Binding of Luciferin to Firefly Luciferase

James H. Vu

University Undergraduate Fellow, 1992-1993

Texas A&M University
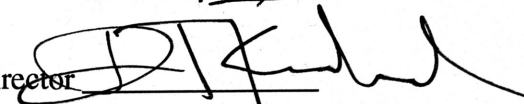
Department of Biochemistry

APPROVED

Fellows Advisor

Honors Director

## ABSTRACT

Firefly luciferase catalyzes a bioluminescent oxidation of luciferin. During the first step of the reaction, when luciferin interacts with ATP, a molecule of luciferyl adenylate is formed and a molecule of pyrophosphate is produced. The luciferyl adenylate is further oxidized by molecular oxygen with the intermediate formation of a cyclic peroxide, dioxetanone, and a molecule of AMP. The dioxetanone is decarboxylated as a result of intramolecular conversions to produce an electron-excited molecule of oxyluciferin which is accompanied with emission of a quantum of visible light ($\lambda_{max}$ = 562-570 nm). The experiments reported here show that luciferase binds to luciferin with an apparent equilibrium dissociation constant of 24.7 µM at 25°C in 100 mM Tris-acetate buffer, pH 7.8, 2 mM EDTA, and 10 mM $MgSO_4$. Analysis of the binding at temperatures between 3° and 25°C indicates an enthalpy of binding ($\Delta H_a$) of -11.5 kcal/mol. Although a solution of luciferin is highly fluorescent, the binding of luciferin to luciferase does not significantly change the fluorescence of the luciferin. However, the enzyme's comparatively weak fluorescence signal does change significantly on the binding of luciferin. This spectral property of the enzyme allows a sensitive method for the determination of the equilibrium dissociation constant of luciferin and luciferase.

# INTRODUCTION

Firefly luciferase is one of the unique enzymes which converts the energy of an enzymatic reaction to light with a high quantum yield. The enzyme is believed to be a dimer with two identical subunits, each having a molecular weight of 64 kDa. Firefly luciferase catalyzes an oxidative reaction involving luciferin, $Mg^{2+}ATP$, and molecular oxygen yielding an electronically excited oxyluciferin species (DeLuca, 1974).
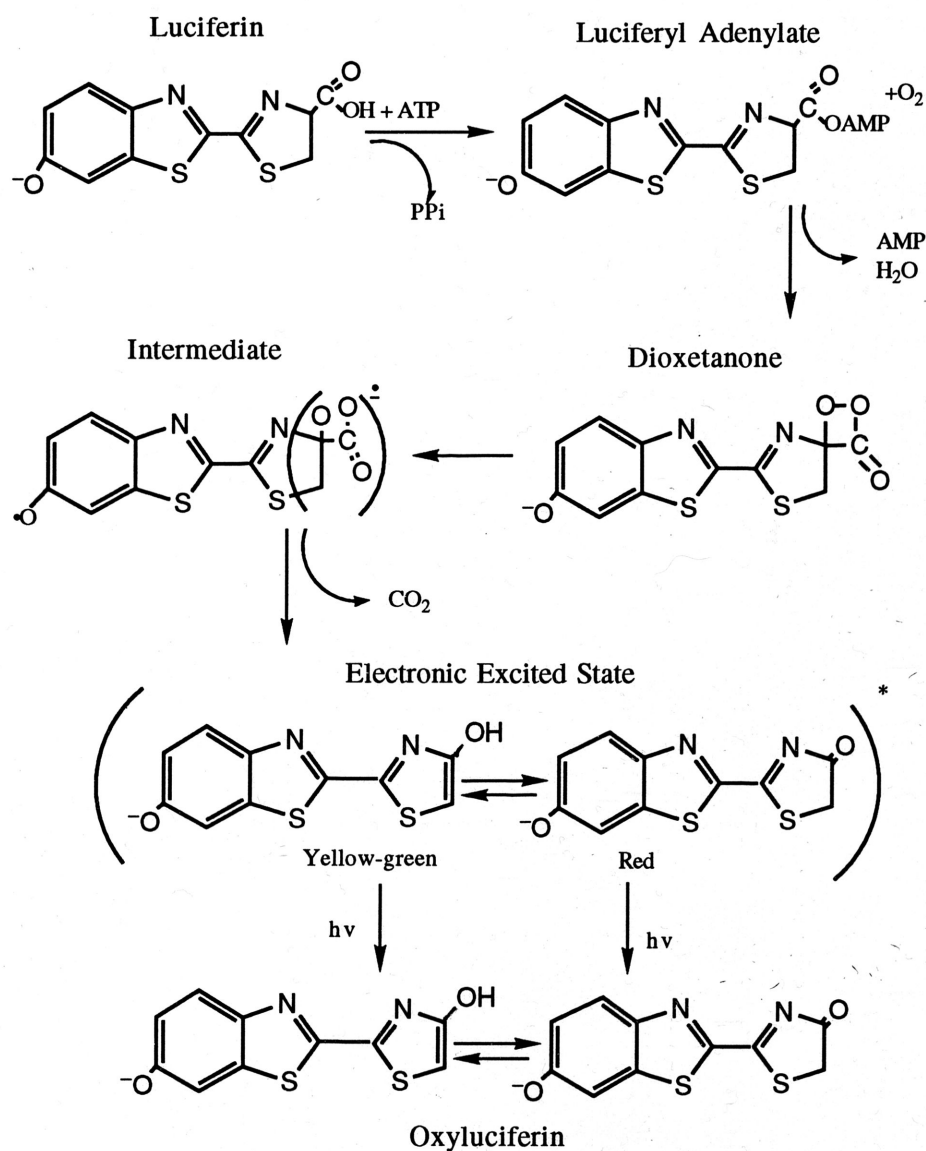


