Isolation and Properties of Bacterial Luciferase–Oxygenated Flavin Intermediate Complexed with Long-Chain Alcohols†

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ABSTRACT: Nonsubstrate long-chain aliphatic alcohols, carboxylic acids, and their methyl esters were found to complex reversibly with and stabilize an oxygenated flavin–luciferase intermediate, with alcohols being more effective in stabilizing the intermediate. Dissociation constants for the binding of alcohols to luciferase intermediate are in the order of $K_8 > K_{10} > K_{12} \simeq K_{14}$ where the subscripts represent the numbers of carbon atoms of various alcohols. Thermodynamic activation parameters for the decay of oxygenated flavin–luciferase intermediate complexed with alcohols or aldehydes were determined, and similarities were noted between alcohol and aldehyde complexes. Luciferase intermediate complexes formed with 1-decanol and 1-tetradecanol were isolated at 0 °C in neutral phosphate buffer, and both showed absorption properties characteristic of 4a-substituted dihydroflavins. The 1-tetradecanol–intermediate species contained one flavin per luciferase molecule. Initially this complex was weakly fluorescent, but upon exposure to 370-nm light it was transformed to a highly fluorescent species. The latter shows a fluorescence excitation peak at 370 nm, and its fluorescence emission (λ_{max} 505 nm) and quantum yield (0.17) closely correspond to that of bioluminescence in vitro. Both the weakly and the highly fluorescent species exhibit full bioluminescence activities when reacted with decanal.

Bacterial luciferase catalyzes the bioluminescent oxidation of reduced flavin mononucleotide ($FMNH_2$)[1] and long-chain aldehyde by molecular oxygen to produce FMN, carboxylic acid, water, and light (Shimomura et al., 1972; McCapra & Hysert, 1973; Dune et al., 1973; Hastings & Balny, 1975). Earlier studies demonstrated that such a reaction involved a long-lived oxygenated flavin–luciferase intermediate, designated II, formed by the reaction of O_2 with the enzyme-bound $FMNH_2$ (Hastings & Gibson, 1963). This intermediate has been isolated at subzero temperatures in 50% ethylene glycol–phosphate buffer and characterized spectroscopically (Hastings et al., 1973; Balny & Hastings, 1975; Ghisla et al., 1978) and with respect to reaction products (Hastings & Balny, 1975). Intermediate II has also been isolated at 0 °C in aqueous medium and found to be fully active in reaction with aldehyde to emit bioluminescence in the absence of free O_2 (Becvar et al., 1978). These studies on the isolated intermediate have contributed significantly to a better understanding of the luciferase reaction mechanism, and further investigations of the molecular identity and properties of II are of fundamental importance in elucidating the nature of luciferase catalysis.

Previously, it has been shown, by using *Photobacterium fischeri* luciferase, that no long-chain aliphatic compounds other than aldehydes are active in reacting with luciferase to produce light, but some of them, such as 1-decanol and 2-decen-1-ol, are inhibitors competitive with aldehyde (Hastings et al., 1966). A recent kinetic study on *Beneckea harveyi* luciferase also indicated the reversible formation of a 1-decanol–II complex which was more stable at 22 °C than II itself (Baumstark et al., 1979). In this report, we describe the effects of various long-chain aliphatic alcohols, carboxylic acids, and their methyl esters on the thermal stability and bioluminescence capacity of *B. harveyi* intermediate II. The isolation of II–alcohol complexes has been achieved, and the spectral properties of the isolated complexes are described.

Experimental Procedures

Materials. Luciferase was purified by the previously described procedure (Gunsalus-Miguel et al., 1972) from cells of *B. harveyi* strain MAV 392 (Reichelt & Baumann, 1973). Luciferase concentrations were determined based on a molecular weight of 79 000 and an absorption coefficient of 1.2 (0.1%, 1 cm) at 280 nm (Tu et al., 1977). Bovine serum albumin and FMN were obtained from Sigma Chemical Co., and long-chain aldehydes were from Aldrich Chemical Co. 1-Decanol, 1-tetradecanol, decanoic acid, lauric (dodecanoic) acid sodium salt, and myristic (tetradecanoic) acid were products of Eastman Kodak Co. 1-Octanol and 1-dodecanol were from Applied Science Laboratories and Matheson Coleman and Bell, respectively. Methyl esters of decanoic, dodecanoic, and tetradecanoic acid were obtained from Supelco. Reagent solutions (0.1–10 mM) of long-chain alcohols, carboxylic acids, and their methyl esters were prepared in 95% ethanol, and stocks of aldehydes (0.1%) were prepared as sonicated suspensions in water.

