Deuterium Kinetic Isotope Effects and the Mechanism of the Bacterial Luciferase Reaction

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ABSTRACT: A combined experimental and theoretical investigation of the deuterium isotope effects on the bacterial luciferase reaction is described. The experimental studies focus on determining if the unusual aldehyde deuterium isotope effect of ~1.5 observed in these reactions is an intrinsic isotope effect resulting from a single rate-limiting step or is a composite of multiple rate-limiting steps. The isotope effect observed is not significantly affected by variation in the aldehyde chain length, changes in the pH over a range of 6–9, use of αC106A and αC106S site-directed mutants, or chloride substitution at the 8-position of the reduced flavin, though the isotope effect is decreased when the 8-methoxy-substituted flavin is used as a substrate. From these observations it is concluded that the aldehydic isotope effect arises from the change in rate of a single kinetic step. A stopped-flow kinetic analysis of the microscopic rate constants for the reactions of 1-[1H]decanal and 1-[2H]decanal in the bacterial luciferase reaction was carried out, and aldehyde hydration isotope effects were determined. From the results it is estimated that the aldehydic isotope effect is ~1.9 after formation of an intermediate flavin C4a-hydroperoxy hemiacetal. Ab initio calculations were used to examine the transformation of the aldehyde into a carboxylic acid and to predict isotope effects for possible mechanisms. These calculations indicate that the mechanism involving rate-limiting electron transfer from the flavin C4a-hydroxide to an intermediate dioxirane is consistent with the enigmatic aldehydic isotope effect and that the intermediacy of a dioxirane is energetically plausible.

Bacterial luciferase catalyzes the production of visible light from the reaction of FMNH2, molecular oxygen, and a long-chain aliphatic aldehyde. The first step of the chemical transformation is the formation of a C4a-flavin hydroperoxide (FMNOOH) from the reaction of FMNH2 and molecular oxygen (1). The FMNOOH is a stable intermediate in the presence of enzyme and the complex can be isolated using low-temperature chromatography (2). In the absence of aldehyde, the intermediate slowly decomposes to FMN and H2O2 with little or no emission of visible light (3). In the presence of aldehyde, the hydroperoxide is thought to form a C4a-flavin hydroperoxy hemiacetal (FMNOOR) (4), although this intermediate has yet to be isolated and structurally characterized. The subsequent decomposition of the hydroperoxy hemiacetal adduct generates an excited-state intermediate which emits a photon of light in the blue region of the spectrum; there is concomitant formation of the carboxylic acid corresponding to the original aldehyde. The ultimate excited-state molecule is thought to be the C4a-flavin hydroxide (FMNOH) (5). The eventual dehydration of the FMNOH affords the final product, FMN. These general aspects of the reaction pathway have involved isotope effect determinations. In general, deuterium substitution within the aldehyde substrate of luciferase has a rather modest effect of about 1.5 on the decay rate of the emitted light. Presswood and Hastings obtained a deuterium isotope effect of 1.7 for the decay of bioluminescence using 1-[2H]decanal as a substrate (7). In an earlier investigation, Shannon et al. reported isotope effects of 1.7, 1.2, and 1.0 for the oxidation of n-decanal, n-dodecanal, and n-tetradecanl, respectively (8). More recently, Tu et al. measured a deuterium isotope effect of 1.5 when n-decanal was utilized as the substrate (9). Macheroux et al. found values of 1.4 and 1.5 for the oxidation of n-octanal and n-dodecanal, respectively (4).

A consistent mechanistic interpretation of these isotope effects has been elusive. The rearrangement of the C4a-hydroperoxy hemiacetal intermediate necessarily involves...
cleavage of the aldehydic proton from the original carbonyl carbon. However, isotope effects of about 1.5 are smaller than the normal range of 2–10 expected for an intrinsic primary isotope effect involving the cleavage of a carbon–hydrogen bond. On the other hand, 1.5 is larger than normally observed for an intrinsic secondary isotope effect. Of course, the observed isotope effect could be a composite of multiple rate-limiting steps, and this is one focus of the current investigation. A second complicating factor is that an inverse secondary deuterium isotope effect is anticipated for the reaction of the C4a-hydroperoxide intermediate with the aldehyde substrate because of the change in hybridization of the central carbon atom from sp² to sp³ upon formation of the putative tetrahedral adduct. This phenomenon has apparently been overlooked in previous investigations. Moreover, a secondary isotope effect by deuterium on the hydration of the aldehyde is also foreseen to play an important factor in the binding of the free aldehyde and/or gem-diol to the protein. Finally, the utilization of D₂O solvent is predicted to perturb the proton-transfer steps during substrate turnover.

In this paper, we describe a comprehensive experimental and theoretical investigation of the deuterium isotope effects on the bacterial luciferase reaction. The detailed kinetic mechanism previously constructed (10, 11) allows here a determination of the substrate and solvent isotope effects on the individual steps of the kinetic mechanism. At the same time, a series of different aldehyde substrates, flavin derivatives, and mutant enzymes were studied with the idea of modulating the rate-determining step(s) of the reaction and observing the intrinsic isotope effects associated with the individual steps. Finally, ab initio calculations were used to examine the transformation of the aldehyde into a carboxylic acid, and the seemingly enigmatic experimental isotope effects were interpreted with the aid of theoretical isotope effects based on the ab initio calculations. The results provide unique and detailed insight into the molecular mechanism of luciferase bioluminescence.

MATERIALS AND METHODS

**General.** Riboflavin-5’-monophosphate (FMN) was purchased from Fluka (97%) and used without further purification. Deuterated aldehydes (1-[2H]octanal, 1-[2H]decanal, and 1-[2H]dodecanal) were synthesized from the correspond-

![Figure 1: Reaction pathway for bacterial-luciferase catalyzed luminescence.](image-url)

**Figure 1:** Reaction pathway for bacterial-luciferase catalyzed luminescence.
coefficients used were 12 200 M⁻¹ cm⁻¹ at 450 nm for FMN (19), 10 100 M⁻¹ cm⁻¹ at 445 nm for 8-Cl-FMN (20), and 24 500 M⁻¹ cm⁻¹ at 445 nm for 8-CH₂O-FMN (20).