Fig. 1. The chemical mechanism for the reaction of luciferin, ATP, and oxygen catalyzed by luciferase.

During the first step (Fig. 1), when luciferin interacts with ATP, a molecule of luciferyl adenylate is formed and a molecule of pyrophosphate is produced. The luciferyl adenylate is further oxidized by atmospheric oxygen with the intermediate formation of the cyclic peroxide, dioxetanone, and a molecule of AMP. The dioxetanone is decarboxylated as a result of intramolecular conversions to produce an electron-excited molecule of oxyluciferin which is accompanied with emission of a quantum of visible light ($\lambda_{max}$ = 562-570 nm). Non-enzymatic oxidation of luciferin also yields oxyluciferin but without luminescence.

Knowledge of the interaction of luciferin with lufciferase is of interest for several reasons. First, the bioluminescence emission spectrum of a particular luciferase is thought to be dependent on the conformational stability of that enzyme and the equilibrium binding constants for substrates and products (Morton *et al.*, 1969). Secondly, an understanding of the interaction of luciferin with luciferase facilitates the study of the kinetics of the reaction. The data presented here gives thermodynamic parameters of the binding of luciferin to the luciferase from the east European firefly *Luciola mingrelica*.

## MATERIALS AND METHODS

*Materials*—ATP and luciferin were from Sigma.

*Luciferase Purification*—Recombinant firefly luciferase from *Luciola mingrelica* was prepared from lysates of *E. coli* bearing the recombinant plasmid pJGR using a procedure developed by Devine *et al.* (1993). The final solution in which the enzyme was purified consisted of 50 mM Tris-acetate buffer, pH 7.8, 2 mM EDTA, 60 mM $MgSO_4$ with 12% glycerol. Concentration of the enzyme was determined by measuring the absorbance at 280 nm and using an extinction coefficient of 0.63 mL/mg-cm.

*Spectroscopy*—Fluorescence measurements were made with an SLM Instruments SLM 8000 fluorescence spectrophotometer. Absorbance measurements were made with a Hewlett Packard 8452A Diode Array Spectrophotometer. All samples that were spectrophotometrically analyzed were in 100 mM Tris-acetate buffer, pH 7.8, 2 mM EDTA, and 10 mM $MgSO_4$.