Assays of Luciferase and of Intermediate Stability. Luciferase activity was measured in a calibrated photometer (Mitchell & Hastings, 1971; Hastings & Weber, 1963) by two nonturnover methods. In the standard assay, the reaction was initiated by the injection of 1 mL of an anaerobic solution containing 5×10^{-5} M $FMNH_2$ (prepared by catalytic reduction of FMN with H_2 over platinum) in phosphate buffer, pH 7, into an equal volume of aerobic phosphate buffer containing luciferase, 0.2% bovine serum albumin, and optimal amounts [$(4–6) \times 10^{-5}$ M; approximately 0.001–0.0015%] of the desired aldehyde. The light intensity reaches a maximum in <1 s after the introduction of $FMNH_2$ and then decreases exponentially. The first-order rate constant of the latter phase of the emission reaction is affected by the type of aldehyde used and thus provides a measure of aldehyde–II stability.

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[1] Abbreviations used: $FMNH_2$ and FMN, reduced and oxidized flavin mononucleotide, respectively; II, oxygenated flavin–luciferase intermediate.

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An alternative assay method, referred to as the intermediate assay, involves the use of intermediate II or its complexes with nonaldehyde aliphatic compounds preformed at a designated temperature. For the formation of the latter complex species, a 10-μL droplet of phosphate buffer containing 40 μg of luciferase and a second droplet (1–20 μL) of a solution of an aliphatic compound were placed without mixing at the bottom of a reaction vial (2.5-cm diameter, 4.5-cm height). The desired complex species was then formed by injecting 1 mL of an anaerobic $FMNH_2$ solution (5×10^{-5} M) into the vial under aerobic conditions. A thorough mixing of all three solutions was achieved by the introduction of the $FMNH_2$ solution. Intermediate II was prepared similarly by introducing the $FMNH_2$ solution into a vial containing luciferase solution only. With each of these preformed intermediate species, the capacity to emit bioluminescence was determined by introducing 50-μL aliquots, withdrawn at different times, into 1 mL of aerobic buffer containing 2.6×10^{-4} M (0.005%) decanal. After the solution of preformed II or its aliphatic compound complex was allowed to stand at a designated temperature, the bioluminescence potential, measured by intermediate assays, decreased exponentially as a function of time. Stabilities of various intermediate species were then determined by following the rates of such decreases.

The amounts of aldehyde used in the intermediate assay were several times larger than those used in the standard assay. This is because luciferase activity is subject to aldehyde substrate inhibition in standard but not intermediate assays (Meighen & MacKenzie, 1973). In both assays, the bioluminescence activity was measured either as the initial maximal intensity (I_0) in quanta s^{-1} or as the integrated total light output (Q) in quanta. Phosphate buffer (0.1 M, pH 7) was used throughout the study, and, unless stated otherwise, all assays were carried out at 23 °C.

Spectral Measurements. Absorption spectra were measured with a Varian Cary 118 spectrophotometer. Fluorescence excitation and emission spectra were recorded with a Perkin-Elmer MPF-44A fluorescence spectrophotometer using a 0.3-cm path length microcuvette. Fluorescence spectra were corrected for wavelength-dependent variations in light source output, phototube response, and monochromator efficiency.

Results

Effects of Long-Chain Aliphatic Compounds on the Stability of II. Stabilities of intermediate II and its complexes with nonaldehyde aliphatic compounds were determined by the intermediate assay described above. A typical experiment illustrating the effect of 1-decanol on the stability of II at 23 °C is shown in Figure 1. The apparent first-order decay of II is progressively retarded at increasing 1-decanol concentrations. At saturating concentrations of 1-decanol (concentrations of 0.1–0.25 mM were tested), the decay rate stabilized at a value of 0.21 min^{-1}, 20-fold slower than the decay of II itself. Similar effects were also observed with the other aliphatic alcohols, carboxylic acids, and their methyl esters tested in this study, and the decay rates of II at saturating levels of these aliphatic compounds are summarized in Table I. Stabilities of aldehyde–II complexes, determined by the decay rates of the capacity for luminescence in the standard assay using different aldehydes, are also shown for comparison. None of the nonaldehyde aliphatic compounds tested reacted with II to emit light. The association of these compounds with II is apparently reversible; all complexes exhibited full bioluminescence light yields when reacted with excess amounts of decanal, confirming the results of earlier, similar studies (Hastings et al., 1966; Baumstark et al., 1979). Alcohols are, in general, more effective in stabilizing II and were chosen for subsequent studies.

