KINETIC MECHANISM OF THE BACTERIAL LUCIFERASE REACTION

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INTRODUCTION

We have undertaken a detailed, multidimensional investigation of the kinetic mechanism of the bacterial luciferase-catalyzed reaction (1-3). Luciferase is a heterodimeric enzyme with a single active center on the α subunit. While the individual subunits exhibit low but authentic bioluminescence activity (4, 5), the active form of the enzyme is the heterodimer. The β subunit is required for the high quantum yield reaction, but its precise function is unknown (6).

Light emission from the enzyme involves reaction of $FMNH_2$, an aliphatic aldehyde and O_2 on the surface of the enzyme to yield an excited state flavin and the carboxylic acid (6). One atom of the oxygen is found in the product carboxylate (7). It is assumed that the other atom from molecular oxygen is converted to water. FMN is the flavin product that is released following bioluminescence (8). It is known that the reaction proceeds through the intermediacy of the C4a-peroxydihydroflavin (9, 10) which can be distinguished from $FMNH_2$ by the characteristic absorbance at 380 nm (10). The formation of FMN can be monitored by absorbance at 445 nm. Bioluminescence resulting from formation of the excited flavin species can likewise be monitored. The lifetimes of singlet excited states are typically in the nanosecond range so that the intensity of light emission at any time is proportional to the rate of formation of the excited state. It has been proposed that the emitter in the bioluminescence reaction is the C4a-hydroxyflavin (11); the FMN product is produced by dehydration of the C4a-hydroxyflavin.

Several chemical mechanisms for the reaction of $FMNH_2$, O_2 and aldehyde have been proposed (6, 12). We proposed a mechanism by which the proposed tetrahedral intermediate formed by reaction of the C4a-peroxyflavin with the aldehyde collapses to form the dioxirane and the C4a-hydroxyflavin (13; Fig. 1). The primary excited state suggested by this mechanism would be formed on the carboxylic acid product by collapse of the dioxirane. The C4a-hydroxyflavin would become excited by energy transfer from the primary excited state. In the presence of lumazine protein (14) or yellow fluorescence protein (15), the secondary emitter would likewise be excited by energy transfer.

The experiments reported here comprise a detailed investigation of the kinetic mechanism of the luciferase catalyzed reaction (1-3). All measurements were made under conditions of 25°, 50 mM Bis-Tris HCl, pH 7.0. The enzyme concentration was maintained at 75 µM for most

C4a-peroxydihydroflavin   Tetrahedral Intermediate   Pseudobase
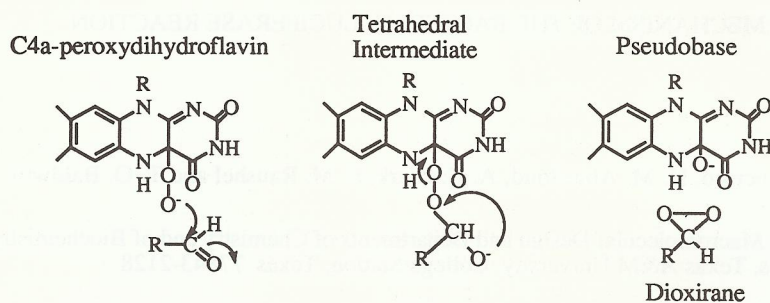
Dioxirane

Figure 1. Schematic representation of the reaction of the flavin C4a peroxide with the aldehyde substrate to yield the proposed dioxirane intermediate and the C4a hydroxyflavin from a tetrahedral intermediate (E'-FMNHOOR in Scheme I) (13).

