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Activity and Stability of the Luciferase–Flavin Intermediate†

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ABSTRACT: A luciferase intermediate in the bacterial bioluminescence system, which is formed by reaction of enzyme with reduced flavin mononucleotide ($FMNH_2$) and oxygen, is shown to emit light with added aldehyde under anaerobic conditions. The reaction with oxygen is thus effectively irreversible under the conditions used. The flavin chromophore has an absorption maximum at about 370 nm and the potential activity (bioluminescence yield) in the further reaction of the isolated intermediate with aldehyde is strictly proportional to the amount of this flavin chromophore.

Hastings et al. (1973) utilized low temperature techniques to isolate an enzymatic intermediate in the bacterial bioluminescence reaction formed by the luciferase-catalyzed reaction of $FMNH_2$ with O_2 which, based on its spectral characteristics, was postulated to be a luciferase–4a-peroxydihydroflavin complex. Bioluminescence occurs simply upon reacting the intermediate with aldehyde at 25 °C. If the reaction with oxygen is effectively irreversible, then the final steps of the reaction, either via decomposition (without aldehyde) or with luminescence, should occur in the absence of free molecular oxygen. The experiments described here demonstrate that this is the case. This contrasts with the report of Entsch et al. (1976) that the reaction of oxygen with the reduced flavin of *p*-hydroxybenzoate hydroxylase is reversible.

Although the initial isolation (Hastings et al., 1973) of the intermediate was accomplished by chromatography at −30 °C in phosphate-buffered 50% ethylene glycol, the lifetime of the intermediate in aqueous solution at 0 °C is such that it can be isolated and studied at this temperature if the steps are executed promptly. Using such techniques, Murphy et al. (1974) described the isolation of an active intermediate, but one reportedly nearly devoid of flavin. In view of this surprisingly different result and of the profound mechanistic implications if bioluminescence potential is indeed maintained in an aproprotein species, we repeated the isolation under solvent conditions and procedures essentially identical with those of Murphy et al. (1974). Using luciferases purified from two different bacterial species, *Beneckea harveyi* and *Photobacterium fischeri* (Becvar et al., 1976, 1977), we are unable to confirm their report. We find that the intermediates from both luciferases possess a flavin moiety with an absorption spectrum peaking at about 370 nm and an activity (bioluminescence) potential which is strictly proportional to the amount of this flavin intermediate. Upon conversion to product (without aldehyde) we observed clearly defined isosbestic points indicating the existence of only two principal flavin chromophores in the reaction under these conditions.

Experimental Procedures

Glucose oxidase (type II dry powder), catalase (twice crystallized from beef liver as crystalline suspension), NADH (grade III), DL-dithiothreitol, and FMN were obtained from Sigma. FMN was not purified further since studies revealed that the use of more purified material did not affect the spectral and quantum yield results. FMN concentrations were based on an absorptivity of 12 500 M^{-1} cm^{-1} at 445 nm (Beinert, 1960).

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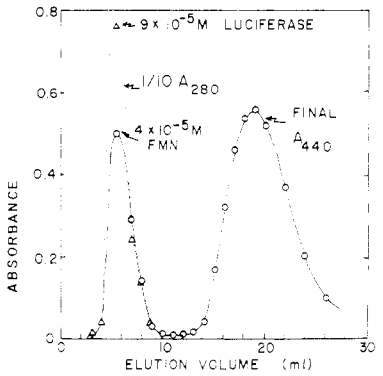

FIGURE 1: Chromatography (isolation) of intermediate II. A 1-mL sample containing 17 mg (220 nmol) of *P. fischeri* luciferase, 500 nmol of FMN, and about 1 mg of sodium dithionite in 0.3 M phosphate, pH 7.0, at 0 °C was applied to a 1 × 18 cm jacketed column of Sephadex G-25 preequilibrated with the same buffer at 0 °C and eluted at a flow rate of 3 mL/min. Samples of 1 mL were collected and maintained on ice until analysis. The absorbance of the fractions which eluted around 20 mL (FMN) did not change with time whereas that of fractions at 5–9 mL (intermediate II), which initially were visibly very pale yellow, increased at 440 nm with time to the final values shown.