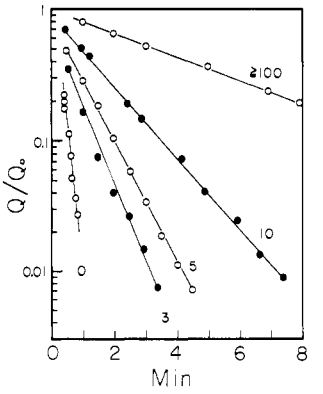

FIGURE 1: Effect of 1-decanol on the decay rate of intermediate II. Intermediate II was prepared and kept at 23 °C in 0.1 M phosphate, pH 7, in the absence or presence of 1-decanol as described in the text. The final concentrations of 1-decanol, in micromolarity, are indicated for each intermediate sample. Aliquots (50 μL each) were withdrawn at different times and injected into 1 mL of aerobic buffer containing 0.26 mM (0.005%) decanal at 23 °C for bioluminescence quantum output (Q) measurements. The rates of the decrease in the remaining bioluminescence capacity provide measures of the apparent first-order decay of intermediate samples. Q_0 is the sample bioluminescence quantum output measured at time zero.

Table I: Decay Rates of Aliphatic Compound–II Complexes at 23 °C

aliphatic compd	k^a (min^{-1})
none	4.33
$CH_3(CH_2)_7OH$	0.12
$CH_3(CH_2)_9OH$	0.21
$CH_3(CH_2)_{11}OH$	0.04
$CH_3(CH_2)_{13}OH$	0.09
$CH_3(CH_2)_8COOH$	1.73
$CH_3(CH_2)_{10}COOH$	1.73
$CH_3(CH_2)_{12}COOH$	0.26
$CH_3(CH_2)_8COOCH_3$	0.14
$CH_3(CH_2)_{10}COOCH_3$	0.63
$CH_3(CH_2)_{12}COOCH_3$	1.28
$CH_3(CH_2)_6CHO$	2.39
$CH_3(CH_2)_8CHO$	17.33
$CH_3(CH_2)_{10}CHO$	2.31
$CH_3(CH_2)_{12}CHO$	6.30

[a] Determined in 0.1 M phosphate, pH 7. In one series of samples, II was prepared in the presence of saturating levels (20–200 μM) of nonaldehyde aliphatic compounds. Aliquots (50 μL each) were withdrawn at different times, and each was introduced into a 1-mL aerobic solution containing 0.26 mM decanal to initiate the light emission. The stability of each complex was determined by following the rate of decrease in bioluminescence potential of the sample as a function of time. The stabilities of aldehyde–II complexes were determined by following the rates of the exponential decay phase of light emission in standard assays using 0.4–0.6 μM aldehydes.

Dissociation Constants for the Binding of Alcohols to II. The observed rates of the decay of II at different alcohol concentrations can be analyzed according to the following scheme to determine the dissociation constant (K_d) of alcohol binding to II.

$$\begin{array}{ccc} \text{II} + \text{H} & \underset{k_2}{\overset{k_1}{\rightleftharpoons}} & \text{IIH} \\ \downarrow k_{\text{II}} & & \downarrow k_{\text{IIH}} \\ \text{fast decay} & & \text{slow decay} \end{array} \qquad (1)$$

In this scheme, II, H, and IIH are intermediate II, alcohol,

and the alcohol–II complex, respectively; k_1 and k_2 are the on and off constants, respectively, for the alcohol binding; k_{II} and k_{IIH} are the first-order constants for the decay of II and IIH, respectively. Assuming that the binding of H to II reaches a fast equilibrium, which requires that k_1 and k_2 are both much greater than either k_{II} or k_{IIH}, the dissociation constant can be expressed as

$$K_d = \frac{k_2}{k_1} = \frac{(II)(H)}{(IIH)} \quad (2)$$

where (II), (H), and (IIH) are the molar concentrations of II, H, and IIH, respectively, at any specified time point. The total concentration of free and complexed intermediate II, $(II)_{total}$, will follow the relationships

$$(II)_{total} = (II) + (IIH) = (II)[1 + (H)/K_d] \quad (3)$$

$$\frac{d(II)_{total}}{dt} = -[(II)k_{II} + (IIH)k_{IIH}] = -(II)_{total}\left[\frac{k_{II} + (H)k_{IIH}/K_d}{1 + (H)/K_d}\right] \quad (4)$$

