the absence of an aldehyde substrate. If this mechanism were correct, then the rates of change of absorbance at 380 nm, following the mixing of $E \cdot FMNH_2$ with O_2, should be similar to the rate measured at 445 nm for the same experiment, as is the case for the nonenzymatic formation of FMN and H_2O_2 from $FMNH_2$ in the presence of molecular oxygen. This is not the case; the data presented in this work are clearly not consistent with the mechanism proposed by Xi *et al.* (1990). The change in the visible absorption spectra following the mixing of $E \cdot FMNH_2$ with air-equilibrated buffer for all four proteins clearly indicates the rapid formation of the $E \cdot$ FMNOOH complex. These intermediates are nearly completely formed within the dead-time for the collection of the first spectrum (Fig. 1, *A* and *B*). The subsequent changes in the absorbance at 445 nm, following the formation of the $E \cdot$ FMNOOH complex, are due to the decomposition of the 4a-hydroperoxy flavin intermediate to FMN and H_2O_2. All three mutant enzymes used in this study clearly catalyze, in high yield, the formation of the $E \cdot FMNOOH$ intermediate, which then decays to FMN and H_2O_2. The second order rate constants (k_5) for the formation of the $E \cdot FMNOOH$ complex with these three mutant proteins, obtained by monitoring the reaction at 380 nm after mixing $E \cdot FMNH_2$ with various amounts of O_2, are very close to the value obtained for the wild-type enzyme. However, the $E \cdot FMNOOH$ intermediates for the three mutant enzyme complexes are extremely unstable when compared to the wild-type enzyme. The first-order rate constants (k_{17}) for the formation of FMN and H_2O_2 from the decomposition of the $E \cdot FMNOOH$ complexes are two orders of magnitude faster with the three mutant proteins than the value observed for the wild-type enzyme. It thus appears that the cysteine at position 106 of the α-subunit of luciferase is critical for stabilization of the hydroperoxy flavin intermediate.

Substrate Inhibition by Excess Aldehyde—The initial maximum light emission from luciferase is reduced at high concentrations of aldehyde substrate when the assay is performed by rapid injection of $FMNH_2$ into an air-equilibrated mixture of enzyme and aldehyde. Plots of the peak light intensity (I_{max}) *versus* the initial *n*-decanal concentration are shown in Fig. 5. To gain a better understanding of the mechanism of substrate inhibition by *n*-decanal, the reaction of E-O_2-decanal with $FMNH_2$ was monitored by following the increase in the absorbance at both 380 and 445 nm. At low levels of aldehyde, the time courses were very similar to those exhibited upon mixing E-O_2 with $FMNH_2$. At high levels of decanal (~500 μM), the time courses were very similar to the nonenzymatic autoxidation of $FMNH_2$. The most plausible explanation for this inhibitory process is that the E-decanal complex cannot bind $FMNH_2$ until the decanal dissociates from the enzyme. In the presence of low levels of decanal, most of the enzyme is free and can thus bind $FMNH_2$ and subsequently form the final products. In the presence of high concentrations of decanal, the apparently slow dissociation of decanal from the E-decanal complex competes with the nonenzymatic aerobic decomposition of $FMNH_2$ to FMN and H_2O_2. Holzman and Baldwin (1983) have previously demonstrated that the inhibition of luciferase activity with aldehyde substrate was fully reversible upon dilution of the inhibited enzyme. They therefore concluded that the inhibitory effects of the aldehyde did not result from an irreversible denaturation of luciferase. Holzman and Baldwin also showed that the stoichiometry of aldehyde to luciferase was 1:1 using the method of continuous variation, but they proposed that the substrate inhibition required two molecules of aldehyde to luciferase to form an enzymatically inactive complex. The data presented here are consistent with a dead-end E-decanal complex that must dissociate before the productive $E \cdot FMNH_2$ complex can form. The inhibition thus results from the rapid nonenzymatic decomposition of the unbound $FMNH_2$ in the presence of O_2. The luciferase enzyme is insensitive to aldehyde inhibition when the $E \cdot FMNH_2$ complex is mixed with increasing amounts of *n*-decanal or when an $E \cdot FMNH_2$-decanal complex is mixed with air-equilibrated buffer. The alanine and valine mutants were less sensitive to decanal inhibition than was the wild-type enzyme. The serine mutant showed the least sensitivity to aldehyde inhibition over the decanal concentration range of 0–500 μM (Fig. 7).

The aldehyde concentration range that causes inhibition of the wild-type enzyme reported here is significantly higher than the inhibitory concentration range reported earlier (Holzman and Baldwin, 1983). Plots of initial maximum light intensity *versus* aldehyde concentration in a normal $FMNH_2$ injection assay show maximal bioluminescence activity at about 10 μM *n*-decanal, while in these studies, the maximal intensity required about 100 μM *n*-decanal. The apparent discrepancy is due to the high concentration of enzyme used in these studies. In the presence of 75 μM luciferase, aldehyde inhibition was not apparent until concentrations above 75 μM (see Fig. 5).