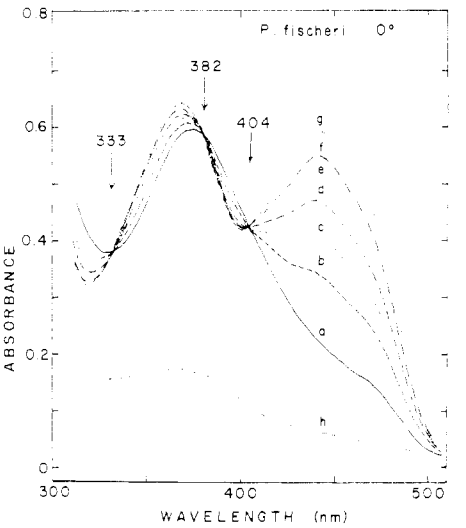

FIGURE 2: Time-dependent spectral changes of isolated *P. fischeri* intermediate II. Fraction 6 from a preparation as in Figure 1 was placed into a cuvette maintained at 0 °C and spectra a to g were recorded successively with time. The minutes which had elapsed since application of the mixture onto the column are (a) 13.1, (b) 22.1, (c) 31.2, (d) 44, (e) 58, (f) 81, and (g) 169. These times represent the moment when 440 nm was recorded for each spectrum. A spectrum of the *P. fischeri* luciferase at the same concentration as in the intermediate sample (A_{280} = 10.5 cm^{-1}) is also shown (h).

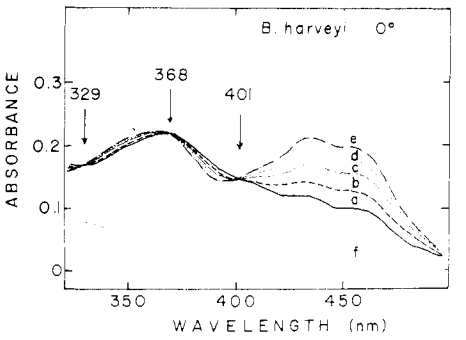

FIGURE 3: Time-dependent spectral changes of isolated *B. harveyi* intermediate II, carried out as in Figure 2. Spectra were recorded at (a) 10.8, (b) 22.7, (c) 52.0, (d) 102, and (e) 184 min after applying the $FMNH_2$-luciferase sample to the column. A spectrum of the *B. harveyi* luciferase at the same concentration as in the intermediate sample (A_{280} = 4.2 cm^{-1}) is shown (f).

Phosphate buffers were the appropriate dilution of a 1 M stock of the dibasic potassium and monobasic sodium salts, pH 7.0 (at 25 °C, 0.1 M). The 0.1% decanal (Aldrich) aqueous suspension was prepared by sonication.

