KINETIC AND MECHANISTIC INVESTIGATION OF THE BACTERIAL LUCIFERASE REACTION

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Introduction

Bacterial luciferase from *Vibrio harveyi* catalyzes the production of visible light from reduced FMN, molecular oxygen, and a long-chain aliphatic aldehyde. The other products are oxidized FMN, water, and the corresponding carboxylic acid (1). The chemical mechanism of this reaction has been previously shown to involve 4a-hydroperoxyflavin (2) and perhaps a dioxirane intermediate (3). Stopped-flow kinetic techniques have now been utilized to obtain microscopic rate constants for the interconversion of the various intermediates and enzyme complexes.

Results and Discussion

Stopped-flow techniques have been used to study the kinetics of the luciferase reaction in the absence and presence of decanal. All studies have been carried out in the dark under nitrogen atmosphere in Bis-Tris buffer, pH 7.0 at 25°C. Changes in the flavin absorption at 380 and 445 nm, in addition to monitoring the production of visible light with time, have been used in the construction of a kinetic model for the bacterial luciferase reaction. These optical probes occur on time scales ranging from <0.1 to 100 seconds during single turnover experiments. Figure 1 shows the time courses for the reaction at 380 and 445 nm after mixing [Enzyme·$FMNH_2$] with [O_2·Decanal] and the corresponding production of light production at saturating decanal. A molar ratio of 1:5 $FMNH_2$

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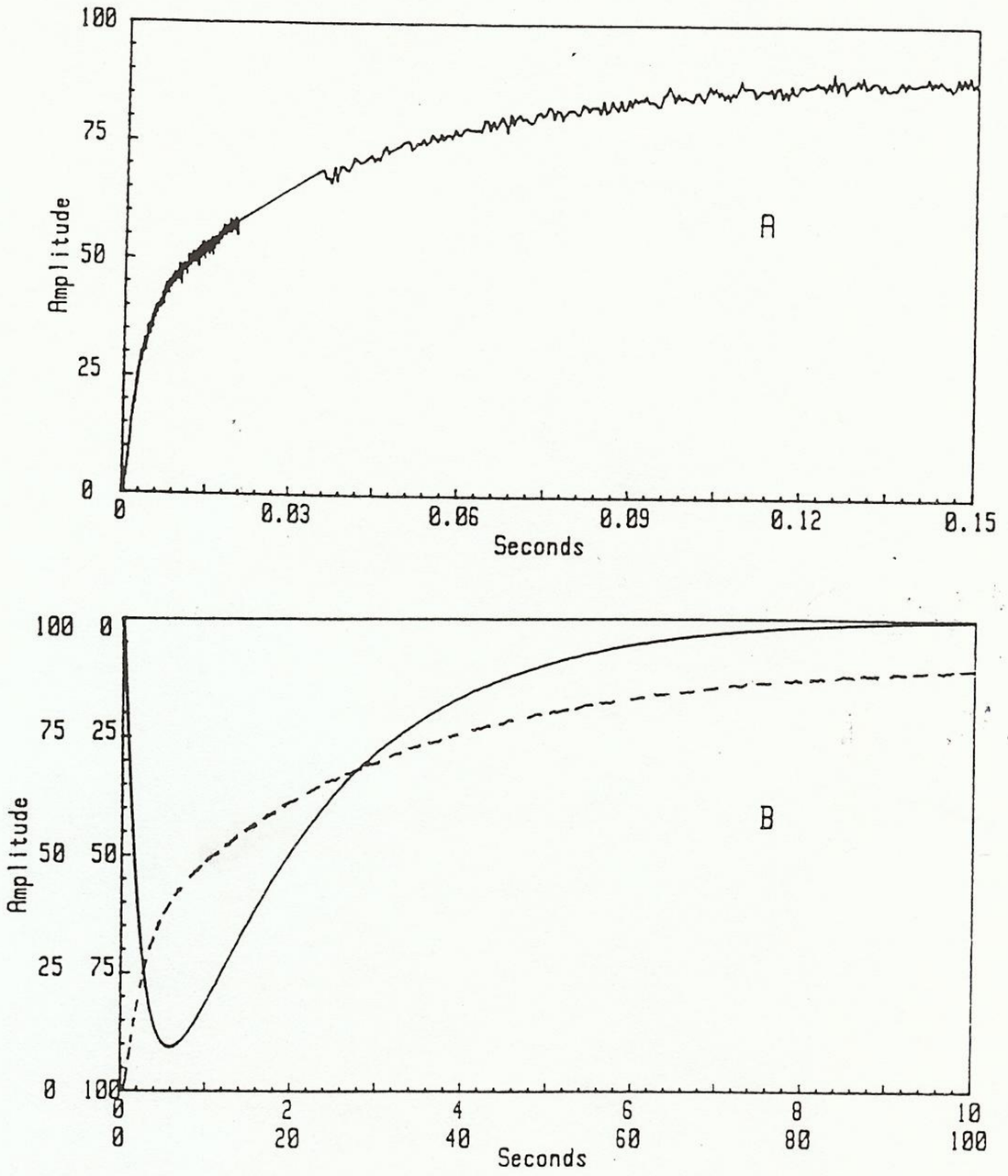