The anaerobic enzyme solutions were prepared using an all-glass vacuum system (21) 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-FMNH₂ solutions were made by mixing the anaerobic enzyme solutions with reduced flavin under a nitrogen atmosphere, and then the mixture was transferred to the stopped-flow instrument using an airtight 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. The stopped-flow experiments were carried out under the same conditions as described previously (10, 11).

Stock solutions of 0.1 M aldehyde 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-squares procedure contained in the software supplied by Hi-Tech Ltd.

\[
y = Ax + C
\]

\[
y = Ae^{-kt} + C
\]

\[
y = Ae^{-k_1t} + Be^{-k_2t} + C
\]

\[
y = A[k_1/(k_2 - k_1)](e^{-k_1t} - e^{-k_2t}) + C
\]

\[
y = A[1 + (1/(k_1 - k_2))(k_2e^{-k_1t} - k_1e^{-k_2t})] + C
\]

Equation 3 represents the sum of two independent exponentials for a parallel process and eqs 4 and 5 describe the time courses for a sequential process (X → Y → Z) monitoring the formation of Y and Z, respectively. In these equations, \(k_1\) and \(k_2\) are the first-order rate constants, \(t\) is time, and \(A\) and \(B\) are amplitude factors. 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 (22) and FITSIM (23) programs, using the comprehensive kinetic model that appears in Figure 2. The microscopic rate constants that appear in this kinetic model were estimated by comparison of the experimental time courses 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.

**Determination of Aldehyde Hydration Constant.** The equilibrium constant and rate of dehydration of \(n\)-octanal were determined enzymatically using horse liver alcohol dehydrogenase as described by Deetz et al. (24). The equilibrium constant for the hydration reaction and the rate constants for the hydration and dehydration reactions were determined for \(1\)-\([1^H]\)octanal and \(1\)-\([2^H]\)octanal in 50 mM TES buffer solutions made with either H₂O or D₂O at 25 °C, pH 7.0. The \(n\)-octanal stock solutions were prepared in acetonitrile and the hydrated solutions were obtained by equilibration following a 5-fold dilution of the aldehyde-acetonitrile solution with TES buffer.

**pH Rate Profiles.** The pH dependence of enzymatic activity and the rate of decay of light emission were studied using \(1\)-\([1^H]\)decanal and \(1\)-\([2^H]\)decanal as substrates. The activity was measured using the standard assay (25) in 25 mM Tris-maleate buffer solutions at 25 °C as a function of pH as described by Nicoli et al. (26). The pH range was from 6.0 to 9.0. The reaction was initiated by vigorous injection of 1.0 mM of a catalytically reduced 50 \(\mu\)M solution of FMNH₂ into a vial containing 1.0 mM of 25 mM Tris-maleate buffer at the desired pH containing luciferase (0.1 \(\mu\)M) and either \(1\)-\([1^H]\)decanal or \(1\)-\([2^H]\)decanal (50 \(\mu\)M). This concentration of aldehyde was found to be optimal and no aldehyde substrate inhibition was observed. The \(pK_a\) values were determined by fitting the data to one of the following equations:

\[
\log k = \log [C/(1 + h/K_a)]
\]

\[
\log k = \log [C/(1 + H/K_a + K_b/H)]
\]

where \(k\) is either the initial maximum light intensity \(I_{max}\) or the rate constant for the decay of light emission \(k_d\), \(C\) is the pH-independent value of \(I_{max}\) or \(k_d\), \(H\) is the hydrogen ion concentration, and \(K_a\) and \(K_b\) are the dissociation constants of the ionizable groups.

**Theoretical Calculations.** The fully optimized structures reported here were obtained with Gaussian 94 (27). (U)-MP2 geometry optimizations and (U)MP4(STDQ) single-point energies were performed as implemented in Gaussian 94, with core orbitals frozen. A 6-31+G** basis set was used for all calculations. Vibrational frequency analyses were carried out on all stationary points. Reduced isotopic
partition functions and isotope effects were calculated by the method of Bigeleisen and Mayer (28–30) using the program QUIVER (31), with frequencies unscaled.

**RESULTS**

**Isotope Effects on Light Production.** Plots of the relative maximum light intensity ($I_{\text{max}}$) and the decay rate for light emission ($k_d$) versus the initial aldehyde concentration when a solution of enzyme and FMNH$_2$ was mixed with air-equilibrated solutions of either 1-$[^{1}\text{H}]$decanal or 1-$[^{2}\text{H}]$decanal are shown in Figure 3, panels A and B. The maximum light intensity increased as the aldehyde concentration was elevated until it reached a maximum value at around 200 $\mu$M and then remained constant at higher concentrations of aldehyde. The first-order rate constant for the decay of light emission also exhibited a similar behavior, reaching a constant value of 0.50 s$^{-1}$ for 1-$[^{1}\text{H}]$decanal and 0.33 s$^{-1}$ for 1-$[^{2}\text{H}]$decanal at high concentrations of aldehyde. The maximum light intensity was diminished and the decay of the light emission was slower when 1-$[^{2}\text{H}]$decanal was used as the substrate. An isotope effect of 1.5 was observed for both parameters throughout the entire aldehyde concentration range. No isotope effect was observed on the quantum yield ($Q$), defined as the total quanta produced under nonturnover conditions, or simply estimated as the ratio of the maximum light intensity and the rate of light decay ($I_{\text{max}}/k_d$).

The maximum light intensity and associated decay rate were also measured after aerobic solutions of enzyme and varying concentrations of either 1-$[^{1}\text{H}]$decanal or 1-$[^{2}\text{H}]$decanal were mixed with FMNH$_2$ (Figure 3, panels C and D). In this type of assay, a reduced enzyme activity was observed at high concentrations of aldehyde. The decay rate of the emitted light exhibited the same type of behavior as in the experiment described above; the rate of light decay increased slightly as the concentration of aldehyde was raised until it obtained a constant value of 0.48 s$^{-1}$ for 1-$[^{1}\text{H}]$decanal and 0.33 s$^{-1}$ for 1-$[^{2}\text{H}]$decanal at high concentrations of aldehyde. An isotope effect of 1.5 was observed on the maximum light intensity and the decay rate of the emitted light throughout the entire aldehyde concentration range. These results are summarized in Table 1.