When the total concentration of alcohol, $(H)_{total}$, is much higher than $(II)_{total}$, i.e., $(H)_{total} \simeq (H)$, the decay of $(II)_{total}$ will follow apparent first-order kinetics

$$\log\left[\frac{(II)_{total}}{(II)_0}\right] = -\frac{k_{II}K_d + (H)k_{IIH}}{2.3[K_d + (H)]}t \quad (5)$$

where $(II)_0$ is the $(II)_{total}$ at time zero and t is the time. In the absence of alcohol, eq 5 reduces to

$$\log\left[\frac{(II)_{total}}{(II)_0}\right] = -\frac{k_{II}}{2.3}t \quad (6)$$

thus describing the exponential decay of intermediate II. When excess amounts of alcohol were used, i.e., under conditions of $(H)/K_d \gg k_{II}/k_{IIH}$, eq 5 can be simplified to

$$\log\left[\frac{(II)_{total}}{(II)_0}\right] \simeq -\frac{k_{IIH}}{2.3}t \quad (7)$$

which defines the kinetics of the IIH decay. In eq 5–7, the ratio of $(II)_{total}/(II)_0$ for a sample can be substituted by Q/Q_0 where Q_0 and Q are the total bioluminescence quantum output of the sample measured at time zero and another defined time point, respectively, by the intermediate assay. It is known that luciferase bioluminescence light output is linearly proportional to the amount of enzyme used in the in vitro assay over a wide enzyme concentration range (Hastings et al., 1966). Furthermore, equal quantities of II and IIH have been found, in both earlier (Hastings et al., 1966; Baumstark et al., 1979) and the present studies, to exhibit the same total light output when reacted with saturating levels of decanal. Therefore, the ratio of Q/Q_0 will be identical in value with that of $(II)_{total}/(II)_0$ for samples containing either II or IIH or both.

The results shown in Figure 1 were analyzed according to eq 5 as described above. Apparent first-order kinetics of decay were observed at various 1-decanol concentrations, strongly supporting the assumption of a fast equilibrium for the complex formation. Values of k_{II} and k_{IIH} were determined at zero and saturating levels of 1-decanol, respectively, and K_d was then calculated based on these values and slopes of the semilogarithmic plot of results obtained at subsaturating alcohol concentrations. Similar experiments were also carried out with other alcohols, each using at least four subsaturating and two saturating alcohol concentrations, and values of K_d are summarized in Table II.

Table II: Dissociation Constants for Alcohol Binding to II at 23 °C

alcohol	K_d[a] × 10^7 (M)
$CH_3(CH_2)_7OH$	24 ± 2
$CH_3(CH_2)_9OH$	12 ± 1
$CH_3(CH_2)_{11}OH$	0.42 ± 0.02
$CH_3(CH_2)_{13}OH$	0.51 ± 0.06

[a] Determined in 0.1 M phosphate, pH 7.

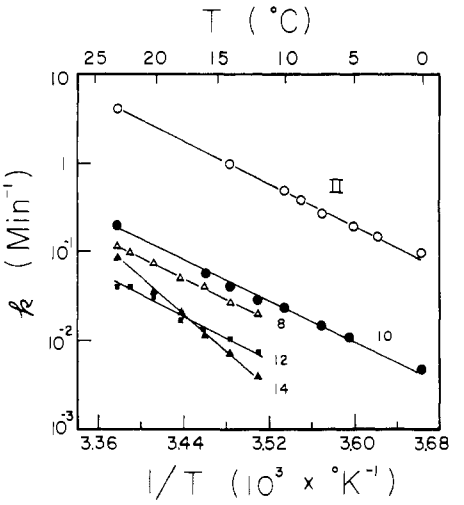

FIGURE 2: Effect of temperature on the decay rates of II and alcohol–II complexes. Decay rates of II (○) and its complexes with 1-octanol (△), 1-decanol (●), 1-dodecanol (■), and 1-tetradecanol (▲) were determined at different temperatures by using saturating levels of alcohols as described in Figure 1. The results are presented as Arrhenius plots.

