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Excited-State Proton Transfer and the Mechanism of Action of Firefly Luciferase†

Lemuel J. Bowie,* Richard Irwin, Michael Loken, Marlene De Luca, and Ludwig Brand

ABSTRACT: The synthesis of a fluorescent substrate analog of firefly luciferin, dehydroluciferol, has been reported in the previous paper of this series. It has been shown to be enzymatically unreactive and binds at the luciferin binding sites. The spectral properties of this analog suggest that it should be a good active-site probe. In the present study our results indicate that the binding of the second substrate for the luciferase bioluminescent reaction, ATP–Mg^{2+}, causes a decrease in the rate of excited state proton transfer from the 6′ hydroxyl of bound dehydroluciferol. These results are interpreted as being due to a change in the "hydrophobicity" of the active site microenvironment. Since formation of dehydroluciferyl adenylate, an analog of the intermediate in the bioluminescent reaction, has been shown to cause a large conformational change in luciferase, our results indicate that this change manifests itself at the active site. The binding of the activator, AMP, causes changes in the fluorescence emission of bound dehydroluciferol which indicate that the microenvironment becomes even more "hydrophobic." In addition, emission occurs almost completely from the excited-state species in which the 6′ hydroxyl is ionized. Since this group is un-ionized in the ground state when natural substrate luciferin, or dehydroluciferol, binds and since bioluminescence emissions occur from an excited-state species of luciferin in which the 6′-hydroxyl group is ionized, ionization of the enzyme-bound chromophore must occur prior to light emission. Therefore, the effect of AMP may have significance in normal firefly bioluminescence since it facilitates this ionization.

Many investigations recently have demonstrated the usefulness of small fluorescent molecules as probes for certain binding sites in proteins. In the case of firefly luciferase the substrate luciferin (LH_2)[1] is a fluorescent molecule. A competitive inhibitor of the enzyme, dehydroluciferin (L), is also fluorescent and the spectral properties of these compounds are sensitive to the composition of the solvent (Morton *et al.*, 1969). Denburg *et al.* (1969) showed that the emission spectrum of L changed when it was bound to luciferase. This change was thought to be a result of dehydroluciferin binding in a very "hydrophobic" [2] site on the enzyme. Previous studies using fluorescent dyes (De Luca, 1969; Turner and Brand, 1968) also indicated the LH_2 or L binding site is very hydrophobic. Both L and LH_2 in the presence of luciferase react with ATP–Mg to form the enzyme-bound acyl adenylate. It was desirable to have a substrate analog which would not react with ATP–Mg in order to study its spectral properties when bound to luciferase in the presence and absence of ATP–Mg. We have synthesized the alcohol analog of dehydroluciferin which does not react with ATP–Mg. The structure and properties of this compound were described in the previous paper. The spectral properties of L-OH have been shown to be sensitive to both pH and solvent composition. L-OH is an inhibitor of luciferase and is competitive with respect to luciferin (Bowie *et al.*, 1972). Using this analog we have been able to study the effect of ATP–Mg and AMP on the spectral properties of the enzyme-bound L-OH. We have found changes in the active site which influence the rate of excited-state ionization of the phenolic hydroxyl group. The apparent hydrophobicity of the binding site also changes in the presence of ATP–Mg or AMP.

Materials and Methods

Firefly luciferase was isolated from lyophilized firefly lanterns as described in the previous paper. However, even after repeated recrystallization, it was not possible to completely remove small amounts of enzyme bound fluorescence. The possibility that this fluorescence might be due to small amounts of enzyme bound L-AMP was suggested by the similarity between the excitation and emission spectra of this fluorescence and the corresponding spectra resulting when enzyme-bound dehydroluciferin is incubated with ATP–Mg. In addition, the K_D for L-AMP is very low, 5×10^{-10}

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[1] Abbreviations used are: LH_2, luciferin; L, dehydroluciferin; L-OH, dehydroluciferol; L-AMP, dehydroluciferyl adenylate; LH_2-AMP, luciferyl adenylate.

[2] The term "hydrophobicity" as used in this paper refers to the sum total of all such environmental effects on fluorescence. For a more complete discussion on hydrophobicity and solvent polarity the reader is referred to the recent review of Brand and Gohlke (1972).