Luciferases from *Photobacterium fischeri* and *Beneckea harveyi* (Reichelt & Baumann, 1973) were purified by methods previously described (Gunsalus-Miguel et al., 1972; Baldwin et al., 1975), but elution of the enzymes from the second DEAE-Sephadex column was accomplished with 0.3 M phosphate buffer, pH 7, to better resolve luciferase and the light-inducible protein form of the enzyme (Mitchell & Hastings, 1970; Tu & Hastings, 1975). The luciferase preparations were judged to be greater than 95% homogeneous by disc gel and sodium dodecyl sulfate gel electrophoresis. Enzyme concentration was determined spectrophotometrically using a weight absorptivity of 1.2 (0.1%, 1 cm) at 278 nm (Tu et al., 1977) and a molecular weight of 79 000, although recent results in this laboratory indicate the actual molecular weight may be slightly (~5%) lower. Luciferase activity was measured in a calibrated photometer (Mitchell & Hastings, 1971; Hastings & Weber, 1963) by injection of 1 mL of 5×10^{-5} M $FMNH_2$ (catalytically reduced by H_2/Pt asbestos) into 1 mL of assay buffer (0.02 M phosphate, pH 7, 0.2% bovine serum albumin, and the optimal amount of a 0.1% (v/v) sonicate of decanal in water) containing enzymes. The specific activities (25 °C) of the luciferases with decanal were 1.6×10^{14} quanta s^{-1} A_{280}^{-1} mL^{-1} from *P. fischeri* and 1.9×10^{14} quanta s^{-1} A_{280}^{-1} mL^{-1} from *B. harveyi*. The activity of intermediate II samples was determined by withdrawing a measured volume (routinely 50 μL) of the sample into a Hamilton syringe (prechilled on ice) and rapidly injecting the sample into 1 mL of assay buffer at 25 °C.

NAD(P)H:FMN oxidoreductase was purified from *P. fischeri* as previously described (Gunsalus-Miguel et al., 1972) and assayed spectrophotometrically by the 340-nm absorbance decrease (Duane & Hastings, 1975) using 10–50 μL of reductase in 1 mL of 0.01 M phosphate, pH 7, containing 0.27 mM NADH and 0.1 mM FMN. The flavin reductase used in the present study had an absorbance at 280 nm of 0.9 cm^{-1} and a specific activity of 20 μmol of NADH oxidized min^{-1} A_{280}^{-1} mL^{-1}, about 120-fold purified from the crude lysate on the basis of specific activity.

Aliquots of intermediate II were first made anaerobic by the reaction of oxygen with glucose, catalyzed by glucose oxidase in 0.8 M phosphate buffer, pH 7, at 0 °C, and then mixed with aldehyde which had been similarly deoxygenated. At the times desired, a 100-μL sample of intermediate II was added to 200 μL of 25 mM glucose and mixed with 300 μL of 3 mg/mL glucose oxidase in a glass syringe. After several minutes of incubation at 0 °C to remove oxygen, the sample was injected into a vial containing deoxygenated aldehyde in buffer at 25 °C. The vial, constructed with a capillary neck to impede diffusion of oxygen, was completely filled (19 mL) with a solution containing: *P. fischeri* luciferase (200 μg), *P. fischeri* flavin reductase (90 μg), NADH (1.8 mM), FMN (100 μM), decanal (0.005%), glucose oxidase (4 mg), glucose (10 mM), and catalase (1 mg), in 0.1 M phosphate buffer, pH 7.

In order to demonstrate that our experimental procedures produced significant oxygen depletion, we utilized an in vitro bacterial bioluminescence assay which is sensitive to even very low (10^{-7} M) oxygen. After 5 min of action of glucose/glucose oxidase the luminescence was less by a factor of 10^4. The mixing of two such deoxygenated solutions by injection, as

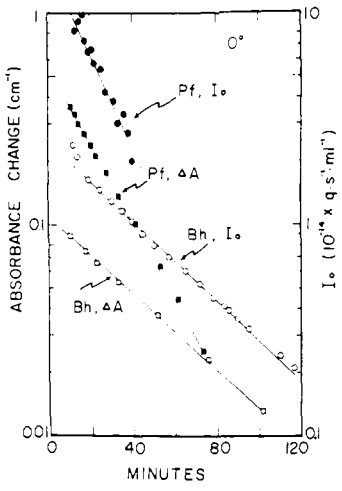

FIGURE 4: Correlation of spectral change and bioluminescence capacity with time of incubation of intermediate II at 0 °C. The data for *P. fischeri* intermediate (■, ΔA_{440} [at 90 min ≃ 6 half-lives] − $A_{440}[t]$; (●) I_0, maximal rate of emission with decanal remaining per mL of sample) came from an experiment similar to the one in Figure 2, whereas the data for *B. harveyi* intermediate (□, ΔA_{435} [at 184 min ≃ 6 half-lives] − $A_{435}[t]$; (○) I_0) came from the experiments in Figure 3.