Figure 1: Time courses for changes in absorption at 380 (A) and 445 (---B, 0-100 sec) nm and the production of visible light (——B, 0-10 sec).

to enzyme was used to ensure the complete formation of the [Enzyme·$FMNH_2$] complex. The flavin was reduced by hydrogen gas in the presence of a few crystals of palladium on activated carbon. The anaerobic enzyme was prepared by several cycles of evacuation and equilibration with nitrogen.

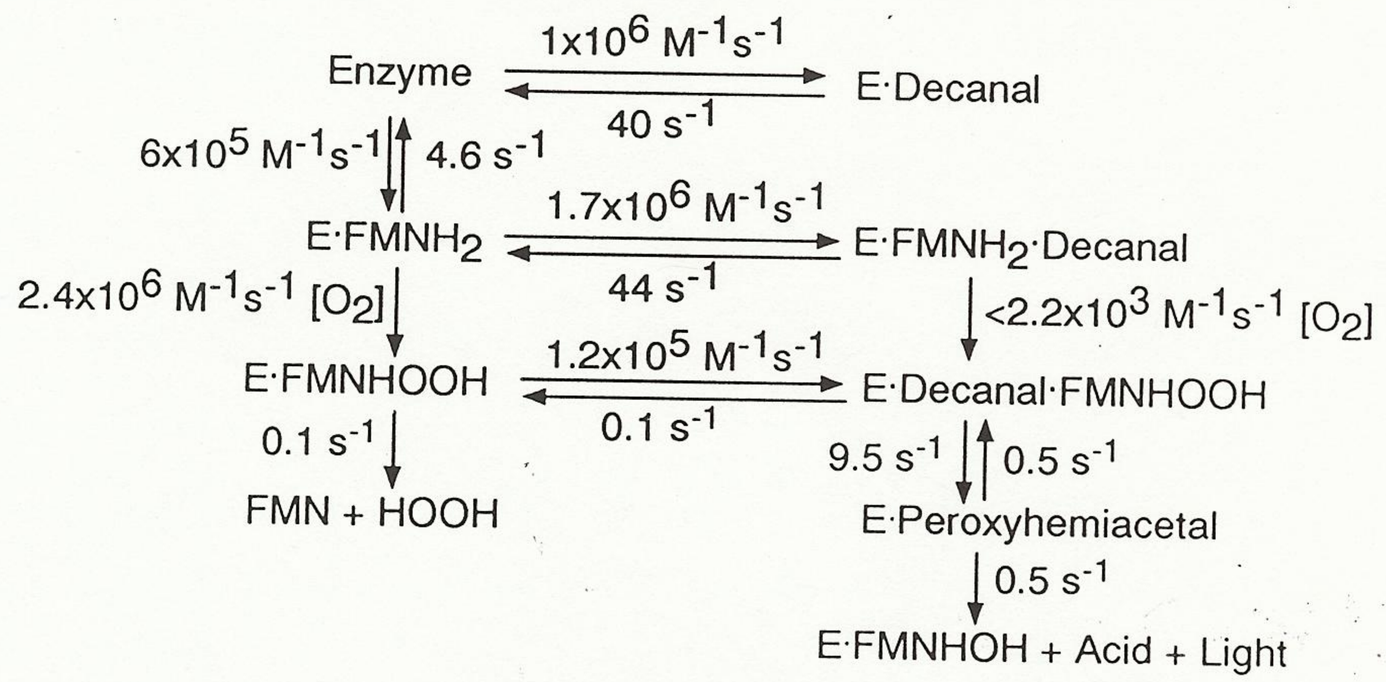
When the [Enzyme·$FMNH_2$] complex is mixed with O_2, the [Enzyme·4a-hydroperoxyflavin] complex is formed and the rate of formation is directly dependent on the O_2 concentration. The second-order rate constant is 2.4×10^6 $M^{-1}s^{-1}$. The decomposition of the enzyme-bound 4a-hydroperoxyflavin intermediate which produces enzyme, FMN, and HOOH, is not a simple process. However, it can be approximated with a first order rate constant of 0.10 s^{-1}.