**Deuterium Isotope Effects as a Function of Aldehyde Chain Length.** The deuterium isotope effects on the maximum light intensity and the decay rate for light emission were also determined for $n$-octanal and $n$-dodecanal. The maximum light intensity observed with these two aldehydes was approximately 10% of that observed with $n$-decanal, and the rate of decay of the emitted light was approximately 10-fold slower. When solutions of enzyme and FMNH$_2$ were mixed with air-equilibrated solutions of either 1-$[^{1}\text{H}]$octanal or 1-$[^{2}\text{H}]$octanal, the maximum light intensity increased as the aldehyde concentration was elevated up to 200 $\mu$M and remained constant at higher concentrations of aldehyde (data not shown). The decay rate for light emission decreased slightly as the aldehyde concentration was increased and reached a limiting value of 0.050 s$^{-1}$ for 1-$[^{1}\text{H}]$octanal and 0.034 s$^{-1}$ for 1-$[^{2}\text{H}]$octanal at an aldehyde concentration of approximately 200 $\mu$M. An isotope effect of 1.5 was observed for both the maximum initial light intensity and the decay rate of light emission. No isotope effect was observed for the quantum yield. The activity profiles for the assays where $n$-dodecanal was used as a substrate were very similar to those observed with $n$-octanal, with the only difference being that lower concentrations of $n$-dodecanal were needed to reach a saturation point ($\leq 50$ $\mu$M). An isotope effect of 1.4 was observed for both the maximum light intensity and the decay rate of light emission when solutions of enzyme and FMNH$_2$ were mixed with aerobic solutions of $n$-dodecanal (data not shown). These results are also summarized in Table 1.

**Deuterium Isotope Effects for $\alpha$C106 Mutants.** The chemically reactive cysteine residue at position 106 of the $\alpha$ subunit of luciferase has been replaced with serine, alanine, and valine (11). All three mutants catalyze the emission of visible light, but the maximum light intensities and light decay rates are different from those of the wild-type enzyme. The isotope effects on the maximum light intensity and on the decay rate of the emitted light were measured for these three mutants in both assay formats using $n$-decanal as the substrate.

The relative maximum light intensity of the $\alpha$C106A mutant was approximately 60% of that of the wild-type enzyme and the decay rate was approximately 1.7-fold slower. The activity profiles for this enzyme were very similar to those of the wild-type enzyme. When a solution of enzyme and FMNH$_2$ was mixed with air-equilibrated solutions of $n$-decanal, the maximum light intensity increased as the concentration of aldehyde was increased, reaching a maximum at about 100 $\mu$M decanal (data not shown). The light decay rate decreased slightly as the aldehyde concentration was raised, reaching a limiting value of 0.30 s$^{-1}$ for 1-$[^{1}\text{H}]$decanal and 0.20 s$^{-1}$ for 1-$[^{2}\text{H}]$decanal at approximately 100 $\mu$M decanal (data not shown). The observed isotope effects for the maximum light intensity and the light decay rate were 1.7 and 1.5, respectively. The maximum

<table>
<thead>
<tr>
<th>enzyme</th>
<th>experiment</th>
<th>$^{d}I_{\text{max}}$</th>
<th>$^{d}k_d$</th>
<th>$^{d}Q$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>E-FMNH$_2$ vs O$_2$-decanal</td>
<td>1.51 ± 0.09</td>
<td>1.50 ± 0.06</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>wild-type</td>
<td>E-O$_2$-decanal vs FMNH$_2$</td>
<td>1.53 ± 0.10</td>
<td>1.45 ± 0.04</td>
<td>1.04 ± 0.07</td>
</tr>
<tr>
<td>wild-type</td>
<td>E-O$_2$-octanal vs 1-$[^{2}\text{H}]$-decanal</td>
<td>1.46 ± 0.05</td>
<td>1.47 ± 0.11</td>
<td>0.98 ± 0.09</td>
</tr>
<tr>
<td>wild-type</td>
<td>E-FMNH$_2$ vs O$_2$-dodecanal</td>
<td>1.36 ± 0.09</td>
<td>1.39 ± 0.03</td>
<td>0.98 ± 0.07</td>
</tr>
<tr>
<td>C106A</td>
<td>E-FMNH$_2$ vs O$_2$-decanal</td>
<td>1.74 ± 0.05</td>
<td>1.46 ± 0.08</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td>C106S</td>
<td>E-FMNH$_2$ vs O$_2$-decanal</td>
<td>1.78 ± 0.09</td>
<td>1.39 ± 0.08</td>
<td>1.28 ± 0.10</td>
</tr>
<tr>
<td>C106V</td>
<td>E-FMNH$_2$ vs O$_2$-decanal</td>
<td>1.55 ± 0.08</td>
<td>1.87 ± 0.11</td>
<td>1.78 ± 0.24</td>
</tr>
<tr>
<td>wild-type</td>
<td>E-8-CH$_2$FMNH$_2$ vs O$_2$-decanal</td>
<td>1.84 ± 0.11</td>
<td>1.46 ± 0.09</td>
<td>1.26 ± 0.11</td>
</tr>
<tr>
<td>wild-type</td>
<td>E-8-CH$_2$FMNH$_2$ vs O$_2$-dodecanal</td>
<td>1.50 ± 0.25</td>
<td>0.95 ± 0.06</td>
<td>1.58 ± 0.28</td>
</tr>
</tbody>
</table>

* Reaction conditions: pH 7.0, 25 °C. $^{d}I_{\text{max}}$ is the deuterium isotope effect on the maximum light intensity, $^{d}k_d$ is the deuterium isotope effect on the decay rate of the emitted light, and $^{d}Q$ is the deuterium isotope effect on the quantum yield.
light intensity observed with the αC106S mutant was approximately 30% of that for the wild-type enzyme, and the decay rate of the emitted light was approximately 1.3-fold faster. The activity profiles for this mutant when solutions of enzyme and FMNH₂ were mixed with aerobic solutions of n-decanal exhibited an isotope effect of 1.8 for the maximum light intensity and 1.4 for the light decay rate.

The αC106V protein was the dimmest of the mutants used. The maximum light intensity was only 2% of the wild-type enzyme. The activity profiles for this mutant when solutions of enzyme and FMNH₂ were mixed with aerobic solutions of n-decanal are shown in Figure 4. The light decay rate for this enzyme was quite dependent on the aldehyde concentration. The rate constant for the decay of the emitted light was decreased from around 2.4 s⁻¹ at low levels of n-decanal (50 μM) to around 0.6 s⁻¹ at high levels of n-decanal (500 μM) (Figure 4B). An isotope effect of 1.6 was observed for the maximum light intensity when solutions of enzyme and FMNH₂ were mixed with aerobic solutions of n-decanal (Figure 4A). Very interestingly, the observed isotope effect for the decay of the emitted light was very near unity or slightly inverse. A summary of the results obtained with the αC106 mutants is presented in Table 1.