experiments, and the $FMNH_2$, aldehyde and O_2 concentrations were varied. The highest flavin concentration used was 15 μM. Experimental data were collected with a stopped flow spectrophotometer. Rate constants were determined either by fitting of the data to a specific equation or by simulation using KINSIM (16). The enzyme used in these experiments was purified from *Escherichia coli* carrying the *luxAB* genes from *Vibrio harveyi* on a pUC-derived plasmid. From this recombinant plasmid, we have been able to isolate about 1 gram of luciferase per liter of culture (17). The high level overproduction of luciferase was essential to the completion of this project, since the complete analysis required over 75 grams of enzyme. In some experiments, mutant forms of luciferase having mutations at position 106 of the α subunit were used. These mutant luciferases, αC106A, αC106S and αC106V, have been described previously (17-19).
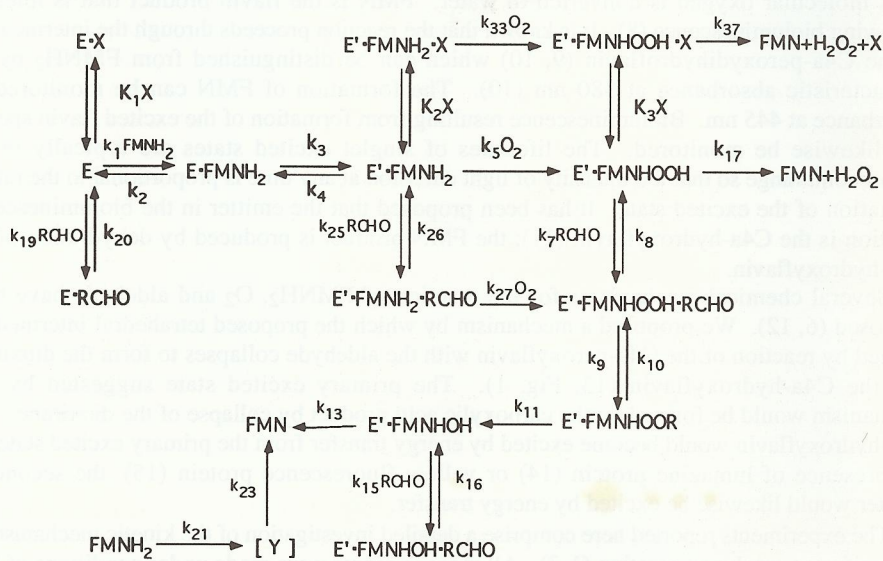
Scheme I representing the bacterial luciferase-catalyzed reaction.

RESULTS AND DISCUSSION

Three spectroscopic signals were utilized to determine the kinetic mechanism of the bacterial luciferase reaction. Absorbance measurements at 380 nm allowed determination of the formation of E-FMNHOOH (10). Emission of visible light allowed measurement of processes occurring following addition of the aldehyde substrate, and absorbance measurements at 445 nm allowed detection of FMN formation from decay of E-FMNHOOH or from dehydration of the pseudobase, E-FMNHOH (8, 11). The time courses for the various transformations were determined as a function of the concentration of $FMNH_2$, O_2, aldehyde and enzyme. The minimal model that satisfies the complete data set is presented in Scheme I. The rate constants presented in Table I were progressively determined by fitting of the data to rate equations and by simulation of more complex reactions (1-3).

The reaction of $FMNH_2$ with O_2 to yield FMN and H_2O_2 in the absence of enzyme was monitored at 380 nm and at 445 nm. The data were fit to the sum of two consecutive first-order reactions ($A \rightarrow B \rightarrow C$) where the two rate constants are $4.7 \ s^{-1}$ and $11.5 \ s^{-1}$; the order of the two rate constants, k_{21} and k_{23}, is arbitrary.

Formation and Decay of the Peroxydihydroflavin Intermediate

The second-order rate constant (k_5) for the formation of E-FMNHOOH was determined by mixing E-$FMNH_2$ with varying concentrations of O_2. The change in absorbance at 380 nm could be fit to a single exponential. The resulting pseudo-first-order rate constants were linearly dependent on the O_2 concentration and the plot passed through the origin, indicating that the reaction is irreversible and that O_2 apparently does not bind to the enzyme prior to reaction. The slope of the linear plot gave the second-order rate constant of $2.4 \cdot 10^6 \ M^{-1}s^{-1}$.