(Rhodes and McElroy, 1958), and, therefore, binds very tightly to luciferase. Treatment of luciferase with coenzyme A and cysteine, a procedure known to remove L-AMP (Airth *et al.*, 1958), failed to remove significant amounts of fluorescence. Thus, the origin of this enzyme-bound fluorescence remains unknown. This fluorescence never contributed more than 10–15% to the emission spectrum, at 425 nm, of enzyme bound dehydroluciferol and contributed less than 5% to the spectra in the presence of ATP–Mg^{2+} or AMP. Nevertheless, all spectra reported here were corrected for enzyme-bound fluorescence by subtracting the spectrum of fluorescent enzyme from the corresponding excitation and emission spectra throughout the region examined.

Luciferase enzymatic activity in the bioluminescent reaction was determined according to the method of McElroy and Seliger (1961) as described in the previous paper of this series.

Firefly luciferin (D form) was synthesized according to the method of Seto *et al.* (1963) by Dr. Peter Plant and dehydroluciferin was obtained from Dr. George Fields. Dehydroluciferol, luciferin, and dehydroluciferin were all checked for purity by thin-layer and paper chromatography as previously described.

Coenzyme A was obtained from Sigma Chemical Co. The nucleotide analog, α,β-methylene-ATP, was obtained from Miles Laboratories, Inc. All other nucleotides were obtained from P-L Biochemicals. The purity of the nucleotides ATP, AMP, and 3′,5′-cAMP were checked by thin-layer chromatography (tlc) on silica gel using 7:3 (95%) ethanol–1 M ammonium acetate (pH 7.5) as the solvent system for development. All nucleotides showed only a single spot on tlc.

Adenosine 5′-methylphosphate was synthesized according to the method of Moffatt and Khorana (1961) and its structure and purity were confirmed by ascending paper chromatography using the three solvent systems reported: solvent I, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2); solvent II, ethyl alcohol–1 M ammonium acetate (pH 7.5) (5:2); solvent III, isobutyric acid–1 M ammonium hydroxide–0.1 M ethylenediaminetetraacetic acid (100:60:1.6). The purity of Me-AMP was also checked using tlc as described above for the other nucleotides.

Fluorescent titrations were performed on a Turner fluorometer under the same conditions as were reported in the previous paper, using the same buffer system, pH, and temperature. Since the position of emission peaks did not change in the presence of ATP–Mg, the same excitation and emission filters were used. Corrections for inner filter effects were again performed as previously described.

All absorption spectra were recorded on a Beckman Acta V spectrophotometer. All steady-state fluorescence excitation and emission spectra were obtained on a Perkin-Elmer MPF-2A (Hitachi) fluorescence spectrophotometer equipped with a constant-temperature cell compartment. All spectra were recorded with 5-nm slits for excitation and emission. The samples used in these studies all had absorbances <0.1 at the exciting wavelength and, as such, effects due to the absorption of exciting light were negligible. Corrected spectra were obtained using a correction curve for the emission monochromator–phototube combination obtained according to the method of Parker and Rees (1960), using Rhodamine B (8 g/l.) in the ethylene glycol as a quantum counter.

Time-resolved emission spectra were obtained with a single photon-counting fluorescence spectrometer similar to that described by Schuyler and Isenberg (1971). Nuclear modules were obtained from Ortec (Oak Ridge, Tenn). These included