**Isotope Effect for 8-X-FMNH₂ Derivatives.** Several 8-substituted FMNH₂ derivatives have been shown to be substrates for the luciferase-catalyzed reaction (32). The maximum light intensity and the decay rate of light emission for these derivatives are very different relative to those observed with FMNH₂ (32). The isotope effects on the maximum light intensity and on the light emission decay rates were determined for 8-Cl-FMNH₂ and 8-CH₃O-FMNH₂ when the enzyme was assayed by mixing solutions of enzyme and reduced flavin analogues with n-decanal.

The decay rate of light emission for the 8-Cl-FMNH₂ derivative was 20-fold slower than with FMNH₂. The activity profiles for the 8-Cl-FMNH₂ derivative exhibited a deuterium isotope effect of 1.8 for the maximum light intensity and 1.5 for the decay rate (data not shown). The activity profiles for the 8-CH₃O-FMNH₂ derivative are shown in Figure 5. The rate of decay of light emission for the 8-CH₃O-FMNH₂ derivative was approximately 9 times faster than with FMNH₂. The rate of decay for light emission with this derivative was very dependent on the total aldehyde concentration. The rate constant for the decay of light emission at low n-decanal concentrations was approximately 7.5 s⁻¹ (15 μM) and 4.3 s⁻¹ at high n-decanal concentrations (500 μM) (Figure 5B). No significant isotope effect was

![Figure 3: Isotope effect on the maximum light intensity and decay rate for light emission for wild-type luciferase with n-decanal.](Image)

![Figure 4: Deuterium isotope effect on the maximum light intensity and decay rate of light emission for the αC106V mutant of luciferase with n-decanal.](Image)

![Figure 5: Deuterium isotope effect on the maximum light intensity and decay rate of light emission for wild-type luciferase with 8-CH₃O-FMNH₂.](Image)
observed for the light emission decay rate. A summary of the results obtained for the 8-substituted flavin derivatives is presented in Table 1.

**Hydration-Dehydration of Aldehydes.** It is known that in aqueous solutions, aldehydes exist as a mixture of the hydrated and unhydrated species, as shown below:

\[
\begin{array}{c}
\text{H(D)} \\
\text{H(D)} \\
\end{array}
\begin{array}{c}
\text{O} \\
\text{R} \\
\end{array}
\begin{array}{c}
\text{O} \\
\text{H(D)} \\
\end{array}
\] 

\[
\begin{array}{c}
\text{H(D)} \\
\text{H(D)} \\
\end{array}
\begin{array}{c}
\text{O} \\
\text{R} \\
\end{array}
\begin{array}{c}
\text{O} \\
\text{H(D)} \\
\end{array}
\]

The extent of aldehyde hydration and the rates of the hydration and dehydration reactions for n-octanal were determined using horse liver alcohol dehydrogenase for the direct measurement of the unhydrated species (24). The enzymatic assays were done with 1-[1H]octanal and 1-[2H]-octanal in H2O and D2O solutions. An equilibrium constant for the hydration reaction \((K_{eq} = [\text{gem-diol}]/[\text{aldehyde}])\) of 0.49 ± 0.03 was measured for 1-[1H]octanal and 0.62 ± 0.03 for 1-[2H]octanal in H2O solutions. This represents an inverse equilibrium isotope effect \((\delta K_{eq} = K_D/K_H)\) of 0.79. The rate constants for hydration \((k_h)\) and dehydration \((k_d)\) of 1-[1H]octanal were 0.17 ± 0.02 min\(^{-1}\) and 0.34 ± 0.02 min\(^{-1}\), respectively. For 1-[2H]octanal, the rates of hydration and dehydration were 0.20 ± 0.02 min\(^{-1}\) and 0.33 ± 0.02 min\(^{-1}\), respectively. The deuteration kinetic isotope effects \((\delta k_h, \delta k_d)\) for the hydration and dehydration rate constants were 0.85 ± 0.10 and 1.06 ± 0.09, respectively. When D2O was used as the solvent, the equilibrium constant for the hydration reaction was found to be 0.52 ± 0.05 for 1-[1H]-octanal. The rates of hydration and dehydration in D2O for 1-[1H]octanal are 0.056 ± 0.008 min\(^{-1}\) and 0.11 ± 0.01 min\(^{-1}\), respectively. These results are summarized in Table 2.

**pH Profiles.** The luciferase-catalyzed enzymatic activity was measured as a function of pH using the standard assay format in Tris-maleate buffer solutions over the pH range of 6.0–9.0. The maximum light intensity and the decay rate of the emitted light were measured at a constant aldehyde concentration at 0.25 pH-unit intervals using either 1-[1H]-decanal or 1-[2H]decanal as substrates. The pH profiles for the emission of visible light for both 1-[1H]decanal and 1-[2H]decanal are shown in Figure 6A. The \(pK_a\) values of the ionizable groups were determined from the pH dependence of \(I_{max}\) by fitting the data to eq 7. The \(pK_a\) values determined when 1-[1H]decanal was used as a substrate were 6.9 ± 0.1 and 8.2 ± 0.1. The \(pK_a\) values determined when 1-[2H]decanal was used as a substrate were 6.7 ± 0.1 and 8.4 ± 0.1. The \(pH\) profiles for the decay rate of light emission for both 1-[1H]decanal and 1-[2H]decanal are shown in Figure 6B. The rate of light emission remained almost constant in the \(pH\) range 6–7.5 and then decreased as the \(pH\) increased. The \(pH\) dependencies of the decay rate for both 1-[1H]decanal and 1-[2H]decanal were fit to eq 6. A \(pK_a\) of 8.1 ± 0.1 was determined when 1-[1H]decanal was used as a substrate and 8.2 ± 0.1 when 1-[2H]decanal was used.