Effects of Temperature on the Decay of Alcohol- and Aldehyde–II Complexes. The stabilities of II complexed with alcohols or aldehydes were determined at different temperatures. Complexes of II with 1-octanol, 1-decanol, and 1-dodecanol are associated with values of the energy of activation for the decay (E_a) similar to that for II whereas the E_a for 1-tetradecanol–II decay is much higher (Figure 2). Consequently, the lifetime of this latter complex is greatly lengthened at lower temperatures, e.g., $t_{1/2} > 7$ h at 0 °C (data not shown). The decay rates (k) of II complexed with 8-, 10-, and 12-carbon aldehydes were similarly affected by temperature, but a significantly higher temperature dependence was observed with tetradecanal–II decay (Figure 3).

From results shown in Figures 2 and 3, thermodynamic activation parameters can be calculated according to the relationships (Lehrer & Barker, 1970)

$$\Delta H^{\ddagger} = E_a - RT \quad (8)$$

$$\Delta S^{\ddagger} = 4.576[\log k - 10.753 - \log T + E_a/(4.576T)] \quad (9)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (10)$$

where $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ are the free energy, enthalpy, and entropy of activation, respectively, k is the first-order decay constant (in s^{-1}), R is the gas constant, and T is the absolute temperature. Results of such calculations are shown in Table III.

Effect of Temperature on Bioluminescence Quantum Yield of Aldehyde–II Complexes. In the $FMNH_2$-initiated assay, the light intensity (reaction rate in quanta s^{-1}) quickly reaches a maximum (I_0) and then decays exponentially. The total

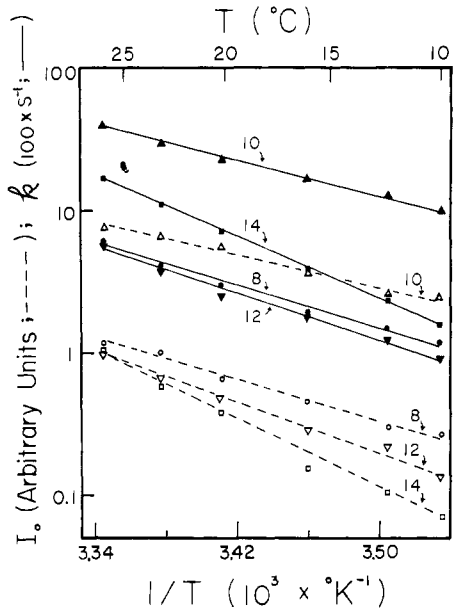

FIGURE 3: Effect of temperature on initial maximal light intensities (I_0; open symbols) and first-order rate constants (k; solid symbols) of the bioluminescent decay of aldehyde–II complexes. The bioluminescence reaction of luciferase samples, each containing 25 μg of enzyme, was initiated by the addition of $FMNH_2$ in the standard assay at different temperatures using optimal amounts of octanal (○, ●), decanal (△, ▲), dodecanal (▽, ▼), or tetradecanal (□, ■). Both I_0 and k are presented as a function of temperature in the form of Arrhenius plots. One arbitrary unit of I_0 is equivalent to 3.9×10^{11} quanta s^{-1}. In assays at ≤20 °C, using octanal and tetradecanal, a fast-decaying flash lasting <1 s was observed at the onset of the reaction, probably due to slight contamination in the aldehydes and/or the enzyme sample. After the brief flash, bioluminescence emission decayed exponentially, allowing an extrapolation to zero time for the determination of I_0. Intensities of flashes were in the range of 5–30% higher than the corresponding I_0 values.

Table III: Thermodynamic Activation Parameters for the Decay of Alcohol- and Aldehyde-II Complexes[a]

		at 296 K		
aliphatic compd	E_a (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	$\Delta G^{\ddagger}$ (kcal/mol)
none	27.2	26.6	26.2	18.9
$CH_3(CH_2)_7OH$	27.8	27.2	21.2	21.0
$CH_3(CH_2)_9OH$	25.0	24.4	12.6	20.7
$CH_3(CH_2)_{11}OH$	27.8	27.2	18.9	21.6
$CH_3(CH_2)_{13}OH$	47.3	46.7	86.3	21.2
$CH_3(CH_2)_6CHO$	16.1	15.5	−12.6	19.2
$CH_3(CH_2)_8CHO$	13.8	13.2	−16.3	18.0
$CH_3(CH_2)_{10}CHO$	18.6	18.0	−4.2	19.2
$CH_3(CH_2)_{12}CHO$	26.5	25.9	24.6	18.6

[a] Determined from results in Figures 2 and 3.

quantum output, Q, of a single assay can be expressed as $Q = I_0/k$ where k is the first-order rate constant (s^{-1}) for the light decay. Values of I_0 and k, measured with different aldehydes over the range of 10–26 °C, are both shown in Figure 3 as Arrhenius plots. Slopes of such plots of both I_0 and k as a function of reciprocal temperature are essentially the same for octanal, decanal, or dodecanal, indicating that the ratio of I_0/k (and, hence, the Q) remained constant for each of these three aldehydes. The Q for the tetradecanal–II complex decreased only slightly at lower temperatures. It should be noted that the bioluminescence quantum yields differ somewhat for different aldehydes.