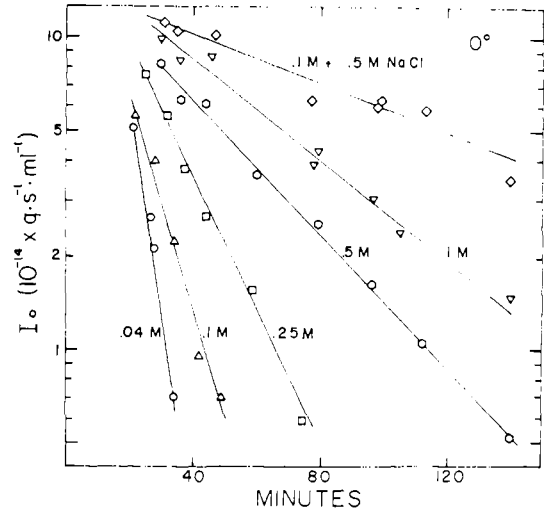

FIGURE 5: The stability of *P. fischeri* intermediate II at 0 °C under various buffer conditions. A sample of intermediate prepared by chromatography was diluted into a series of test tubes kept on ice in the dark under the following buffer conditions: 0.04 M phosphate (○), 0.1 M phosphate (△), 0.25 M phosphate (□), 0.5 M phosphate (○), 1 M phosphate (▽), and 0.1 M phosphate + 0.5 M NaCl (◇). At the times indicated, aliquots were removed in a precooled syringe and assayed for light activity remaining, expressed as maximal emission rate per mL of sample at 25 °C with decanal. The semilogarithmic plots are no longer linear after about 140 min.

described above, resulted in light emission equal to about 1% (never more than 5%) of that with oxygen present. Experiments with and without added catalase indicate that the H_2O_2 produced had no effect on the stability or activity of the luciferase intermediate.

Results

Intermediate II was prepared and isolated by chromatography of an anaerobic mixture of luciferase + $FMNH_2$ on an aerobic column. To 1 mL of luciferase (>1 mg/mL) in 0.35 M phosphate buffer, pH 7.0, at 0 °C, was added an excess of FMN (approximately twofold on a molar basis) and then enough solid sodium hydrosulfite (dithionite) to bleach the solution. The mixture was promptly applied to a small Sephadex G-25 column (1 × 18 cm) preequilibrated at 0 °C with 0.3 or 0.8 M phosphate buffer, pH 7.0, and chromatographed in this buffer at a flow rate of 1-2 mL/min. Fractions (1 mL) were collected and kept in the dark at 0 °C (on ice) until analysis. Protein (A_{280}) elutes with the void volume, well separated from free low molecular weight species such as oxidized flavin (A_{440}) and dithionite oxidation products (Figure 1). The luciferase intermediate, eluting with the void volume, has bioluminescence activity and carries flavin with it as measured by the absorbance at 440 nm, which appears only after its activity has fully decayed. Based on a stoichiometry of one flavin per luciferase (Hastings & Balny, 1975; Becvar & Hastings, 1975), only about half of the luciferase molecules were in the intermediate form in the experiment shown. This is attributed to the presence of unreacted or unreactive protein.

The spectra for the isolated intermediates of both *P. fischeri* (Figure 2) and *B. harveyi* (Figure 3) luciferases exhibit absorbance maxima in the 370-nm region. Incubation at 0 °C in the absence of aldehyde results in a relatively slow loss of the potential for luminescence activity, involving a decomposition to yield H_2O_2 and FMN (Hastings & Balny, 1975), with concomitant absorbance increases in the 450-nm region (Figure 4). During these reactions three clear isosbestic points are observed at 333, 382, and 404 nm (Figure 2) for the *P. fischeri* intermediate and at 329, 368, and 401 nm (Figure 3) for the *B. harveyi* intermediate.[1] The identity of flavin as a product in decomposition (Figure 3) is substantiated by the spectral structure which develops near 435 and 460 nm which closely resembles that for FMN bound to this luciferase (Baldwin, 1974).