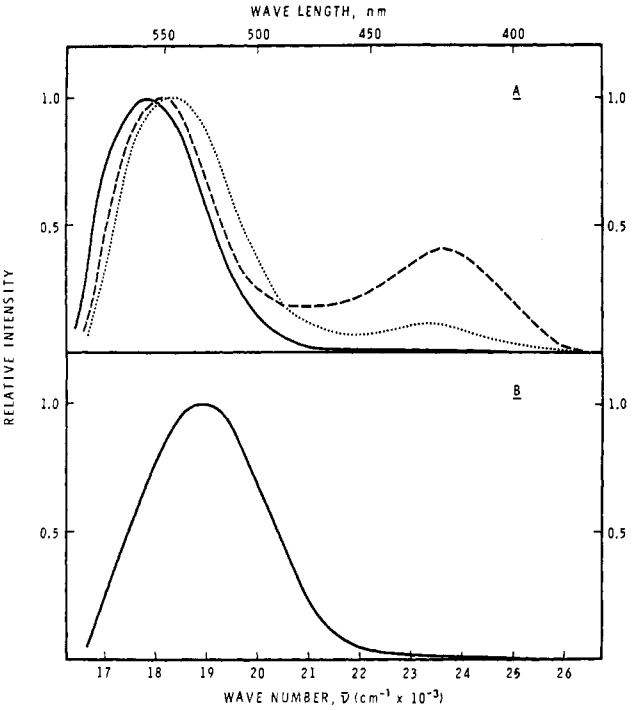

FIGURE 1: Fluorescence emission spectra. (a) Normalized to the peak of the phenolate emission, of dehydroluciferol 4×10^{-7} M in 0.05 M phosphate buffer (pH 7.5) (—); dehydroluciferol 4×10^{-7} M plus 4×10^{-6} M luciferase (. . . . . .); dehydroluciferol 4×10^{-7} M, luciferase 4×10^{-6} M, ATP–Mg 2×10^{-3} M (- - - - -). (b) Of dehydroluciferol, 4×10^{-7} M in the presence of 4×10^{-6} M luciferase and 2×10^{-3} M AMP, in 0.05 M phosphate buffer (pH 7.5).

two 454 timing filter amplifiers, two 453 constant fraction discriminators, a 437A time to pulse height converter, and a 425 timing delay. The signal from the pulse height analyzer was processed with a Hewlett-Packard (Santa Clara, Calif.) 5402A multichannel pulse height analyzer. The multichannel analyzer was directly interfaced with a Hewlett-Packard 2100 computer. An Amperex (Hicksville, N. Y.) 56DUVP electron multiplier phototube was used for photon detection. A thyratron pulsed lamp (Ware *et al.*, 1971) run in air at 15 cm of Hg was used for excitation. The light was focused onto the sample through a 340-nm band pass filter (Baird-Atomic, Inc., Cambridge, Mass.). The fluorescence emission was observed through a Basuch and Lomb $^1/_2$ meter monochromator (Rochester, N. Y.). Decay curves were obtained under single photon-counting conditions or were corrected for photon pileup by the method of Coates (1968).

Nanosecond time-resolved emission spectra were generated in the following way. Decay curves were obtained at each wavelength of interest, corrected for photon pileup, and normalized to the same number of lamp flashes. The number of counts obtained in the bin corresponding to the desired time was then plotted as a function of wavelength.

Results

Fluorescence of Dehydroluciferol. In buffer at pH 7.5, dehydroluciferol exhibits a single fluorescence emission having a maximum at 555 nm (17,850 cm^{-1}) which is due to emission from the excited state form of dehydroluciferol in which the 6′-hydroxyl group of the benzothiazole portion of the molecule is ionized (Figure 1) (Bowie *et al.*, 1972). The absorption

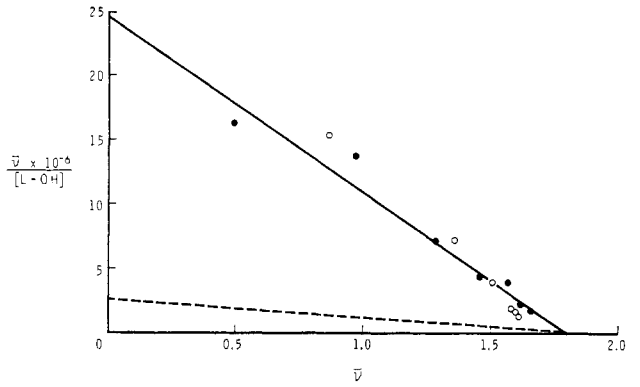

FIGURE 2: Scatchard plot of a representative titration of luciferase with dehydroluciferol in the presence of 2×10^{-3} M ATP–Mg (——). The luciferase concentration was varied from 2.1×10^{-7} M (○) to 8.2×10^{-7} M (●). Under these conditions $n = 1.8$ and $K_b = 14 \times 10^6$. A titration in the absence of ATP–Mg is included for comparison (- - - - - - -).

and excitation spectrum of this compound at pH 7.5 indicated that the ground-state form existing under these conditions is the *phenol* form (*i.e.*, the 6′-hydroxyl group is not ionized). These results are similar to those obtained by Morton *et al.* (1969) and indicate that the 6′-hydroxyl group is undergoing excited-state ionization or excited-state proton transfer (Weller, 1961).