Isolation and Spectral Properties of the Alcohol–II Complex. The 1-tetradecanol–II complex was isolated at 0 °C by

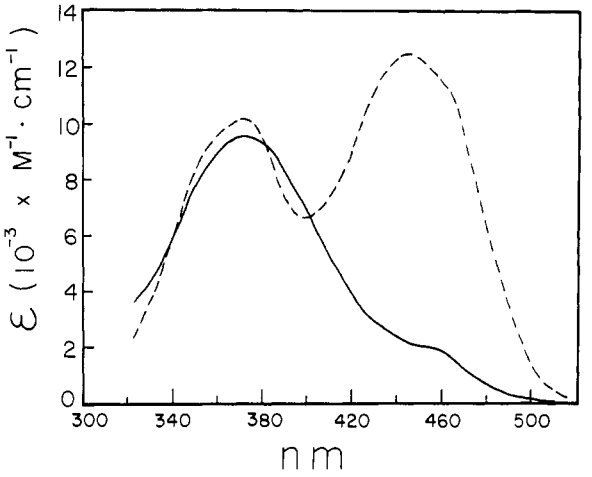

FIGURE 4: Absorption spectra of 1-tetradecanol–II and the flavin decay product. The 1-tetradecanol–II species was isolated at 0 °C in neutral phosphate buffer as described in the text, and the absorption spectrum (—) was recorded immediately at the same temperature by using a blank containing the same concentration of luciferase. Subsequently, the sample was warmed to and kept at 23 °C for 3 h for a complete decay of the intermediate and cooled down to 0 °C, and the spectrum (---) was recorded against the blank described above. The spectrum of the flavin decay product is identical with that of authentic FMN. The extinction coefficients of the 1-tetradecanol–II species are calculated based on the known extinction coefficients of FMN.

Sephadex G-25 column chromatography in 0.1 M phosphate, pH 7, by a modification of the previously described method (Becvar et al., 1978). In a typical run, a 0.5-mL buffer solution containing 1.5 mg of luciferase and 5×10^{-5} M FMN was mixed aerobically at 0 °C with 5 μL of 10 mM 1-tetradecanol in 95% ethanol. Some precipitates of 1-tetradecanol were formed, but no significant precipitation of protein species was encountered. To this, a few milligrams of sodium dithionite were added to reduce the FMN, and the solution was quickly applied, under aerobic conditions, to a Sephadex G-25 column (1 × 16 cm) preequilibrated and eluted at 0 °C with aerobic buffer (0.1 M phosphate, pH 7) containing approximately 5 μM 1-tetradecanol. The protein species was eluted in the void volume and separated from smaller molecular weight reagents, such as free FMN, dithionite, and its oxidized products. The entire operation was carried out under red light. A 1.5-mL fraction containing the highest protein concentration was used for bioluminescence activity and spectral measurements. The absorption spectrum of the isolated intermediate is shown in Figure 4, together with that of the final product of the decay reaction, i.e., FMN, for comparison. Based on the FMN content (determined after standing at 23 °C for 3 h) and the protein concentration of several 1-tetradecanol–II samples, the molar ratio of flavin to luciferase was found to be approximately 0.9. The 1-decanol–II complex was also similarly isolated and found to exhibit an absorption spectrum essentially identical with that of 1-tetradecanol–II.