Calculation of Microscopic Rate Constants—The microscopic rate constants for the kinetic model that appears in Scheme II for the three mutant enzymes and the wild-type protein were obtained by direct correlation of the experimental time courses for intermediate and product formation with the calculated time courses. To determine the rate constants for the formation of the $E \cdot FMNH_2$ complex (k_1, k_2, k_3, and k_4), it was necessary to simultaneously fit the complete set of time courses at 380 nm for the experiments where $E \cdot FMNH_2$ was mixed with O_2 and also where E-O_2 was mixed with $FMNH_2$ using the model which appears in Scheme II. The second-order rate constant for the formation of the $E \cdot$ FMNOOH complexes (k_5) was held constant during the simulation. The rate constants for the autoxidation of $FMNH_2$ in the presence of O_2 were obtained as previously described (Abu-Soud *et al.*, 1992).

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 $E \cdot$ FMNOOH complex through the chemical formation of $E \cdot$ FMNOH (k_7–k_{11}) were established by obtaining the time courses for the emission of visible light that followed the mixing of air-equilibrated *n*-decanal with $E \cdot FMNH_2$ and also air-equilibrated E-decanal with $FMNH_2$. These rate constants were estimated by the simultaneous fit of the time courses for the emission of light from both types of experiments to the model that appears in Scheme II. The rate constant (k_{17}) for the decomposition of the $E \cdot FMNOOH$ complex to FMN and

H_2O_2 was obtained by monitoring the formation of FMN at 445 nm in the absence of *n*-decanal. To estimate the association and dissociation rate constants of the $E \cdot$FMNOH complex with *n*-decanal (k_{15} and k_{16}), and the rate constant for the final dehydration of $E \cdot$FMNOH complex to FMN (k_{13}), the time courses at 445 nm for the final formation of FMN (obtained by mixing the $E \cdot FMNH_2$ complex with air-equilibrated *n*-decanal solutions) were also simultaneously simulated with the time courses for the emission of visible light. All of the simulated time courses for the mutant and the wild-type enzymes were generated from the self-consistent set of rate constants listed in Table I. The standard error for each individual rate constant has been estimated to be less than 15% using the FITSIM program.

The rate constants for the association of decanal to the three mutants and the wild-type enzyme are nearly the same, but the dissociation rate constants for the *E*-decanal complex for the serine mutant is 10-fold faster than for the wild-type enzyme, and 5-fold faster for the alanine mutant. The dissociation rate constant of the *E*-decanal complex is about the same for the wild-type and valine mutant enzymes. This explains why the serine mutant is less sensitive to inhibition by decanal. The dissociation of decanal from the $E \cdot$FMNOH-decanal complex to produce $E \cdot$FMNOH is approximately 50 times faster with the serine mutant than with the wild-type protein. For the alanine mutant, the rate constants for the formation and dissociation of the $E \cdot$FMNOH-decanal complex are very similar to the wild-type enzyme. Data were not available with the valine mutant to estimate the rate constants k_{19}, k_{20}, and k_{21}, because very little of the tetrahedral adduct is converted to product via a light-emitting pathway. When a complex of αC106V $E \cdot FMNH_2$ is rapidly mixed with increasing amounts of decanal in air-equilibrated buffer, the increase in FMN absorbance (monitored at 445 nm) at low concentrations of aldehyde was very similar to that observed upon mixing $E \cdot FMNH_2$ with O_2. In the presence of high concentrations of decanal, the change in the flavin absorbance at 445 nm was fit to the sum of two exponentials (Equation 3) with rate constants of 8 and 0.4 s^{-1}. The fast phase is similar to that observed for the formation of FMN and H_2O_2 from the decomposition of $E \cdot$FMNOOH complex, while the second phase is attributed to the decomposition of the $E \cdot$FMNOH-decanal complex. The dissociation rate constant for the $E \cdot$FMNOOH-decanal complex to produce $E \cdot$FMNOOH and decanal for the valine mutant is 18-fold faster than for the wild-type protein. In addition, the rate of formation of the light-emitting species (k_{11}) is 55-fold slower than observed for the wild-type enzyme. These observations suggest that, for the valine mutant, unlike the serine and alanine mutants and the wild-type enzyme, the rate of decay of bioluminescence under conditions of saturating aldehyde (0.6 s^{-1}) is dominated by the back-reactions (k_{10}, k_8, and k_{17}) leading to FMN and H_2O_2, rather than the forward reaction leading to the light-emitting species. The data presented above suggest that the loss in activity for the valine mutant is due to a combination of instability of both the $E \cdot$FMNOOH and the $E \cdot$FMNOOH-decanal intermediates and the very slow formation of the $E \cdot$FMNOH complex from the putative tetrahedral intermediate.

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