For intermediates prepared from both luciferases, the bioluminescence potential remaining at any time disappears in a first order process which parallels the amount of the as-yet-undeveloped absorbance increase at 440 nm (Figure 4). Although this is not absolute proof that the two are related (i.e., they may be fortuitously following the same kinetics), there are arguments against this possibility. First, the bioluminescence quantum yield with respect to unreacted flavin in the intermediate is very nearly the same as that for the overall bioluminescence reaction. For the intermediate of *B. harveyi* and *P. fischeri* we measure bioluminescence quantum yields of 0.19 and 0.27 per flavin, compared with values of about 0.18 (Becvar & Hastings, 1975) and 0.3 (Hastings & Gibson, 1963), respectively, for the overall reaction. Secondly, the two processes still occur in parallel at other temperatures and with different buffer composition. For example, the decomposition rate of the intermediate is slower at higher phosphate and NaCl concentrations. The half-life of the intermediate from *P. fischeri* luciferase at 0 °C can be as short as 5 min or as long as 90 min (Figure 5). For the *B. harveyi* intermediate, the anion effect on half-life is much less dramatic, ranging from about 10 to 40 min. The rate of breakdown is independent of the concentration of the intermediate itself over a thousand-fold range (10^{-5} to 10^{-8} M) and is also insensitive to mM concentrations of hydroxylamine, hydrogen peroxide, dithiothreitol, ethylenediaminetetraacetic acid, and FMN. Exchanging potassium for sodium also produced no change.

[1]Note Added in Proof: These values for the isosbestic points with *B. harveyi* luciferase (especially the one at 368 nm) are lower than those earlier reported (Hastings et al., 1973). A recheck of this has indeed indicated a higher value of about 380 nm. The lower value obtained is difficult to explain; it may be due to the very high luciferase and flavin concentrations used in these experiments, which result in a higher proportion of the oxidized FMN being bound to luciferase.

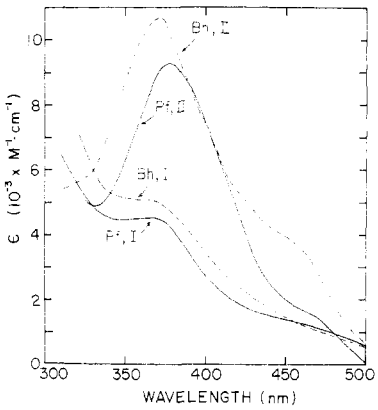

FIGURE 6: Spectra for flavin chromophores of intermediate I and intermediate II of both *P. fischeri* (—) and *B. harveyi* (---). Spectra of intermediate I were obtained as described previously (Tu et al., 1977). Spectra of intermediate II were determined by an extrapolation to zero time of the data from experiments of Figures 2 and 3. For all spectra presented, the absorption of the protein components was subtracted. (Ordinate) Molar extinction coefficient; (abscissa) wavelength in nanometers.