When bound to luciferase, dehydroluciferol, excited at 353 nm, exhibits two emissions (Figure 1), one at 540 nm (18,300 cm^{-1}) and the other at 425 nm (23,400 cm^{-1})). The 425-nm emission band is similar in band position to the emission observed from dehydroluciferol in nonpolar solvents, where excited-state proton transfer is inhibited and all emission occurs from the excited-state phenol. We, therefore, attribute this 425-nm band to emission from the excited-state phenol form of enzyme-bound dehydroluciferol. The 540-nm emission is due to emission from the excited state *phenolate* form of enzyme-bound dehydroluciferol. The blue shift in the phenolate emission from 555 to 540 nm and the enhancement of the phenol emission at 425 nm which accompanies the binding of dehydroluciferol to luciferase suggest that dehydroluciferol is bound in a "hydrophobic" region of the enzyme. The presence of emission from both the excited-state phenol form of dehydroluciferol and the excited-state phenolate form indicates that the 6′-hydroyxl group of enzyme-bound dehydroluciferol is capable of undergoing ionization (proton transfer) in the excited state; however the rate is slower.

Effect of ATP–Mg^{2+} on the Fluorescence of Dehydroluciferol Bound to Luciferase. The addition of excess ATP–Mg^{2+} causes an even further enhancement of 425 nm emission (Figure 1). The intensity at 425 nm is increased by a factor of approximately 2.5. The peak position of the 425-nm emission, however, remains unchanged. A small red shift in the phenolate emission is observed (λ_{max} 548 nm, $\bar{\nu}_{max}$ 18,200 cm^{-1}) with no change in bandwidth. The presence of ATP–Mg^{2+} has no effect on the excitation or emission spectra of L-OH when both are free in solution.

In order to determine whether this additional enhancement due to binding of ATP–Mg^{2+} was solely due to an increase in quantum yield, or whether there was also a change in the association constant possibly causing more molecules to be bound, fluorescent titrations with L-OH were performed in the presence of excess ATP–Mg^{2+}. The data in Figure 2, plotted according to the method of Scatchard (1949), show that, under these conditions, the number of L-OH binding sites does not change when ATP–Mg^{2+} is present ($n = 2$, both in the presence and absence of ATP–Mg^{2+}). There is, however, a 10-fold change in the association constant. These results tend to support the conclusion that more molecules of L-OH are bound under the same conditions in the presence of ATP–Mg^{2+}, giving rise to the observed increase at 425 nm. However, the ratio of $F_b:F_{free}$, indicated otherwise (where F_b is the fluorescence at 425 nm of totally bound L-OH and F_{free} is the 425-nm fluorescence of L-OH free in solution). The value of F_b is determined both in the presence of a 10-fold excess of luciferase and by the double-reciprocal extrapolation method of Weber and Young (1964). The value of F_{free} is taken as the intensity of the small 425-nm shoulder of the same concentration of L-OH in buffer. The increase in fluorescence when L-OH binds in the absence of ATP–Mg^{2+} as judged by $F_b:F_{free}$ is 10–15-fold. In the presence of ATP–Mg^{2+}, however, the ratio $F_b:F_{free}$ is 28–30. Therefore, ATP–Mg^{2+} causes a 2–2.5-fold increase in the fluorescence of completely bound material. As in the case with the luciferase light-emitting reaction, ATP alone (uncomplexed with Mg^{2+}) is not the substrate and causes only a small enhancement at 425 nm.