The rate constants for formation of E-$FMNH_2$ were extracted by simulation from data obtained by mixing of either enzyme and $FMNH_2$ with O_2 or enzyme and O_2 with $FMNH_2$. The second order rate constant (k_5) for reaction of E-$FMNH_2$ with O_2 was known from the previous experiments, so it was not allowed to vary in the simulations. When increasing

Table I: Rate Constants and Equilibrium Constants for the Model in Scheme I[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[b]	$1.9 \times 10^7 \ M^{-1} s^{-1}$
k_8[b]	$120 \ s^{-1}$
k_9[b]	$1.6 \ s^{-1}$
k_{10}[b]	$1.2 \ s^{-1}$
k_{11}[b]	$1.1 \ s^{-1}$
k_{13}	$0.60 \ s^{-1}$
k_{15}[b]	$3.0 \times 10^3 \ M^{-1} s^{-1}$
k_{16}[b]	$0.06 \ s^{-1}$
k_{17}	$0.10 \ s^{-1}$
k_{19}[b]	$9.1 \times 10^5 \ M^{-1} s^{-1}$
k_{20}[b]	$5.8 \ s^{-1}$
k_{21}	$4.7 \ s^{-1}$
k_{23}	$11.5 \ s^{-1}$
k_{25}[b]	$1.2 \times 10^6 \ M^{-1} s^{-1}$
k_{26}[b]	$37 \ s^{-1}$
k_{27}[b]	$5.1 \times 10^4 \ M^{-1} s^{-1}$
k_{33}[c]	$7.7 \times 10^4 \ M^{-1} s^{-1}$
k_{37}[c]	$0.004 \ s^{-1}$
K_1[d]	$3.9 \times 10^3 \ M^{-1}$
K_2[d]	$6.1 \times 10^3 \ M^{-1}$
K_3[d]	$3.5 \times 10^4 \ M^{-1}$

[a] Determined at pH 7.0, 25°C.
[b] Determined with *n*-decanal.
[c] Determined with *n*-decanol.
[d] Equilibrium constants determined with *n*-decanol.

concentrations of air-equilibrated enzyme were mixed with $FMNH_2$, the rate constant for formation of E-FMNHOOH reached a limiting value of about 85 s^{-1}, significantly below that observed when $E\text{-}FMNH_2$ was mixed directly with O_2. These results demonstrate that the initial complex of $E\text{-}FMNH_2$ does not react directly with O_2 until after a unimolecular reaction occurs yielding $E'\text{-}FMNH_2$. The rate constants k_1, k_2, k_3, and k_4 presented in Table I allowed the best simulation of the experimental data with the value of k_5 fixed at $2.4 \bullet 10^6$ $M^{-1}s^{-1}$.

The decay of E-FMNHOOH to yield FMN was monitored by absorbance at 445 nm. The time course following mixing of luciferase (75 μM) and $FMNH_2$ (15 μM) with O_2 (120 μM) fit a single exponential with a rate constant of 0.10 s^{-1} (k_{17} in Scheme I). The formation of E-FMNHOOH is complete within 10 ms under most experimental conditions. By comparison, the decay to yield FMN occurs on a time scale of many seconds.

Binding of Aldehyde to the Various Enzyme Species

In the presence of n-decanal, light emission is observed (Fig. 2). In the range up to about 500 μM n-decanal, increased aldehyde results in increased light emission when aldehyde and O_2 are mixed with $E\text{-}FMNH_2$. When the reagents are mixed, light emission rises rapidly to a peak and then decays exponentially over a period of several seconds. The decay rate is strongly dependent on the chainlength of the aldehyde and the source of the enzyme (6, 12).

Figure 2. Effect of concentration of n-decanal on the peak light emission from reactions initiated by mixing $E\text{-}FMNH_2$ with aldehyde and O_2. The concentrations were 30 μM ($\bullet$), 40 μM ($\bigcirc$), 100 μM ($\square$) and 500 μM ($\blacksquare$).