**Solvent Isotope Effects.** The effect of deuteriation of the solvent was studied for various reactions. No measurable effect was observed on either the rate of autoxidation of FMNH2 or the formation and decay of the C4a-hydroperoxyflavin intermediate. The effect of deuteriation was also measured for the decay rate of light emission for 1-[1H]-decanal and 1-[2H]decanal. When an H2O solution of enzyme (75 μM) and FMNH2 (15 μM) was mixed with an aerobic solution of n-decanal (200 μM), a rate constant for the decay of the emitted light of 0.50 s\(^{-1}\) was observed with 1-[1H]decanal and 0.30 s\(^{-1}\) for 1-[2H]decanal. When the same experiment was performed in 95% D2O, a rate constant of 0.36 s\(^{-1}\) was measured for 1-[1H]decanal and 0.20 s\(^{-1}\) for 1-[2H]decanal.

**Calculation of Microscopic Rate Constants.** The microscopic rate constants for the kinetic model that appears in Figure 2 for 1-[1H]-decanal and 1-[2H]decanal with wild-type luciferase and FMNH2 are presented in Table 3. These rate constants were obtained by direct comparison of the experimental time courses for light production with the calculated time courses using the KINSIM program. The rate constants for the hydration of the aldehyde \((k_{33}\) and \(k_{36}\)) were those obtained for the case of n-octanal. The rate constants for the formation \((k_1\) to \(k_5\)) and decay \((k_{12}\) and \(k_{17}\)) of E'FMNOOH, the rate of decomposition of E’FMNOOH \((k_{33}, k_{35}\) and \(k_{36}\)), and the rate of autoxidation of FMNH2 have been reported previously \((10, 11)\) and were held constant during the simulation.

The rate constants associated with the binding of aldehyde to free enzyme \((k_{19}\) and \(k_{30}\)) and the rate constants for the processes associated with the binding of aldehyde to the E’FMNOOH complex through the formation of E’FMNOH \((k_7\) to \(k_{11}\)) were established by the simultaneous fit of the
these rate constants were held constant during the simulation of the light emission profiles obtained with the series of intermediates and transition states in the model mechanisms are summarized in Table 4, along with the calculated reduced isotopic partition functions for substitution of deuterium for hydrogen in each structure at the position corresponding to the aldehydic C–H, and appropriate calculated isotope effects.

**DISCUSSION**

The discussion here will first try to pin down the nature of the isotope effects observed in the bacterial luciferase reaction, then use the isotope effects in concert with theoretical calculations and other observations to formulate a consistent detailed molecular mechanism.

**Isotope Effects.** In the critical mechanistic steps between the time of formation of the hydroperoxy hemiacetal and the formation of the excited state of the flavin pseudo-base (Figure 1), the fate of the aldehydic hydrogen has not been defined. Since this atom is ultimately found in the solvent, the C–H bond of the aldehyde must be broken sometime during the course of the overall reaction. However, it has not yet been ascertained whether the C–H bond cleavage is heterolytic or homolytic, or if the bond breakage is part of a transition state. It has been observed that at least one atom, possibly the aldehydic hydrogen, must be transferred to the solvent. The study of isotope effects on the decay of luminescence have provided data that are consistent with the hypothesis that the hydrogen transfer is a modulator of the rate of the reaction.

**Theoretical Results.** Figure 7 shows two model mechanisms examined in ab initio calculations. The first is based on the proposed intermediacy of a dioxirane (33, 34) while the second is based on the direct cleavage of FMNOOR on electron transfer as proposed by Eckstein et al. (32). In these calculational models, the long alkyl chain of the starting aldehyde, final acid, and all intermediates was replaced by a methyl group, and 1-hydroxyethyl hydroperoxide (2) was used to model FMNOOR. Stationary points corresponding to the radical anion of methyl dioxirane (3) and the transition state for its ring opening could not be located, apparently the O–O bond of methyl dioxirane is broken without barrier on reduction to afford the dioxyethane radical anion 4. A transition structure (5) was located for the rearrangement of 4 to acetic acid radical anion. This rearrangement is predicted to be exothermic by 24.6 kcal/mol at the UMP4/6-31+G**//UMP2/6-31+G** level with a barrier of only 1.7 kcal/mol, while the barrier to rearrangement of 8 to 10 via transition structure 9 predicted at the same level is 27.5 kcal/mol. The overall rearrangement of methyl dioxirane to acetic acid is predicted to be exothermic by 90.0 kcal/mol. The energies of the series of intermediates and transition states in the model mechanisms are summarized in Table 4, along with the calculated reduced isotopic partition functions for substitution of deuterium for hydrogen in each structure at the position corresponding to the aldehydic C–H, and appropriate calculated isotope effects. 

**Table 4:** Energies, Isotopic Fractionation Factors, and Isotope Effects for Calculated Structures

<table>
<thead>
<tr>
<th>structure</th>
<th>(UMP2) (U)MP4</th>
<th>reduced isotopic partition function$^a$</th>
<th>calculated isotope effect$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−153.38908</td>
<td>12,000</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>−304.58454</td>
<td>17,390</td>
<td>0.69$^c$</td>
</tr>
<tr>
<td>3</td>
<td>−228.32326</td>
<td>−228.37395</td>
<td>16.214</td>
</tr>
<tr>
<td>4</td>
<td>−228.36467</td>
<td>−228.42134</td>
<td>9.268</td>
</tr>
<tr>
<td>5</td>
<td>−228.36258</td>
<td>−228.41855</td>
<td>3.662</td>
</tr>
<tr>
<td>6</td>
<td>−228.41115</td>
<td>−228.46052</td>
<td>4.8$^d$</td>
</tr>
<tr>
<td>7</td>
<td>−228.46999</td>
<td>−228.51732</td>
<td>12.063</td>
</tr>
<tr>
<td>8</td>
<td>−228.5943</td>
<td>−229.01732</td>
<td>1.24$^e$</td>
</tr>
<tr>
<td>9</td>
<td>−228.91888</td>
<td>−228.97332</td>
<td>4.784</td>
</tr>
<tr>
<td>10</td>
<td>−228.98111</td>
<td>−229.03208</td>
<td>4.9$^c$</td>
</tr>
</tbody>
</table>

$^a$ Temperature 25 °C. $^b$ Equilibrium isotope effects were calculated from the ratio of reduced isotopic partition functions. Kinetic isotope effects also include a factor for the ratio of calculated imaginary frequencies for isotopomers at the transition state (28). $^c$ Equilibrium isotope effect ($k_{D}/k_{H}$) predicted for formation from acetaldehyde (1). $^d$ Equilibrium isotope effect for formation from 2. $^e$ Kinetic isotope effect based on 2 as the starting material.