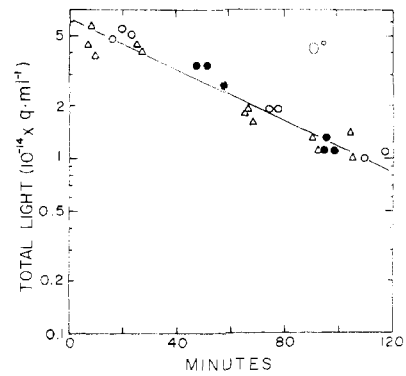

FIGURE 7: This experiment shows that molecular oxygen is not required for the emission of light from intermediate II plus aldehyde. A stock of *P. fischeri* intermediate II was prepared by chromatography in 0.8 M phosphate, pH 7, at 0 °C and kept on ice. The total light yield remaining per mL of stock (△) was monitored by injecting 50 μL of sample into 1 mL of assay buffer with decanal. At 41 and 85 min three aliquots were placed into syringes under anaerobic incubation conditions at 0 °C (see Experimental Procedures). After incubation for the times shown, the content of each syringe was injected into an anaerobic decanal containing vial at 25 °C producing the yields of light per mL shown (●). Similar incubations and assays under aerobic conditions commenced at 14, 70, and 106 min, producing the yields shown (○).

With the *B. harveyi* intermediate, the absorbance changes are essentially complete after 180 min at 0 °C. There is, at most, a very small absorbance increase (<4%) over the 420–460-nm region and no apparent loss in isosbesticity upon much longer incubation. Assuming the final sample represents an equilibrium mixture between free FMN, free luciferase, and the luciferase-FMN complex studied by Baldwin (1974), the degree of complexation and therefore the K_D value can be determined from the final spectrum. Baldwin found that FMN and the complex are essentially isosbestic at 432 and 460 nm with respective absorptivity values of 1.15×10^4 and 1.11×10^4 M^{-1} cm^{-1}, whereas the complex has a distinct minimum at 445 nm with absorptivity of 1.07×10^4 M^{-1} cm^{-1}. Making the minor correction in the spectrum resulting from the small absorbance contribution of the light inducible chromophore (shown in curve f in Figure 3), the apparent degree of complexation is 79% based on comparison of A_{445} and A_{432}, and 62% based on comparison of A_{445} and A_{460}. The total concentrations of flavin and luciferase in the sample are 1.6×10^{-5} and 3.7×10^{-5} M, respectively, and thus the K_D for the complex is 0.7×10^{-5} M (if 79% bound) or 1.7×10^{-5} M (if 62% bound). Under these conditions *B. harveyi* luciferase thus has an affinity for oxidized flavin which is an order of magnitude tighter than that previously determined for the binding of exogenously added FMN by apoluciferase (Baldwin et al., 1975; Baldwin, 1974). The two methods are evidently also very different.

With the *P. fischeri* intermediate, secondary spectral changes occur after the intermediate has decomposed to FMN and H_2O_2, i.e., after standing for about 200 min, or 10 half-lives. With incubation at 0 °C an increase in absorbance occurs over the next 12 h at all wavelengths above 300 nm, causing a slight blue shift in the spectrum, and a deviation from the isosbestic points in Figure 2. These changes, which can be speeded up by warming the sample to 30 °C for 10 min, are not due to precipitation of the enzyme. The molecular basis of this process may reside in a conformation change of the *P. fischeri* enzyme which could, for example, affect the environment of bound FMN or result in the dissociation of oxidized flavin from luciferase.

The appearance of isosbestic points in Figures 2 and 3 indicates that in both cases only two principal forms of the flavin chromophore are present during the time of the spectral changes: intermediate II and its inactive decomposition product. A graphical determination of the spectrum of the flavin moiety of intermediate II for the two species of luciferase was made (Figure 6) by extrapolation of the spectral changes at each wavelength to zero time. The spectra of the reduced flavin-luciferase intermediates for luciferases from both species are also shown.