Effect of AMP. Lee and McElroy (1971) recently found that adenosine 5-phosphate (AMP), a product of the bioluminescent reaction, is also an activator of yellow green light emission at pH 6.5 or below. It was of interest, therefore, to see if the presence of AMP affected the bound L-OH. Figure 1b shows the emission spectrum of enzyme-bound L-OH in the presence of excess AMP. In contrast to the previous cases, there is now only one emission which occurs at 523 nm. There is also an increase in bandwidth (FWHM = 2850 cm^{-1}) and a small (5 nm) red shift in the excitation spectrum. One hypothesis which could explain these results is that binding of AMP increases the hydrophobic character of the environment around the 6′-hydroxyl group of bound L-OH. Therefore the single emission at 523 nm observed under these conditions comes from the excited state phenolate which is shifted to the blue. The absence of emission from the excited-state phenol would suggest also that the binding of AMP enhances excited-state proton transfer so that only phenolate emission is observed.

Fluorescent titrations were attempted using the 523-nm emission to determine if there was a change in the number of L-OH molecules bound; however, difficulties in determining an accurate value of $F_b:F_{free}$ made these results ambiguous. The data suggested that n was approximately 2–3, however. In addition, there is no release of L-OH from luciferase since there is no increase in fluorescence at 555 nm. The binding of AMP + Mg^{2+} produced the same effects as AMP alone.

Effect of Various Solvents on the Fluorescence of L-OH. In order to further investigate the origin of the new emission at 523 nm, fluorescence emission spectra of L-OH were recorded in a number of solvents of different dielectric constant and Kosower "Z" values (Kosower, 1958). In all the nonpolar solvents tested there was only one emission present which was attributed to emission from the excited-state phenol. The addition of a suitable proton acceptor was required in order to obtain emission from the phenolate in these solvents. One such acceptor used by Morton *et al.* (1969), with luciferin, was triethylamine. Using triethylamine (3%) as the proton acceptor, the position of the phenolate emission wavelength maximum shifted to lower and lower wavelengths as the polarity of the solvent was decreased. The results are summarized in Table I. The emission spectrum of L-OH in chloroform in the

TABLE I: Effect of Solvent Polarity on Phenolate Emission.

	λ_{max} (Uncor),[a] nm	D[b]	Kosower
Water	536	80	94.6
Methanol	531	33	83.6
1-Propanol	529	20	78.3
Ethyl acetate	520	6	
Chloroform	508	4.8	63.2

[a] All solvents contain 3% triethylamine (except water). [b] D, dielectric constant.

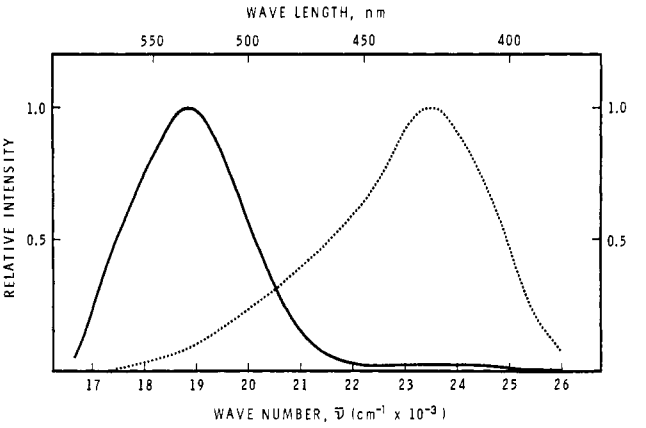

FIGURE 3: Fluorescence emission spectra of dehydroluciferol in chloroform; no triethylamine (. . . . .); 3% triethylamine (——); dehydroluciferol concentration 4×10^{-7} M.

presence and absence of triethylamine (3%) is shown in Figure 3. The phenol emission occurs at 425 nm while the phenolate emission (in the presence of 3% triethylamine) occurs at 527 nm, thus mimicking almost exactly the steady-state emission spectra of enzyme-bound L-OH in the presence of AMP. The quantum yields for the phenol emission and phenolate emission, as judged by the areas under the corrected curves, are approximately the same.

Surprisingly, although AMP does not react with L bound to luciferase, the addition of AMP causes only very small changes in the emission spectrum of L bound to luciferase.