When the reactions described above were carried out in the presence of n-decanal, numerous alterations were observed in the reaction time courses. When enzyme, $FMNH_2$ and aldehyde were mixed with O_2, the formation of E-FMNHOOH appeared biphasic at intermediate aldehyde concentrations, and could be fit to the sum of two exponentials; at very low concentrations of aldehyde, the time course approached that observed in the absence of aldehyde, while at very high aldehyde concentration, the rate of formation of E-FMNHOOH was again monophasic, but much slower (Fig. 3A). This observation suggested the existence of a ternary complex $E'\text{-}FMNH_2\text{-}RCHO$ that reacts more slowly with O_2 than the binary complex $E'\text{-}FMNH_2$. The equilibrium constant for the formation of the ternary complex from $E'\text{-}FMNH_2$ and the associated rate constants were determined by analysis of the formation of the 380 nm chromophore as a function of n-decanal concentration. The same experiment done at constant enzyme (75 μM), $FMNH_2$ (15 μM) and n-decanal (500 μM) with O_2 varying from 120 μM to 600 μM allowed determination of the second-order rate constant for reaction of the ternary complex with O_2. This reaction (k_{27}) appeared to be about 100-fold slower than the reaction of O_2 with the binary complex.
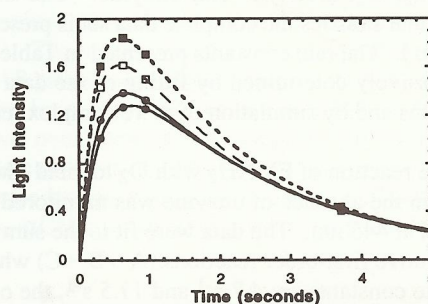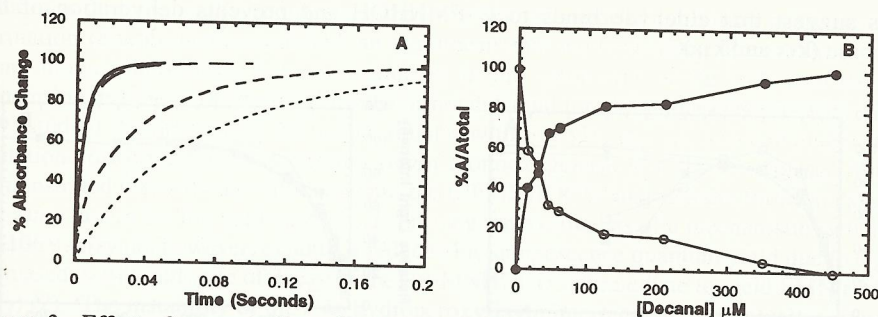
Figure 3. Effect of concentration of *n*-decanal on the time course of formation of the 380 nm chromophore. Panel A shows the % absorbance change versus time for reactions in which enzyme, $FMNH_2$ and various concentrations of aldehyde were mixed with O_2-containing buffer. The solid line represent the reaction with no aldehyde, the long dashed line is for 10 μM *n*-decanal, the intermediate dashed line is for 100 μM *n*-decanal, and the short dashed line is for 400 μM *n*-decanal. Panel B shows the effect of *n*-decanal concentration on the relative amplitudes of the fast phase (open symbols) and the slow phase (filled symbols) of the reactions depicted in Panel B.