## RESULTS

*Spectra of luciferin-bound enzyme*—A single tryptophan on each subunit is responsible for the fluorescence of the enzyme. The binding of luciferin to luciferase causes a significant quenching of the enzyme fluorescence. This is shown in Fig. 2, where the fluorescence emission spectra of 220 nM luciferase is shown in the absence and presence of 12 μM luciferin. To a first approximation, this concentration of luciferin quenches the fluorescence of the enzyme by about 30%, and uniformly so at all wavelengths.
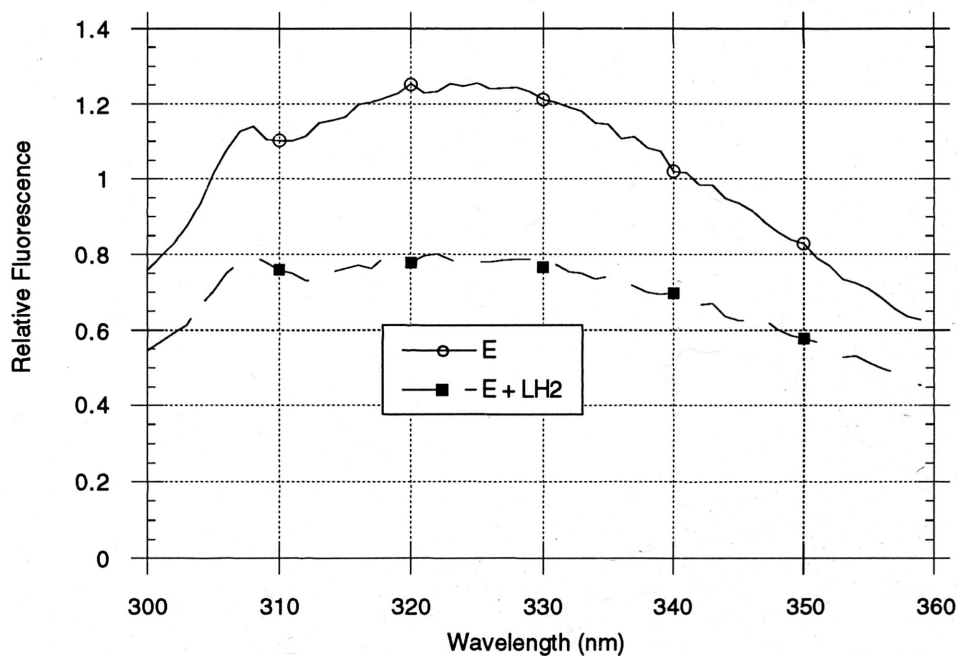


Fig. 2. Fluorescence emission spectra of luciferase in the absence and presence of luciferin. Samples (220 nM luciferase ± 12 μM luciferin) in 100 mM Tris-acetate, pH 7.8, 2 mM EDTA, 10 mM MgSO$_4$, at 25.0°C were excited at 280 nm.

Although luciferin has a very strong fluorescence emission spectra, titration of luciferin by excess enzyme altered neither the form nor intensity of the luciferin

fluorescence (data not shown). However, quenching of enzyme fluorescence upon binding to luciferin permits a sensitive measurement of the extent of luciferin binding during the titration of enzyme by increasing luciferin concentrations. By relating the observed fluorescence with the initial concentrations of enzyme and luciferin, and the intrinsic fluorescence of free enzyme, a value for the $K_d$, the dissociation constant for the enzyme-luciferin complex, can be calculated. First, an equation for the concentration of the enzyme-luciferin complex, [EL], must be found:

$$K_d = \frac{[E][L]}{[EL]} \tag{1}$$

$$K_d\,[EL] = [E]\,[L] \tag{2}$$

$$K_d\,[EL] = ([E]_t - [EL])\,([L]_t - [EL]) \tag{3}$$

In equations 1-3, [E], [L], and [EL] are equilibrium concentrations whereas $[E]_t$ and $[L]_t$ are the total concentrations of enzyme and luciferin, respectively. By multiplying the terms in Equation 3 together and rearranging, the following quadratic is produced:

$$0 = [EL]^2 - ([L]_t + [E]_t + K_d)[EL] + [L]_t[E]_t \tag{4}$$

Using the quadratic formula, the concentration of the enzyme-luciferin complex can be solved:

$$[EL] = \frac{[E]_t + [L]_t + K_d - \sqrt{([E]_t + [L]_t + K_d)^2 - 4[E]_t[L]_t}}{2} \tag{5}$$

The enzyme fluorescence in the absence of luciferin, $F_E$, and the fluorescence of the fully quenched (i.e. $[EL] = [E]_t$) enzyme-luciferin complex, $F_{EL}$, are related to the intrinsic fluorescence of free and bound enzyme, $f_E$ and $f_{EL}$, by the following equations:

$$F_E = [E] \, f_E \tag{6}$$

$$F_{EL} = [EL] \, f_{EL} \tag{7}$$

The observed fluorescence, $F_{obs}$, of a sample is as follows:

$$F_{obs} = [E] \, f_E + [EL] \, f_{EL} \tag{8}$$

Since $[E] = [E]_t - [EL]$, Equation 8 can be expressed in the following manner:

$$F_{obs} = [E]_t \, f_E - [EL] \, f_E + [EL] \, f_{EL} \tag{9}$$

Substituting Equation 6 into Equation 9 and rearranging gives:

$$F_{obs} = F_E - [EL] \, f_E + [EL] \, f_{EL} \tag{10}$$

$$F_E - F_{obs} = (f_E - f_{EL}) \, [EL] \tag{11}$$

And finally substituting Equation 5 into Equation 11 to relate the observed fluorescence with the initial concentrations of enzyme and luciferin, and the intrinsic fluorescence of free and bound enzyme:

$$F_E - F_{obs} = (f_E - f_{EL}) \, \frac{[E]_t + [L]_t + K_d - \sqrt{([E]_t + [L]_t + K_d)^2 - 4[E]_t[L]_t}}{2} \tag{12}$$