Specificity of Effects. A variety of other nucleotides structurally related to ATP and AMP were tested for their ability to induce changes in the emission spectrum of enzyme-bound L-OH. The analogs of ATP tested were: uncomplexed ATP, α,β-methylene-ATP, ADP, GTP, TTP, CTP, and UTP. Of these, the only nucleotides which gave any significant enhancement or change in the emission spectrum were free ATP and α,β-methylene-ATP for which the enhancement was only slight in both cases. In the case of uncomplexed ATP, the spectra were determined in the presence of 5×10^{-3} M EDTA. Several nucleoside monophosphates were also examined for their ability to cause changes in the fluorescence of bound L-OH. Of the nucleoside monophosphates UMP, GMP, CMP, and 3′,5′-cAMP, only the latter nucleotide produced any significant effect, and the interesting observation was that the binding of cAMP produced changes in the fluorescence of bound L-OH nearly identical with those observed when ATP–Mg^{2+} was bound. One possible explanation arises from the observation that the presence of a negative charge on the α phosphate of ATP–Mg^{2+} is important for binding (Lee *et al.*, 1970). It becomes obvious that the similarity electrostatically between ATP–Mg^{2+} and 3′,5′-cAMP could be the presence of one negative charge at the α phosphate in contrast to either no charge there for ADP–Mg^{2+} or two negative charges for AMP. If these electrostatic interactions were responsible for the specificity of ATP–Mg^{2+} for its site as opposed to the uncomplexed ATP or AMP site, then the monomethyl ester of the AMP 5′-phosphate group should also produce a nucleotide which should elicit a response like that of ATP–Mg^{2+} or 3′,5′-cAMP. The 5′-monomethyl ester of AMP was synthesized according to the method of Moffatt and Khorana (1961) and tested for its ability to mimic ATP–Mg^{2+}. The emission spectrum obtained, however, showed that the methyl ester had little or no effect on emission at all.

Coenzyme A which reacts with enzyme-bound L-AMP to form dehydroluciferyl coenzyme A, releasing AMP (Airth *et al.*, 1958), also has no effect on the emission of enzyme-bound L-OH. The relative quantum yields, as judged by the areas under the peaks of the corrected and unnormalized spectra (not shown), are approximately the same for all these cases. Therefore, these ionizations seem to reflect only the relative *distributions* between phenol and phenolate.

Nanosecond Spectrofluorometry. Time-resolved emission spectroscopy has been shown to be an important approach for obtaining direct kinetic evidence about processes occurring during the lifetime of the excited state (De Luca *et al.*, 1971; Brand and Gohlke, 1971; Ware *et al.*, 1968). We, therefore, used this technique to follow directly the relative rates of proton transfer from the excited-state phenol to solvent. In order to get a better idea of the time-dependent shift in emission, the spectra are normalized at or near the isoemissive point for the two emissions. The reason for the normalization is that at longer times the actual intensity is decreasing because the points taken are on the tail of the decay curve. Normalizing at the isoemissive point has the effect of showing only the changes in the ratio of phenol to phenolate emission while the intensity at the isoemissive point remains constant with time.

Figure 4a shows the results of such a plot of the time-dependent shift in the emission of L-OH in buffer at pH 7.5. It should be noted that these spectra are uncorrected for photomultiplier response. The times for which the individual curves were calculated are given in the upper left-hand corner of each curve. The time zero point (0.0 nsec) is arbitrarily taken as the peak of the lamp flash, and subsequent time points are taken at the specified times after this point. At 0.0 nsec, there is a significant amount of phenol emission which rapidly decays with an associated rise in the phenolate emission and by 5 nsec the phenol emission is almost completely gone. No subsequent spectral changes occurred after 5 nsec.

Figure 4b shows the time-resolved emission spectrum of enzyme-bound L-OH in buffer. Note that at zero time the phenol emission is significantly higher than in the case of free L-OH in buffer. In addition, after 5 nsec, there is still a large amount of phenol emission present, the intensity at 440 being approximately 50% of the intensity at 525 nm. It should be remembered that in the case of free L-OH, the phenol emission was almost completely absent at this time. At the end of approximately 13 nsec, a significant amount of phenol emission still persists.