The isolated 1-tetradecanol–II complex initially exhibited weak fluorescence at 480 nm upon excitation at 370 nm but increased 13-fold to a maximal level in about 1 min at 3 °C (Figure 5 inset) and remained at this level for at least 10 min. The corrected fluorescence excitation (λ_{max} 370 nm) and emission (λ_{max} 505 nm) spectra of this photoinduced fluorescent species are shown in Figure 5 in comparison with the emission spectrum (λ_{max} 535 nm) of FMN. The fluorescence quantum yield of this photoinduced species was calculated to be 0.17 by using free FMN (fluorescence quantum yield 0.24) as a standard (Moore et al., 1977). The true emission spectrum of the initially weakly fluorescent 1-tetradecanol–II

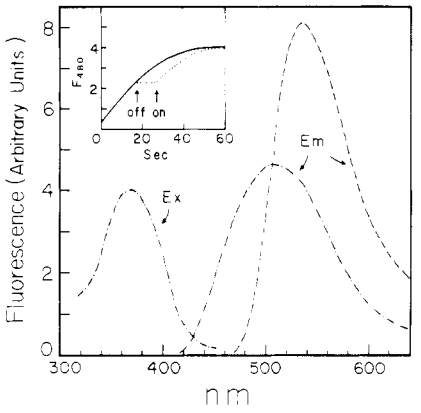

FIGURE 5: Corrected fluorescence spectra of FMN and 1-tetradecanol–II_y obtained from 1-tetradecanol–II_x by irradiation at 370 nm. The inset shows the time course (—) for the conversion of 1-tetradecanol–II_x to the corresponding II_y species upon exposure to 370-nm light, followed by measuring the increase in fluorescence emission at 480 nm. The transformation of alcohol–II_x to –II_y species can be stopped by cutting off the excitation light and resumed at a later time by reexposing to light (···). After 1 min of exposure to 370-nm light, the 1-tetradecanol–II_y species was used for the determination of fluorescence excitation and emission spectra (–·–), with emission detected at 480 nm for the former and excitation set at 370 nm for the latter measurement. The fluorescence emission of FMN (- - -) upon excitation at 370 nm is shown for comparison. Fluorescence intensities of 1-tetradecanol–II_y and FMN are normalized to correspond to the same absorbance at 370 nm for both samples. All measurements were carried out at 3 °C. Em, emission spectrum; Ex, excitation spectrum.

complex was not obtained due to its instability in the presence of the excitation light. However, an emission spectrum recorded during a 45-s exposure to 370-nm excitation light showed a substantial shoulder at 535 nm in addition to a 505-nm peak. Although light affected both the fluorescence intensity and emission spectrum of the tetradecanol–II complex, the total bioluminescence capacities of the complex (determined by reacting with excess amounts of decanal) remained unchanged before and after 1 min of exposure to 370-nm light, both being identical with that of the same amount of luciferase determined in standard assays.

Discussion

Although the specificity of different long-chain aldehydes in the bacterial bioluminescence reaction has been studied for both *P. fischeri* (Hastings et al., 1963; Spudich & Hastings, 1963) and *B. harveyi* (Hastings et al., 1969) systems, relatively little is known about the nature of interactions of other aliphatic compounds with luciferase (Hastings et al., 1966; Baumstark et al., 1979). In the present study we found that long-chain aliphatic alcohols, carboxylic acids, and acid methyl esters all associate, reversibly, with *B. harveyi* intermediate II and stabilize it (Table I). In this respect, the luciferase system is quite interesting in comparison with other external flavoprotein monooxygenases (or hydroxylases). In addition to luciferase, the involvement of oxygenated flavin species as reaction intermediates has been noted for several other hydroxylases such as *p*-hydroxybenzoate hydroxylase (Spector & Massey, 1972a,b; Entsch et al., 1976), melilotate hydroxylase (Strickland & Massey, 1973), phenol hydroxylase (Massey & Hemmerich, 1975), and salicylate hydroxylase (Presswood & Kamin, 1976). Although the binding of substrates and nonsubstrate effectors to hydroxylases has been known to enhance the rate of reduction of enzyme-bound flavins and, in some cases, the subsequent oxygenation of reduced flavins (Massey & Hemmerich, 1975), the stabilization of an oxygenated flavin intermediate by nonsubstrate effectors has so far only been observed with luciferase.

Aliphatic alcohols are more effective than acids and acid methyl esters in stabilizing II (Table I), but this apparently cannot be ascribed to differences in charge. Although the stability of all the complexes tested so far is sensitive to the chain length of aliphatic compounds, no clear correlation was obtained.