Because the absorption spectra of luciferin overlaps the emission spectra of the enzyme-luciferin complex, an "inner-filter effect" arises (Kirby, 1971). Fig. 3 shows the absorption spectra of 22 µM luciferin and 330 nM luciferase, and the emission spectrum of 330 nM luciferase. The wavelength of maximum absorbance for luciferin, 330 nm, is also the wavelength of maximum fluorescence emission of the enzyme.
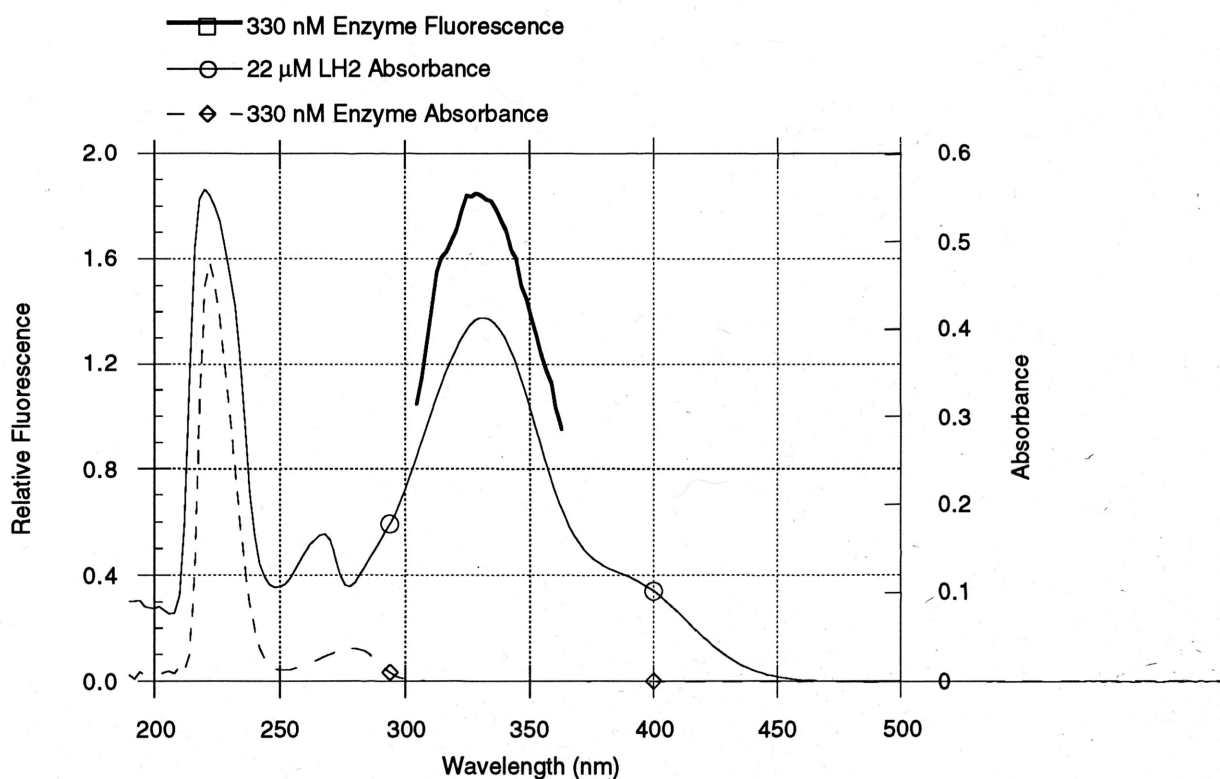


Fig. 3. The absorbance spectrum of luciferin overlaps with the fluorescence emission spectrum of luciferase. 330 nM luciferase and 22 µM luciferin were in 100 mM Tris-acetate, pH 7.8, 2 mM EDTA, and 10 mM MgSO$_4$ at 25°C. The enzyme was excited at 280 nm.