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 was mixed with a solution of enzyme and aldehyde to the kinetic model that appears in Figure 2.

The time courses for the reactions with 1-[1H]decanal were simulated first. The rate constants obtained for 1-[1H]decanal were used, after some modifications, as initial estimates for the chemical events and the sequence of transformations. However, it has not yet been ascertained whether the C–H bond cleavage is heterolytic or homolytic, or if the bond breakage is part of a transition state. It has been observed that at least one atom, possibly the aldehydic hydrogen, must be transferred to the solvent. The study of isotope effects on the decay of luminescence have provided data that are consistent with the hypothesis that the hydrogen transfer is a modulator of the rate of the reaction.

**Theoretical Results.** Figure 7 shows two model mechanisms examined in ab initio calculations. The first is based on the proposed intermediacy of a dioxirane (33, 34) while the second is based on the direct cleavage of FMNOOR on electron transfer as proposed by Eckstein et al. (32). In these calculational models, the long alkyl chain of the starting aldehyde, final acid, and all intermediates was replaced by a methyl group, and 1-hydroxyethyl hydroperoxide (2) was used to model FMNOOR. Stationary points corresponding to the radical anion of methyl dioxirane (3) and the transition state for its ring opening could not be located, apparently the O–O bond of methyl dioxirane is broken without barrier on reduction to afford the dioxyethane radical anion 4. A transition structure (5) was located for the rearrangement of 4 to acetic acid radical anion. This rearrangement is predicted to be exothermic by 24.6 kcal/mol at the UMP4/6-31+G**//UMP2/6-31+G** level with a barrier of only 1.7 kcal/mol, while the barrier to rearrangement of 8 to 10 via transition structure 9 predicted at the same level is 27.5 kcal/mol. The overall rearrangement of methyl dioxirane to acetic acid is predicted to be exothermic by 90.0 kcal/mol. The energies of the series of intermediates and transition states in the model mechanisms are summarized in Table 4, along with the calculated reduced isotopic partition functions for substitution of deuterium for hydrogen in each structure at the position corresponding to the aldehydic C–H, and appropriate calculated isotope effects.

**DISCUSSION**

The discussion here will first try to pin down the nature of the isotope effects observed in the bacterial luciferase reaction, then use the isotope effects in concert with theoretical calculations and other observations to formulate a consistent detailed molecular mechanism.

**Isotope Effects.** In the critical mechanistic steps between the time of formation of the hydroperoxy hemiacetal and the formation of the excited state of the flavin pseudo-base (Figure 1), the fate of the aldehydic hydrogen has not been defined. Since this atom is ultimately found in the solvent, the C–H bond of the aldehyde must be broken sometime during the course of the overall reaction. However, it has not yet been ascertained whether the C–H bond cleavage is heterolytic or homolytic, or if the bond breakage is part of a transition state. One approach to this problem is to measure kinetic isotope effects upon substitution of a deuterium for protium at this site within the aldehyde substrate. However, all previous investigations of the deuterium isotope effects on the decay of luminescence have obtained a value of approximately 1.5 when n-decanal and FMNH₂ were used as substrates. This modest value is larger than normal α-secondary effects and is significantly smaller than expected for a primary deuterium kinetic isotope effect.

The series of chemical steps that must occur prior to the emission of visible light from the active site of luciferase is reasonably complex. The actual size of any apparent kinetic isotope effect may, in fact, be modulated by the nature of the chemical events and the sequence of transformations leading up to the moment for actual light emission. For example, an inverse secondary equilibrium isotope effect of about 0.80 is expected on the formation of the hydroperoxy hemiacetal (35). This α-secondary isotope effect is due to the change in bond stiffness that accompanies the change in hybridization of the carbonyl carbon from sp² to sp³. An estimate for the magnitude of this effect in the luciferase catalyzed reaction was made by determining the deuterium isotope effect on the equilibrium constant for the hydration...
of n-octanal to the corresponding gem-diol. The experimental α-secondary deuterium effect on the equilibrium constant for this reaction with n-octanal is 0.79. An isotope effect of similar size would also be anticipated for the analogous addition of the hydroperoxide intermediate to the carbonyl group of the aldehyde substrate. This inverse effect (whether kinetic or equilibrium) would then tend to diminish the observed effect of isotopic substitution on the decay of light emission. The small spectral changes in the flavin nucleus observed by Macheroux et al. (4) during substrate binding and turnover have provided direct spectral support for the formation of an intermediate prior to the excited state. These absorbance changes are formed with an apparent inverse isotope effect of 0.90 and 0.75 when n-octanal and n-dodecanal, respectively, are used as substrates. However, these inverse α-secondary isotope effects were not recognized at the time (4) but, in fact, supply the best direct evidence for the formation of the hydroperoxy hemiacetal intermediate during the luciferase catalyzed reaction. Our simulations of the time courses for light emission are fully consistent with an inverse isotope effect during the formation of this intermediate.

Our calculations indicate that the deuterium kinetic isotope effect on the decay of light emission (k1) is 1.9 for n-decanal with FMNH2. In the mechanism presented in Figure 2, the step depicted by the rate constant k1 is a composite for a number of steps that occur after the formation of the hydroperoxy hemiacetal. It is not clear whether this rate constant represents a single rate-limiting mechanistic step or multiple rate-limiting steps. Therefore, the calculated kinetic isotope effect of 1.9 might represent an intrinsic isotope effect or, alternatively, might be the result of a larger primary isotope effect diminished due to the mechanistic and kinetic complexities of the luciferase catalyzed reaction. One approach to simplify these apparent complexities has been articulated by Cleland (6) and involves the measurement of isotope effects with modified substrates, extremes of pH and the utilization of sluggish mutants of the native enzyme. These changes in reaction conditions can be utilized for the purpose of altering the rate-limiting step(s) and/or modulating the structure of the transition state. Therefore, we have utilized this approach to exploit the information that can be extracted from the use of heavy atom isotopes to elucidate the mechanism for light emission in the bacterial luciferase catalyzed reaction.