In Figure 4c the time resolved emission spectrum of enzyme-bound L-OH in the presence of ATP–Mg^{2+} is shown. Note that at early times (0–2 nsec) there is almost pure phenol

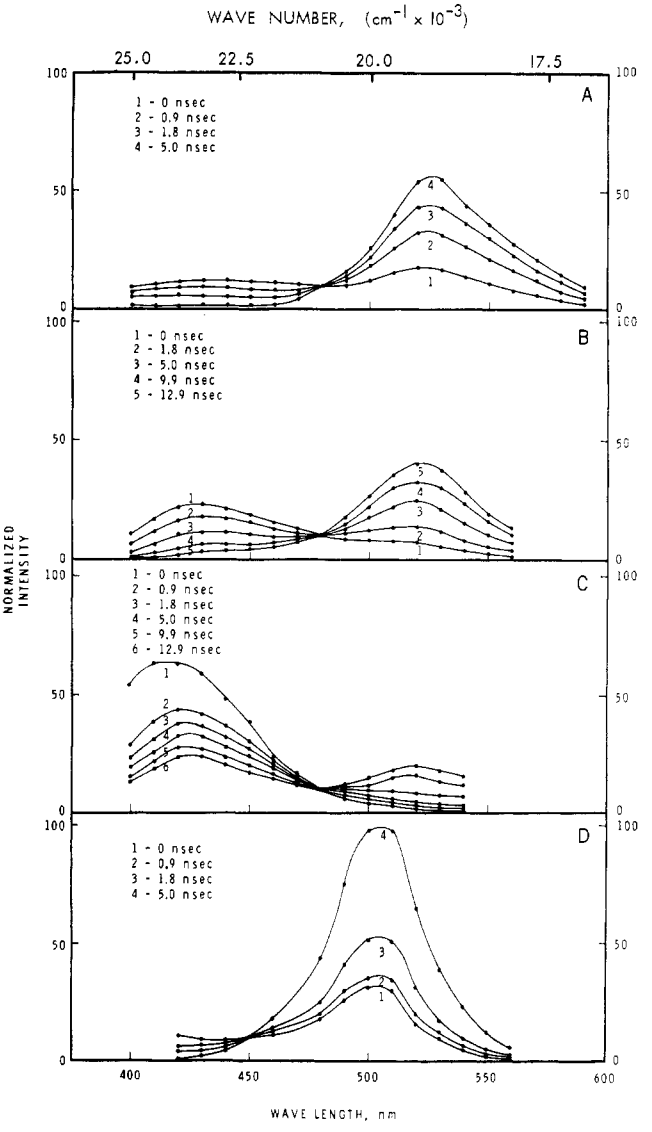

FIGURE 4: Time-resolved emission spectra of: (a) dehydroluciferol, 4 $\times$ 10^{-6} M, in 0.05 M phosphate buffer (pH 7.5); (b) dehydroluciferol plus 5 $\times$ 10^{-5} M luciferase; (c) same as part b plus 2 $\times$ 10^{-3} M ATP–Mg; (d) same as part (b) plus 2 $\times$ 10^{-3} M AMP.

emission with very little emission from the phenolate (at 525 nm). Even after 13 nsec the predominant emission is still from the phenol form.

However, as shown in Figure 4d, AMP causes a shift toward emission from the phenolate form of enzyme-bound L-OH. At zero time there is only a small amount of phenol emission and as was the case with free L-OH in buffer, the phenol emission has completely decayed after 5 nsec. It was not possible to get lower wavelength points due to technical problems involved with collecting data on such low intensity emissions.

Lifetimes. Fluorescent lifetimes for the emitting species were calculated from the decay curves according to the method of moments as described by Isenberg and Dyson (1969) using a computer program written by R. Schuyler.

Although the estimated accuracy of the numbers given in Table II is approximately $\pm 15\%$, the data give strong evidence that the rate of excited-state proton transfer changes both when L-OH binds to luciferase and when substrates and

TABLE II: Average Lifetimes (τ_0) of the Excited-State Phenol Form of Dehydroluciferol.