In measuring fluorescence, if the sample solution has a sufficiently high absorbance, an appreciable amount of the emitting light will be absorbed before it leaves the cuvette. The inner-filter effect can be corrected with the following equation,

$$I_{corr} = I_{obs} \times antilog \left( \frac{A_1 + A_2}{2} \right) \tag{13}$$

where $I_{obs}$ and $I_{corr}$ are the observed and corrected fluorescence intensities, and $A_1$ and $A_2$ are the absorbances of the solution at the wavelengths of excitation and emission.
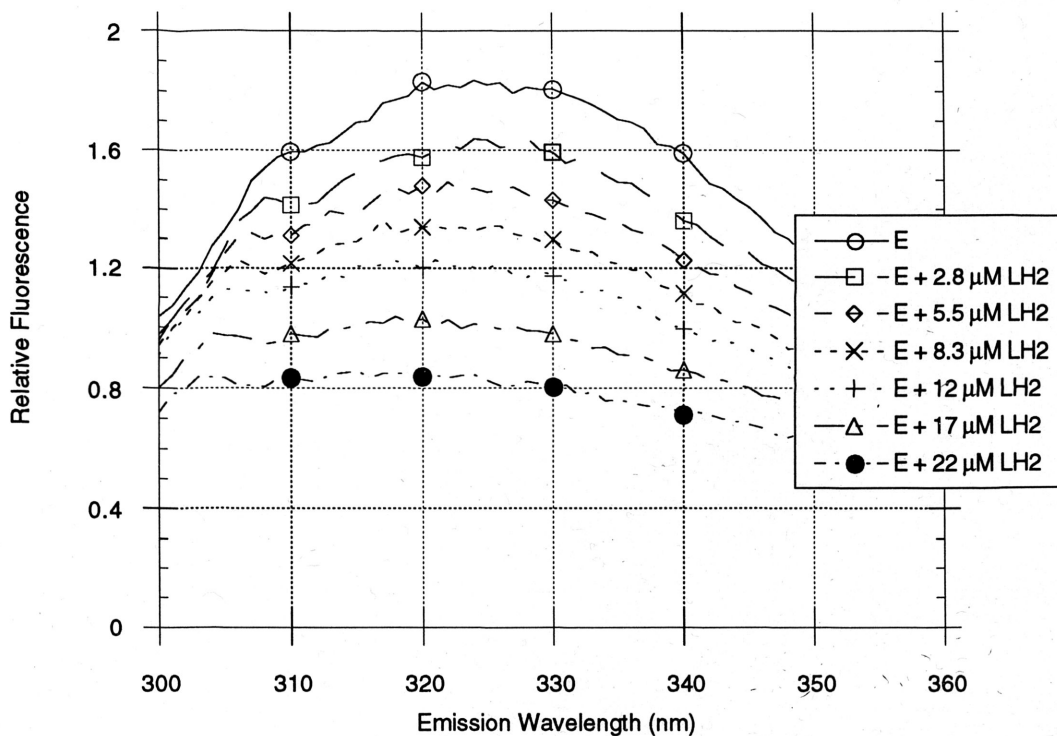


Fig. 4. Fluorescence emission spectra from the titration of 220 nM enzyme with increasing concentrations of luciferin. Samples were in 100 mM Tris-acetate, pH 7.8, 2 mM EDTA, 10 mM MgSO$_4$, at 25.0°C and were excited at 280 nm.

Fig. 4 shows the uncorrected emission spectra obtained from a typical titration experiment. Starting with a luciferase concentration of 220 nM, increasing amounts of luciferin are added into a cuvette for fluorescence measurements. The titration was performed at 25.0°C. The concentration of luciferin was determined by monitoring the absorbance at 330 nm. The extinction coefficient for luciferin is 18,450 $M^{-1}cm^{-1}$ (Morton *et al.*, 1969). The absorbance spectrum of luciferin does not change significantly on binding to the enzyme (data not shown). The solution containing enzyme only was used as a baseline in the determination of luciferin concentration. A graph of relative fluorescence versus enzyme concentration was plotted and the slope of the straight line is the intrinsic fluorescence of luciferase (data not shown). The initial concentration of luciferase in the titration was determined from the observed fluorescence, $F_E$, and the intrinsic fluorescence, $f_E$, as in Equation 7.