Variation of the Aldehyde Chain Length. The apparent deuterium kinetic isotope effects were first measured using deuterated aldehydes of different carbon chain lengths. Quite curiously, changing the carbon length from C-10 to C-8 and C-12 alters the rate constants for light emission from 0.50 to 0.05 s\(^{-1}\) and 0.034 s\(^{-1}\), respectively. The changes in rate constant may originate from the nonproductive binding of aldehydes of different chain length. These are substantial effects considering that the additional methylene groups are being added to the substrate at a position rather remote from the carbonyl group of the n-decanal parent. This reduction

in the decay rate for light emission is not, however, accompanied by any significant change in the observed deuterium isotope effect. This observation must mean that either the change in aldehyde chain length has diminished equally all of the rate-limiting steps after the formation of the hydroperoxy hemiacetal or, alternatively, that there is a single rate-limiting step.

Variation of the Flavin Structure. The change in the redox potential and electron-withdrawing properties of the reduced flavin substrate was also used in an attempt to modulate the magnitude of the observed deuterium kinetic isotope effect. With an electron-withdrawing chloride substituent at the 8-position of the flavin, the rate of decay for light emission is 20-fold slower than with a methyl substituent at this same position. However, the observed deuterium isotope effect on the decay of light emission is essentially unchanged relative to FMNH\(_2\) itself. Since the substantial reduction in the rate of decay of light emission observed with the electron-withdrawing substituent has been proposed to be due to a requirement for an electron transfer from the flavin to the aldehyde/acid (32), then the constancy of the deuterium isotope effect with the flavin substrate would argue that the isotope effect is associated with the electron-transfer process as a single rate-limiting step. Otherwise, the observed isotope effect would be anticipated to diminish in magnitude with the slower substrate, but this is not observed. In support of this conclusion are the results obtained with the methoxy substituent at this same site in the flavin nucleus. The rate constant for the decay of light emission with the 8-MeO-FMNH\(_2\) is an order of magnitude faster than with FMNH\(_2\). The observed deuterium isotope effect is 0.95 (slightly inverse). With this faster substrate, the apparent electron-transfer step is no longer as rate limiting as with the native FMNH\(_2\). If the electron-transfer step and a step having a large primary isotope effect (i.e., C–H bond cleavage) occurred sequentially in partially rate-limiting steps, then the observed isotope effect would have been predicted to increase. The reduction in the observed isotope effect is therefore consistent with a single rate-limiting step after the formation of the hydroperoxy hemiacetal. With the 8-MeO-FMNH\(_2\) analogue, it is tempting to speculate that the formation of the hydroperoxy hemiacetal has now become partially rate limiting since a slightly inverse effect is observed.

Variation of the Active-Site Residues. Site-directed mutants of active-site residues of bacterial luciferase were utilized to probe the origin of the deuterium isotope effects. The best characterized of these mutants are those constructed for the chemically reactive residue at αC106 (11). The mutants, αC106A and αC106S, have rate constants for the decay of light emission that are 1.7-fold slower and 1.3-fold faster, respectively, than the wild-type enzyme. Although these changes in rate are rather modest, there is no observable effect on the measured deuterium kinetic isotope effects (1.5 and 1.4, respectively). However, a significantly different picture emerges with the αC106V mutant. The observed deuterium isotope effect on the decay of light emission is slightly inverse, but the observed quantum yield is only 2% that of the wild-type enzyme under identical reaction conditions. The decay rate is slightly faster (0.6 versus 0.5 s\(^{-1}\)). Why is the deuterium isotope effect so low when the apparent decay rate for light emission is identical

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2 The apparent isotope effects for the first phase of the absorbance changes in the visible region of the spectrum during the luciferase-catalyzed reaction were calculated from the half-times reported in Table 1 of Macheroux et al. (4). For n-octanal the deuterium isotope effect is 0.90 (210 s/235 s) and for n-dodecanal the deuterium isotope effect is 0.75 (150 s/200 s).
with the wild-type enzyme? Previous characterization of the kinetic behavior of the αC106V mutant has clearly demonstrated that the decay of light intensity is not limited by $k_{11}$ (11). The observed value is an artifact that is the direct result of an extremely unstable hydroperoxide intermediate and a rather unfavorable equilibrium constant for the formation of the hydroperoxy hemiacetal (11). Thus, the low quantum yield is due to a dominant off-pathway dark reaction that masks any intrinsic isotope effect on the actual step for light formation.

**Alteration in pH.** The deuterium isotope effects on the maximum light intensity and on the decay rate for light emission were measured as a function of pH. At a fixed aldehyde concentration, there is observed a bell-shaped pH profile for the maximum emitted light intensity. The maximum light intensity drops off at both high and low pH, but the observed deuterium isotope effect does not vary significantly over the pH range 6–9. The drop in the quantum yield at low pH (with a $pK_a$ of 6.9) is proposed to be due to the protonation of the reduced flavin. The $pK_a$ value for the hydrogen at N1 of FMNH$_2$ is 6.2. Either the protonated FMNH$_2$ species does not bind to the enzyme or the protonated species does not react with molecular oxygen on the enzyme to form the hydroperoxide intermediate at a significant rate. In these single turnover experiments, either of these effects would result in the reduction in the observed light intensity and apparent quantum yield. At higher pH values, the $I_{max}$ values and the decay rates for light emission decrease together, and thus, the apparent quantum yield is relatively constant. The drop in the light decay rate at high pH may be the result of ionization of a residue that protonates or hydrogen bonds to the light-emitting complex prior to the rate-limiting step.

If a proton transfer is involved in the rate-limiting step prior to the emission of visible light, then one might expect to observe a significant solvent isotope effect on the decay rate of light emission. When the luciferase catalyzed reaction is conducted in 95% D$_2$O, an isotope effect of 1.4 is observed on the decay rate of light emission using 1-[^1]H]decanal as a substrate and 1.5 using 1-[^2]H]decanal as a substrate. These modest isotope effects are consistent with proton-transfer equilibria before the rate-limiting step.

**The Molecular Mechanism of Luciferase Luminescence.** In sum, the observations above strongly support the contention that the isotope effect on $k_{11}$ of 1.9 is the result of a change in rate of a single kinetic step. Any mechanism proposed for the central steps in luciferase luminescence should account for this isotope effect as well as the observed correlation of the decay rate for light emission with the redox potential of the dihydroflavin. It was noted by Eckstein et al. (32) that a Baeyer–Villiger type mechanism (36, 37) would predict a wrong trend for rate versus redox potential. We note here that a Baeyer–Villiger type mechanism (Figure 8) also does not account for the observed isotope effects since a large primary effect would be expected.