Measurement of bioluminescence following mixing of enzyme, $FMNH_2$, O_2 and aldehyde allowed investigation of the processes from aldehyde binding through the formation of E-FMNHOH (k_7 through k_{11}). In addition, these measurements demonstrated the binding of aldehyde to the free enzyme (k_{19} and k_{20}) and confirmed the binding of aldehyde to E'-$FMNH_2$ to form the ternary complex E'-$FMNH_2$-RCHO. When E'-$FMNH_2$ was mixed with air-equilibrated aldehyde, light emission increased to a maximum about 1 s after mixing and decayed exponentially over the next 10 s. The peak intensity increased as the aldehyde concentration was increased up to about 100 μM, remaining constant thereafter. However, when enzyme, aldehyde and O_2 were mixed with $FMNH_2$, the peak light intensity decreased at aldehyde concentrations above about 100 μM. This phenomenon has been described as aldehyde inhibition (20), and is strongly dependent upon the chainlength of the aldehyde. Inhibition is virtually absent with *n*-heptanal and becomes progressively more pronounced as the aldehyde chainlength is increased. This behavior appears to be due to binding of aldehyde to the enzyme to form a binary E-RCHO complex that does not bind $FMNH_2$ (Fig. 4). The inhibition reflects the reaction of $FMNH_2$ with O_2 in solution, a competing process that consumes $FMNH_2$ that would otherwise react on the surface of the enzyme. The order of addition is therefore crucial to the process of inhibition. If E'-$FMNH_2$ is mixed with air equilibrated aldehyde, aldehyde inhibition is not observed, since O_2 reacts quickly with the flavin on the surface of the enzyme.

The formation of the product FMN following dehydration of the pseudobase, E'-FMNHOH, was detected by measurement of absorbance at 445 nm. Fixed concentrations of enzyme (75 μM) and $FMNH_2$ (15 μM) were mixed with various concentrations of air equilibrated *n*-decanal. At low concentrations of aldehyde, the formation of FMN was essentially complete after about 15 s, while in the presence of 500 μM *n*-decanal, the reaction became distinctly biphasic, with a fast phase with the same rate as that observed in low aldehyde concentrations, and a slow phase that continued to change after 50 s. These obser-

vations suggest that aldehyde binds to E'-FMNHOH and prevents dehydration of the pseudobase (k_{15} and k_{16}).
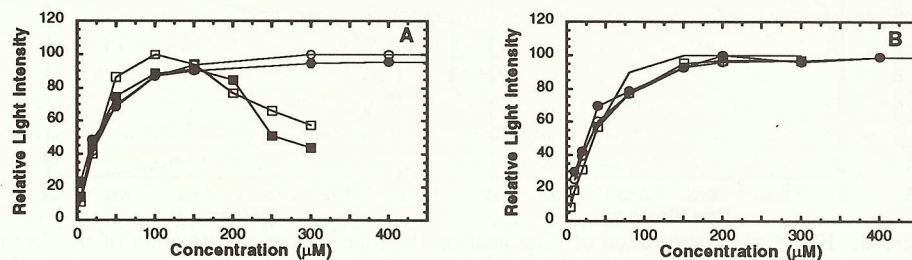
Figure 4. Effect of order of addition and aldehyde chainlength on aldehyde substrate inhibition. In Panel A, reactions were initiated by mixing enzyme, aldehyde and O_2 with $FMNH_2$. In Panel B, reactions were initiated by mixing enzyme and $FMNH_2$ with aldehyde and O_2. Circles represent the relative peak light emission with *n*-heptanal as substrate and squares represent relative peak light intensity with *n*-undecanal as substrate. The filled symbols represent the experimental data and the open symbols were calculated peak light intensities based on the rate constants given by Franscisco et al. (3).

Mode of Binding of Aliphatic Inhibitors

Luciferase is known to be inhibited by a variety of aliphatic compounds (21-23), including *n*-alkyl alcohols, carboxylic acids, amines and trifluoromethylketones. We have determined the effects of these compounds on the rate of formation of E-FMNHOOH (absorbance at 380 nm following mixing of E-$FMNH_2$ ± inhibitor with O_2) and on the rate of formation of FMN from E-FMNHOOH (absorbance at 445 nm following mixing of E-$FMNH_2$ ± inhibitor with O_2 ± inhibitor). The results of these experiments (3) demonstrate that the aliphatic inhibitors decrease the rate of reaction of O_2 with enzyme-bound $FMNH_2$ in the same manner as the aldehyde substrates, suggesting that the mode of inhibition by these compounds is similar to aldehyde substrate inhibition. Furthermore, these compounds decrease the rate with which the E-FMNHOOH intermediate decays to FMN and H_2O_2, demonstrating the existence of a ternary complex of E-FMNHOOH-inhibitor. Tu has demonstrated that *n*-decyl alcohol has a strong stabilizing influence on E-FMNHOOH and has used *n*-decyl alcohol as a buffer additive for isolation of E-FMNHOOH by column chromatography (22). Aldehyde binding to E-FMNHOH appears to stabilize the product complex and prevent or slow the dehydration reaction (k_{13}, k_{15} and k_{16}); inhibitor binding to E-FMNHOOH appears to exert a similar influence.