Since the volume change on adding luciferin is very small, a proportionality constant can be used to correct for the decrease in fluorescence due to dilution of the enzyme. After correction for the inner-filter effect and the dilution of the enzyme, a graph of $F_E - F_{obs}$ versus luciferin concentration is plotted (Fig. 5), where $F_E$ is the relative fluorescence of the solution containing only enzyme and $F_{obs}$ is the relative fluorescence of the solutions containing enzyme with varying concentrations of luciferin. The data points in the graph in Fig. 5 are derived from the fluorescence data from Fig. 4.
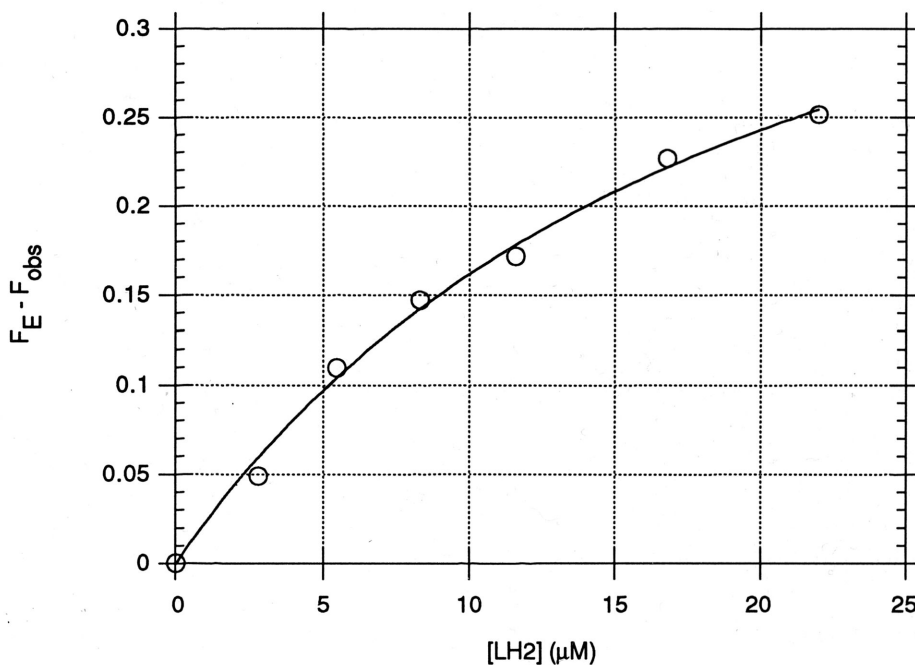
Fig. 5. Plot of $F_E - F_{obs}$ versus $[LH_2]$ for the titration described in Fig. 4.

With respect to Equation 12, only the equilibrium dissociation constant, $K_d$, and the intrinsic fluorescence of the enzyme-luciferin complex, $f_{EL}$, are unknown:

$$F_E - F_{obs} = (f_E - f_{EL}) \frac{[E]_t + [L]_t + K_d - \sqrt{([E]_t + [L]_t + K_d)^2 - 4[E]_t[L]_t}}{2} \qquad (12)$$

A computer is used to fit the data points from the $F_E - F_{obs}$ versus [luciferin] plots to Equation 12. The curve fit gives values for $K_d$ and $f_{EL}$. From values for $f_{EL}$ derived from the curve fits, the fraction of the enzyme's fluorescence that is quenched can be calculated from the following equation:

$$\% \text{ quenching} = \left(1 - \frac{f_{EL}}{f_E}\right) \times 100 \qquad (14)$$

The fluorescence of luciferase is quenched by 33% on binding of luciferin. The luciferin-luciferase complex has 67% of the fluorescence of the free enzyme. Apparent values for $K_d$, obtained from the curve fitting of the titration data, at various temperatures are presented in Table I.

Table I. Temperature dependence of luciferin binding to luciferase.

| Temperature (°C) | $K_d$ ($\mu M$)[a] | $\Delta G_a$ (kcal/mol)[b] |
|---|---|---|
| 3.0 | 4.33 | -6.75 |
| 7.0 | 9.32 | -6.42 |
| 18.0 | 14.9 | -6.40 |
| 25.0 | 24.7 | -6.26 |

[a]Derived from curve fits of Equation 12 with data from titrations.
[b]$\Delta G_a = -RT \ln K_a$, where $K_a = 1/K_d$.

The enthalpy of association, $\Delta H_a$, for binding of luciferin to luciferase was determined by analysis of the temperature dependence of $K_d$ as described by the van't Hoff equation,