Previously, it has been shown that II decays in the absence of aldehyde to yield stoichiometric amounts of FMN and H_2O_2 or, alternatively, reacts with aldehyde to form light, carboxylic acid, and FMN (and presumably water), but no H_2O_2 (Hastings & Balny, 1975). Since nonaldehyde aliphatic compounds are inactive in the light-emitting monooxygenation reaction, the detailed mechanisms of the decay of alcohol– and aldehyde–II complexes must be different. Nevertheless, interesting similarities are observed between the properties of these two types of complexes. Previous studies on *P. phosphoreum* (Yoshida et al., 1974) and *P. fischeri* (Hastings & Nealson, 1977) luciferases have demonstrated that longer chain aldehydes are associated with higher affinities in binding with II, as indicated by lower K_m values in activity assays. Similarly, the present study showed that the values of K_d for alcohol binding to *B. harveyi* intermediate II are in the order of $K_8 > K_{10} > K_{12} \simeq K_{14}$ with respect to alcohol carbon atoms (Table II). For both alcohol– and aldehyde–II complexes, little differences in thermodynamic activation properties are observed when the aliphatic chain lengths are 12 carbons or less, whereas complexes containing a 14-carbon alcohol or aldehyde exhibit substantially higher values in E_a, $\Delta H^{\ddagger}$, and, especially, $\Delta S^{\ddagger}$ (Table III). The high $\Delta S^{\ddagger}$ values for the decay of 1-tetradecanol– and tetradecanal–II complexes indicate that their thermal activations to transition states are associated with substantial increases in the disorder of molecular structures.

Intermediate II has been postulated to be a 4a-peroxydihydroflavin species based on absorption (Hastings et al., 1973), fluorescence (Balny & Hastings, 1975), and nuclear magnetic resonance (Ghisla et al., 1978) characterizations of the isolated II in organic–aqueous mixtures at subzero temperatures. Chemical model studies also demonstrated that 4a-hydroperoxyflavins reacted with aldehydes to emit chemiluminescence (Kemal & Bruice, 1976; Kemal et al., 1977). However, Murphy et al. (1974) reported the isolation, in an aqueous solution at 0 °C, of a long-lived luciferase intermediate which was free from flavin but fully active in reacting with aldehyde for light emission. Attempts to confirm the latter results were unsuccessful (Becvar et al., 1978); the isolated species again exhibited an absorption characteristic of 4a-substituted dihydroflavins, and the bioluminescence capacity was found to be proportional to the flavin content. Questions concerning the fact that the isolated II had a flavin/luciferase molar ratio of only about 0.5 were recently raised (Hart & Cormier, 1979). This is perhaps related to the slow but appreciable decay of II at 0 °C. In the present study, similar procedures have been followed to isolate II as alcohol complexes. The isolated 1-tetradecanol–II complex exhibited an absorption spectrum (Figure 4) essentially identical with that of II at −20 °C, contained very nearly one flavin per luciferase molecule, and showed full bioluminescence activity, with respect to the flavin content, upon reacting with excess amounts of decanal. The flavoprotein nature of active intermediate II thus appears to be certain.

Balny & Hastings (1975) have observed that exposure of II to light at 370 nm resulted in about a fivefold enhancement of fluorescence intensity and a blue shift of the fluorescence emission from a peak at 505 to 485 nm (both uncorrected).

The terms II_x and II_y were proposed for the species prior to and after the phototransition, respectively. Although the 370-nm light illumination affected the fluorescence emission of intermediate II, no differences were observed in the absorption spectra (λ_{max} at 370 nm) for II_x and II_y. Furthermore, both II_x and II_y reacted with decanal at 23 °C to emit about the same total amount of light. Three alternative schemes have been postulated to describe possible mechanistic roles of II_x and II_y, but differences in the molecular structure of these two species remain obscure (Balny & Hastings, 1975). Interestingly, the fluorescence properties and the bioluminescence potential of 1-tetradecanol–II are closely similar to those of II. Exposure to 370-nm light also transforms the alcohol–II complex from the II_x to the II_y type, except that a 13-fold fluorescence increase was observed (Figure 5). The fluorescence emission of II_y, previously reported as an uncorrected spectrum (Balny & Hastings, 1975), was found to be identical with the uncorrected emission spectrum of 1-tetradecanol–II_y. As is the case with II_x and II_y, no differences in absorption spectra for 1-tetradecanol–II_x and –II_y complexes were found. An important finding is that both the emission spectrum (λ_{max} 505 nm, or 485 nm uncorrected) and the quantum yield (0.17) of alcohol–II_y fluorescence correlate well with those of the in vitro bioluminescence (Hastings & Nealson, 1977). This suggests that the chemical nature and the surrounding microenvironment of the light-excited flavin species in alcohol–II_y are similar to those of the bioluminescence emitter generated from the decay of the aldehyde–II complex.

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