Two other mechanisms based on previous proposals are shown in Figures 9 and 10. The first involves the proposed dioxirane intermediate (33, 34). Rate-limiting electron transfer to the dioxirane in 12 to form 13 would be consistent with the observed correlation of rate with dihydroflavin redox potential. This is also true of the mechanism of Figure 10 involving a direct electron transfer within FMNOOR to cleave the O–O bond and form 16, as proposed by Eckstein et al. (32). However, a critical problem was that it was unclear how these mechanisms or any other mechanism could account for the aldehyde isotope effect.

A key insight in this regard came with the observation that the reduced isotopic partition function calculated for the radical anion 4 is anomalously low for H/D on an sp$^3$ carbon. This low fractionation factor is related to the very low barrier predicted for 1,2-hydrogen migration in 4, in that the C–H stretching frequency in 4 is quite low [2556 cm$^{-1}$ compared to 3120 cm$^{-1}$ in 2 (the calculated frequencies are unscaled)]. The result is that the calculated equilibrium isotope effect for formation of 4 from 2 is 1.88. This is extraordinarily high for a secondary isotope effect but is understandable if one views 4 as having, in effect, a partially broken C–H bond.

The predicted kinetic isotope effect if electron transfer to 3 to form 4 is rate limiting depends on the energetics of the electron transfer. Since there is no barrier predicted for ring opening of the radical anion of the dioxirane 3 to form 4, this conversion may be considered a single kinetic step. In the actual system (electron transfer in 12 to form 13), it is reasonable to assume that this step is moderately endothermic, if it were thermoneutral or exothermic, a different trend in rate versus redox potential of the dihydroflavin would be expected, and the electron transfer would likely be too fast to be rate limiting. The equilibrium isotope effect for a reaction is equal to the ratio of kinetic isotope effects for the forward and reverse reactions, i.e., $K_{dR} = k_{dR}/k_{dD}$. For highly exothermic electron transfers, $k_{dR}/k_{dD}$ increases as $\Delta C_{el}$ increases in the Marcus “inverted region”, but this quantum effect falls off to unity for moderately exothermic electron transfers (38). Thus, for a moderately endothermic electron transfer, $k_{dR}/k_{dD}$ (forward/ reverse) $\approx$1. The expected kinetic isotope effect for electron transfer to 3 to form 4 would then be equal to the equilibrium isotope effect of 1.88. This is in excellent agreement with the observed isotope effect on $k_{11}$ of 1.9, indicating that the unusual experimental isotope effect is consistent with rate-limiting electron transfer in 12 to form 13.

The direct electron-transfer-mediated cleavage of FMNOOR in Figure 10 might be expected to exhibit a similar isotope effect since 16 differs from 13 only by the position of a proton. However, the presence of an extra proton in the theoretical model 8, compared to 4, raises the predicted barrier to 1,2-rearrangement in 8 to 27.5 kcal/mol. The reduced isotopic partition function in 8 is not unusual and the predicted kinetic isotope effect for electron-transfer mediated formation of 8 from 2 is only 1.24. (This assumes that there would still be no barrier from O–O cleavage in
the radical anion of 2.) Thus, the mechanism of Figure 10 does not account for the observed isotope effect. However, either a similar electron-transfer-mediated cleavage of the O–O bond in 11 or a direct formation of 13 from FMNOOR would be predicted to exhibit an isotope effect identical with that of the Figure 9 mechanism, without the intermediacy of a dioxirane. Neither mechanism can be rigorously excluded, though the former would involve an unlikely intramolecular electron transfer to an already negatively charged part of 11, and the latter would require that both the O–O cleavage in FMNOOR and a proton transfer occur simultaneously with electron transfer and without a barrier to the bonding changes.

It would be difficult to predict from qualitative considerations the isotope effects expected for rate-limiting 1,2-hydrogen rearrangements of 4 or 8 via transition states 5 or 9, respectively, though such nonlinear hydrogen transfers are sometimes associated with low primary deuterium isotope effects. The ab initio predicted isotope effects for these rearrangements are 4.8 and 4.9, much higher than the experimental value. The inclusion of a correction for tunneling effects [not taken into account in these predictions based on conventional transition state theory (28)] would generally increase the predicted isotope effects for these primary hydrogen transfers. Rate-limiting rearrangement of 4 or 8 would therefore not account for the observed isotope effects.

The theoretical calculations support the energetic plausibility of the intermediacy of a dioxirane, and give insight into why a back electron transfer would favor formation of the excited state of the dihydroflavin for this CIEEL (chemically induced electron exchange luminescence) mechanism (39). The energy available for back electron transfer to the flavin C4a-hydroxide would be equal to the strain energy in the dioxirane compared to the carboxylic acid (90.0 kcal/mol based on 3 versus 7), minus the exothermicity of the 1,2-rearrangement (24.6 kcal/mol based on 4 versus 6), plus the endothermicity of the initial electron transfer. This gives at least 65.4 kcal/mol for the back electron transfer, compared to an energy of 58.4 kcal/mol for the excited state of the FMNOH, based on emission at 490 nm. The modestly exothermic back electron transfer to form the excited-state FMNOH would be expected to occur much faster than electron transfer to form the ground state FMNOH, because the latter would be highly exothermic and well into the Marcus inverted region.

Summary. We have utilized deuterium isotope effects under a variety of reaction conditions to probe the molecular details of the bacterial luciferase catalyzed reaction. Upon the basis of the constancy of the observed isotope effects obtained under a variety of reaction conditions, we have concluded that the isotope effects arise from a single rate-limiting step. Theoretical calculations indicate that the mechanism in Figure 9 involving rate-limiting electron transfer to an intermediate dioxirane is consistent with the unusual aldehydic isotope effect, and that the intermediacy of a dioxirane is energetically plausible. It should be stressed that the existence of the dioxirane intermediate and the radical pair has not been observed directly. However, Kazakov et al. have recently observed the emission of visible light during
the decomposition of dimethyl dioxirane in the presence of aromatic hydrocarbons (40). Experiments to spectroscopically detect these proposed intermediates are currently in progress.

ACKNOWLEDGMENT

We thank Dr. Ravindra Topgi for the synthesis of the modified flavins.

SUPPORTING INFORMATION AVAILABLE

Structural drawings, geometries, and energies of structures 1–10 (11 pages). See any current masthead page for ordering information.

REFERENCES