$$\frac{d\ln K_a}{d(1/T)} = -\frac{\Delta H_a}{R} \tag{15}$$

where $K_a = 1/K_d$. A van't Hoff plot is shown in Fig. 6. The straight line indicates that $\Delta H_a$ is independent of temperature over the experimental range and has a value of 11.5 kcal/mol.
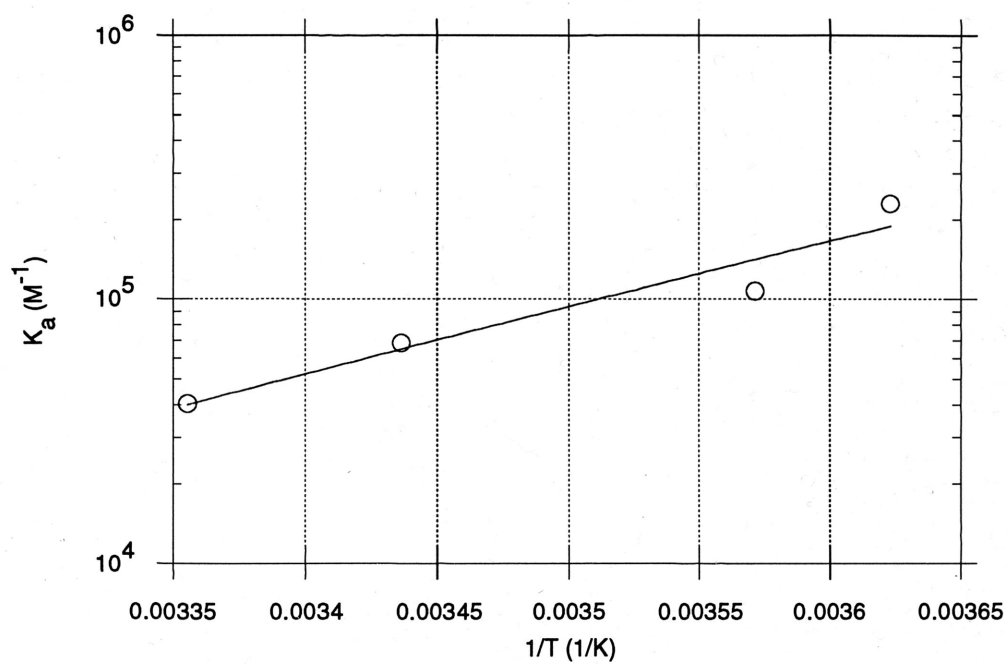
Fig. 6. Van't Hoff plot for the binding of luciferin to luciferase. Values of $K_a$ were obtained as $1/K_d$ from Table 1, and the enthalpy of binding, $\Delta H_a$, was calculated from the slope of the line (Equation 13).

## DISCUSSION

Binding of luciferin to luciferase resulted in a partial quenching of the protein's fluorescence. The dissociation constant for the luciferin-luciferase complex obtained in this work (24.7 $\mu M$ at 25°C) is close to the value measured by Brovko *et al.* (1989), who give a value of 11 $\mu M$ (temperature not reported). They also report that the $K_d$ is pH independent over the whole pH range of enzyme catalytic activity (5.8-8.4).

The use of fluorescence for the determination of the luciferin-luciferase dissociation constant is fairly straight forward. Once an equation relating observed fluorescence to the total concentrations of enzyme and substrate is derived and proper spectral and volumetric corrections are made, a very accurate value for $K_d$ can be determined. However, limitations exist on the concentration of luciferin that can be used in fluorescence studies. Because luciferin has such a high extinction coefficient, concentrations above about 30 $\mu M$ produce an intense inner-filter effect. Fluorescence values obtained under such conditions can not be accurate. In addition, the fluorescence measurements obtained at 3.0°C may not be accurate due to condensation of water on the cuvette. However, this technical problem can be rectified by flooding the sample cuvettes with dry nitrogen.

The bioluminescence emission spectra of several species of fireflies have been determined. These emission spectra are smooth, unstructured, single bands with peak intensities ranging from 546-594 nm. It has been demonstrated that the luciferin molecule for several of these species is the same (Seliger and McElroy, 1964). The emitting molecule in the reaction, oxyluciferin, is also the same for each enzyme. The energy difference between the ground state and excited state enzyme-oxyluciferin complex is reflected in the color of the emitted light. Because the luciferases of different species emit different colored light, the microenvironment of the ground state and excited state enzyme-oxyluciferin complexes in the various enzymes must be different. Therefore, the binding affinities of the various enzyme-substrate complexes may give

information about the bioluminescence spectra. If the $K_d$'s of the luciferin-enzyme complex from several species were determined, correlations between the $K_d$'s and the bioluminescnece spectra may be found. Furthermore, correlation between the $K_d$'s and specific amino acid residues may be found if the sequence for the various enzymes were known. The genes for the luciferases from *Photinus pyralis, Luciola cruciata, Pyrophorus plagiophthalamus*, and *Luciola mingrelica* have been cloned and sequenced (de Wet *et al.*, 1985; Masuda *et al.*, 1989; Wood *et al.*, 1989; Devine *et al.*, 1993).