	Phenol[a] (nsec)
(A) L-OH	0.59
(B) Luciferase + L-OH	1.58
(C) Luciferase + L-OH + ATP–Mg^{2+}	2.6
(D) Luciferase + L-OH + AMP	1.46

[a] All phenol emissions were monitored at 425 nm.

effectors bind. Since, in buffer, the steady-state spectra of L-OH indicate emission almost entirely from the excited-state phenolate, under conditions where the predominant ground-state form is the phenol, there must be rapid proton transfer occurring during the lifetime of the excited state. The effect of such proton transfers from excited-state phenol to solvent is to make the lifetime of the excited phenol quite short. When L-OH binds to luciferase, the rate of excited-state proton transfer seems to decrease since the lifetime of the excited phenol is increased by more than 2.5-fold. The addition of ATP–Mg^{2+} causes a further slowing of the rate of proton transfer in the excited state as evidenced by the further increase in the lifetime of the excited-state phenol. In the presence of AMP there is again rapid proton transfer, and a concomitant decrease in the lifetime of the excited-state phenol. The lifetime of the excited phenolate remains fairly constant at 3.7–4.0 nsec in all cases. These results strongly suggest that the observed steady-state and time-resolved spectral changes are rate effects, affecting the ionization of phenolic hydroxyl during the lifetime of the excited state.

Discussion

It has been reported that there is a significant conformation change which occurs when L and ATP–Mg^{2+} are added to luciferase (De Luca and Marsh, 1967). In addition, Lee and McElroy (1971) suggest that the binding of AMP also causes a conformation change in luciferase. The results of our studies indicate that these conformation changes are significant and their effects are manifested at the active site of luciferase.

The steady-state and time-resolved emission spectra of enzyme-bound L-OH in the presence of ATP–Mg^{2+} indicate that the binding of ATP–Mg^{2+} increases the hydrophobic character of the L-OH binding site as evidenced by the large enhancement of fluorescence at 425 nm and by the blue shift of the phenolate emission (from 555 to 548 nm). The enhancement at 425 nm is due *both* to an increase in K_a when ATP–Mg^{2+} is present and also due to a change in the quantum yield of bound L-OH as evidenced by the 2–2.5-fold increase in the ratio $F_b:F_{free}$. Since this analog does not react to form the adenylate, the mere binding of ATP–Mg^{2+} to luciferase when L-OH is bound is sufficient to cause this change in the active site microenvironment. The steady-state and time-resolved emission spectra of enzyme-bound L-OH in the presence of AMP indicate that although the binding of AMP apparently increases the hydrophobic character around the 6′-hydroxyl group, the proton can still be removed from this group with relative ease in the excited state.

Both these observations (active-site hydrophobicity and analog ionization) seem quite logically to fit into a scheme de-

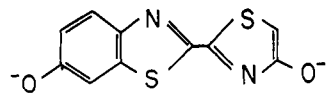

FIGURE 5: Structure of the emitter in luciferase bioluminescence.

picting molecular events leading to light emission. That the dehydroluciferol or luciferin binding site should be more hydrophobic, from the time of adenylate formation through that of O_2 attack and release of AMP, which leads to the chemically produced excited state, is very reasonable if these highly reactive intermediates are not to be destroyed by hydrolytic mechanisms. In addition, such a hydrophobic environment might also decrease the probability of quenching the bioluminescence emitter since the quantum yield for bioluminescence is known to be very high (approximately 1). Whether the AMP portion of L-AMP is sufficient to cause this change at the active site cannot be determined from these results.

The fact that AMP allows transfer of the hydroxyl proton from the excited-state phenol also has possible significance in bioluminescence, inasmuch as yellow-green emission is believed to occur from the excited state of the dianion structure shown in Figure 5, in which the 6′-phenolic hydroxyl is ionized (White *et al.*, 1971). If this dianion is the true emitter, ionization at the 6′ position must occur subsequent to binding, but prior to light emission, since the excitation spectrum of enzyme-bound luciferin (also dehydroluciferin and dehydroluciferol) indicates that the phenol form is the species present in the ground state. The effect of AMP would then be to make this ionization more efficient. It is tempting to speculate that the binding of AMP also causes a proton acceptor to be brought into close proximity to the 6′ hydroxyl causing accelerated proton transfer. The need for a proton acceptor is dictated by the poor proton accepting character of the binding-site microenvironment. The surprising finding that AMP had little or no effect on the emission of luciferase bound L or LH_2 probably attests to the fact L-OH is a better analog of the bioluminescence emitter since it has no highly charged carboxyl group as is the case with L and LH_2. Also, since the proposed emitter is an enol, similar types of interactions might be experienced by both the alcohol function of dehydroluciferol and the OH of this enol when either binds to luciferase.

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