When the [Enzyme·$FMNH_2$·decanal] complex is mixed with O_2 in the presence of low levels of decanal, there is a rapid formation of the enzyme bound 4a-hydroperoxyflavin intermediate similar to that previously assigned in the reaction of [Enzyme·$FMNH_2$] with O_2, followed by a slower phase (23 s^{-1}). The second phase is attributed to the dissociation of decanal from the [Enzyme·$FMNH_2$·decanal] complex. The amplitude of the first phase decreases and the amplitude of the second phase increases upon increasing the decanal concentration. In the presence of high levels of decanal the first phase disappears while the second phase predominates. In this assay the maximum light intensity produced during the course of the reaction increased upon increasing the decanal concentration and saturates at levels above 100 μM decanal.

The maximum light intensity produced during the course of the reaction is inhibited at a high concentration of decanal (>100 μM) when the [Enzyme·decanal·O_2] complex is mixed with $FMNH_2$, but not when the [Enzyme·$FMNH_2$] complex is mixed with [decanal·0_2]. These results are consistent with the formation of a dead-end enzyme·aldehyde complex which must dissociate before the productive [Enzyme·$FMNH_2$] complex can form. The inhibition thus results from the rapid nonenzymatic decomposition of the unbound $FMNH_2$ with O_2. The decomposition of $FMNH_2$ with O_2 is not a simple process. The time course for the appearance of FMN exhibits with a distinctive lag phase followed by a single exponential. The time course of this reaction at fixed oxygen concentrations can be fit to a two step consecutive reaction (A -> B -> C) with values for k_1 and k_2 of 11.5 and 4.7 s^{-1}, respectively.

The time course the production of light with decanal proceeds with a short lag phase which parallels hydroperoxyflavin formation, increases to a maximum intensity at approximately 0.5 seconds and then decays with a rate constant of 0.39 s^{-1} at saturating decanal.

The stopped-flow kinetic analysis described above provided evidence for each enzyme species and leads to the construction of the basic kinetic mechanism (Scheme 1). Based on this kinetic mechanism, the KINSIM kinetic simulation program has been used to simulate the experimental data (4). The kinetic parameters and time courses, including the relative maximum light intensity,



Scheme 1: Kinetic mechanism for luciferase reaction.

collected from this study were used as initial estimates in the simulation. These rate constants were evaluated by trial and error until the best fit between the simulation and the experimental data was obtained. One set of kinetic and relative light intensity parameters accounts for every kinetic trace in the presence of low and high levels of decanal. The simulated lines fit the raw data reasonably well indicating that the proposed kinetic mechanism accurately describes the kinetic mechanism of bacterial luciferase.

Acknowledgements

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References

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