The experiments described in this paper define a method to quantitatively determine the binding of luciferin to luciferase in a simple system. In the presence of the other substrates ($Mg^{2+}$ATP and molecular oxygen) and product molecules (oxyluciferin, AMP, pyrophosphate, and $CO_2$), the system becomes much more complex. Furthermore, several effector molecules affect the kinetics and bioluminescence spectra of the reaction. Airth *et al.* first described the stimulation of the firefly luciferase reaction by coenzyme A (1958). The presence of certain metal ions also can change the bioluminescence spectrum dramatically (Seliger and McElroy, 1964). More recently, Ford, Hall, and Leach have described how cytidine nucleotides enhance firefly luciferase activity (1992).

The simplest system in which bioluminescence occurs, that is, the reaction of luciferin, $Mg^{2+}$ATP, and $O_2$ catalyzed by luciferase, has not been clearly characterized to this day. Studies of ATP binding to luciferase suggests that the luciferase dimer has two catalytically active sites (DeLuca and McElroy, 1984). These putative high and low affinity ATP binding sites on the enzyme are thought to be responsible for the observed biphasic time course of light emission. More recently, Ugarova has proposed that luciferase has only one catalytically active ATP binding site and two allosteric binding sites (1989). Thus, the literature concerning the binding of ATP to luciferase gives confusing hypotheses.

The time course of light emission shows an ATP concentration dependence (DeLuca and McElroy, 1984). This requires the incorporation of non-identical ATP

binding sites in models that attempt to explain this effect. However, varying the concentration of luciferin does not affect the kinetics of the reaction (data not shown). This observation suggests that there is only one type of luciferin binding site. However, the question of whether each dimer has one or two luciferin binding sites remain. DeLuca and McElroy hypothesized that only one luciferyl adenylate is produced per dimer (1984). Ugarova's model, in which there is only one active ATP binding site per dimer, suggests that only one luciferin binding site exists per dimer.

The binding of ATP *and* luciferin to the enzyme to form the luciferyl adenylate is the next logical step in the analysis of the reaction. Reported equilibrium dissociation constants for ATP binding to luciferase show that ATP binds about ten times tighter than luciferin (Ugarova, 1989). Thus, the most likely reaction mechanism would be that ATP binds first to the enzyme, luciferin binds next, and then ATP reacts with luciferin to form the luciferyl adenylate. Using fluorescence to study the binding of the luciferyl adenylate to luciferase shows much promise. However, equilibrium experiments involving luciferyl adenylate must be performed anaerobically otherwise oxygen would react with the adenylate to form oxyluciferin.

Work using fluorescence can also be useful in the kinetic analysis of the firefly reaction. For example, the rate constant for the binding of luciferin to luciferase can be determined by stopped-flow kinetics using the quenching of the enzyme's fluorescence as a probe. Because the firefly reaction is so complex, an unambiguous reaction mechanism will require the analysis of each step in the reaction.

## ACKNOWLEDGMENTS

## REFERENCES

Airth, R. L., Rhodes, W. C., and McElroy, W. D. (1958). *Biochim. Biophys. Acta* **27**, 519-532.

Brovko, L., Gandelman, O., and Ugarova, N. N. (1989). *Biological Luminescence* **1**, 293-308.

Devine, J., Kutuzova, G., Green, V., Ugarova, N. N., and Baldwin, T. O. (1993). *Biochem. et Biophys. Acta.*, in press.

de Wet, J. R., Wood, K. V., Helinski, D. R., and DeLuca, M. (1985). *Proc. Natl. Acad. Sci. USA* **82**, 7870-7873.

DeLuca, M., and McElroy, W. D. (1974). *Biochemistry* **13**, 921-925.

DeLuca, M., and McElroy, W. D. (1984). *Biochem. Biophys. Res. Commum.* **23**, 765-770.

Ford, S. R., Hall, M. S., and Leach, F. R. (1992). *Anal. Biochem.* **204**, 283-291.

Kirby, E. P. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., and Weinryb, I., Eds.) Plenum Press, New York, 39-40.

Masuda, T., Tatsumi, H., and Nakano, N. (1989). *Gene* **77**, 265-270.

Morton, R. A., Hopkins, T. A. and Seliger, H. H. (1969). *Biochemistry* **8**, 1598-1607.

Seliger, H. H., and McElroy, W. D. (1964). *Proc. Natl. Acad. Sci. USA* **52**, 75-81.

Ugarova, N. N. (1989). *J. Biolumin. Chemilumin.* **4**, 406-418.

Wood, K. V., Lam, Y. A., and McElroy, W. D. (1989). *J. Biolumin. Chemilumin.* **4**, 289-301.