Effect of Mutations at α106 on the Enzyme-Catalyzed Reaction

Luciferase is known to possess an "essential" thiol (24) that resides at position α106 (25). Modification of this residue with even the very small nonpolar -SCH_3 group renders the enzyme inactive (25). By site-directed mutagenesis, we demonstrated that this thiol is not essential for activity (18). The αC106S, αC106A and αC106V variants were created and the enzymes analyzed and shown to be active in the bioluminescence reaction (17); the αC106S variant had essentially wild-type activity and appeared to be less sensitive to aldehyde substrate

inhibition than the wild-type enzyme, implying that the mode of inhibition might be through formation of a thiohemiacetal (18), an hypothesis that we have since discounted (17). The same mutant luciferases have been studied in the laboratory of Tu (19) confirming the conclusion that the α106 cysteinyl residue is not essential for bioluminescence activity. Xi et al (19) studied the reaction of the valine mutant with $FMNH_2$ and O_2 and concluded that the mutation converted luciferase from a flavin monooxygenase to a flavin oxidase. We have demonstrated that with the valine mutant, the E-FMNHOOH intermediate forms at essentially the same rate as for the wild-type (2), disproving the hypothesis of a mechanistic switch. The αC106V enzyme, however, exhibits a reduced bioluminescence quantum yield due to a greatly increased (>100 fold) rate of decay of the E-FMNHOOH intermediate to yield FMN and H_2O_2 (k_{17}) (2). The instability of the C4a-hydroperoxyflavin intermediate for the valine mutant (2) probably accounts for the results of Xi et al. (19).

CONCLUSIONS

The results of these studies (1-3) comprise a set of rate constants defining the primary reactions catalyzed by the bacterial luciferase from *Vibrio harveyi*. These rate constants were determined under a single set of well-defined experimental conditions. It is clear from the complexity of the reaction that few valid conclusions can be drawn about the effects of inhibitors, mutations, buffer conditions, etc., on the reaction without performing a detailed kinetic analysis. The discovery of an isomerization of the E-$FMNH_2$ complex to yield the O_2-reactive E'-$FMNH_2$ was unexpected, but is consistent with reports of a two step mechanism for binding of $FMNH_2$ to the enzyme of *Photobacterium phosphoreum* (27) and a conformational change that occurs in the *Vibrio harveyi* enzyme during the catalytic cycle (28).

The mechanism of aldehyde substrate inhibition appears to reside simply in the ordered binding of substrates (1-3). If enzyme and aldehyde are mixed prior to addition of $FMNH_2$, $FMNH_2$ binding cannot occur until after aldehyde release. The inhibition is due to loss of the free $FMNH_2$ to reaction with O_2 prior to binding to the enzyme. Formation of the ternary complex E-$FMNH_2$-RCHO reduces the rate of formation of E-FMNHOOH, but does not greatly reduce the bioluminescence quantum yield. Oxygen can react directly with the complex, albeit at a reduced rate, and if the aldehyde temporarily dissociates, O_2 can react with the E'-$FMNH_2$ very rapidly (1).

The rate constants shown in Table I allow simulation with high precision of the various reaction time courses that occur on the *V. harveyi* enzyme. These results should serve as a foundation for investigations into the details of the chemical mechanism of bacterial luciferase.

ACKNOWLEDGEMENTS

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