Free molecular oxygen is postulated to be required only for the formation of intermediate II, not for any subsequent steps in the emission process. To test this, we compared the bioluminescence potential of *P. fischeri* intermediate under aerobic and anaerobic conditions. Isolated II was maintained aerobically at 0 °C and assayed from time to time, as in Figure 4, for the bioluminescence capacity remaining (Figure 7, open symbols). For the anaerobic assays (closed circles) similar aliquots were taken from time to time, depleted of oxygen at 0 °C, and assayed for luminescence by mixing with oxygen-free aldehyde at 25 °C, while maintaining strictly anaerobic conditions all the time (see Experimental Procedures). Incubations of II with glucose and glucose oxidase required several minutes at 0 °C to remove all dissolved oxygen, during which time the reaction of II by way of the "dark" processes are occurring. Thus the comparisons between aerobic and anaerobic reaction of II with aldehyde were conducted alternately over the relatively long period of time shown in Figure 7. The points fall on the same decay curve, characteristic of the decomposition of the intermediate at 0 °C, showing no difference between the total light produced aerobically and anaerobically. Moreover, the kinetics of light emission in individual in vitro assays remained the same under both aerobic and anaerobic conditions. It can be concluded that the bioluminescent reaction of II with aldehyde occurs without the intervention of molecular oxygen.

Discussion

These experiments provide the first direct demonstration that an isolated enzyme intermediate contains a stabilized oxygen moiety. The fact that samples were held under oxygen-free conditions for longer than 10 min and yet gave the full bioluminescence permits an estimate of an upper limit of 3×10^{-4} s^{-1} for the rate constant, k_{-1}, for the back reaction of II formation from oxygen and the luciferase-reduced flavin

complex. The calculation is based on the estimate that, after oxygen depletion, the overall rate of decomposition of the intermediate certainly does not increase by as much as twofold. Stopped-flow experiments have also shown that the reaction of luciferase-$FMNH_2$ complex with O_2 is associated with a fast absorbance increase at 380 nm whose rate is directly proportional to O_2 tension (Presswood, 1977). This further suggests that the formation of intermediate II is an irreversible bimolecular reaction.

The existence of an oxygenated flavoprotein intermediate with luciferase was indicated some years ago (Hastings & Gibson, 1963), and later shown to exhibit absorption in the 375-nm range (Hastings et al., 1973). There are some interesting similarities and also some differences between the luciferase intermediate and those of other flavoprotein hydroxylases (Spector & Massey, 1972a,b; Strickland & Massey, 1973; Presswood & Kamin, 1976; Entsch et al., 1974, 1976). Although absorption in the 375-nm range has been documented for *p*-hydroxybenzoate hydroxylase (Entsch et al., 1974, 1976), the spectrum, with a maximum at about 383 nm ($\epsilon = 9.3 \times 10^3\ M^{-1}\ cm^{-1}$), is a highly transient one; species absorbing at longer wavelengths appear rapidly (Entsch et al., 1976). For the luciferases the long wavelength absorptivity maxima of the intermediates in this study at 0 °C are 377 nm ($\epsilon = 9.3 \times 10^3\ M^{-1}\ cm^{-1}$) for *P. fischeri* and 372 nm ($\epsilon = 10.7 \times 10^3\ M^{-1}\ cm^{-1}$) for *B. harveyi*. In previous studies, carried out in phosphate-buffered 50% ethylene glycol, the maximum for the *B. harveyi* luciferase intermediate (−24 °C) was at 372 nm with absorptivity of $10.5 \times 10^3\ M^{-1}\ cm^{-1}$ (Hastings et al., 1973). For the same luciferase intermediate from *P. phosphoreum*, an absorption maximum at about 380 nm ($\epsilon = 9.5 \times 10^3\ M^{-1}\ cm^{-1}$) has recently been reported (Ashizawa et al., 1977). With the luciferase intermediate II as well as with synthetic N^5-alkyl-4a-peroxy dihydroflavins (Kemal & Bruice, 1976), there is also a minor absorption shoulder near 460 nm (Figure 6); this is not apparent with *p*-hydroxybenzoate hydroxylase in the absence of substrate or effector (Entsch et al., 1976).

The lack of (or very slow) reversibility of oxygen addition to the reduced flavin-luciferase complex is also different from *p*-hydroxybenzoate hydroxylase. Entsch et al. (1976) concluded from kinetic analysis that the reaction of oxygen with the reduced flavin complex of this enzyme is significantly reversible in the presence of (hydroxylatable) substrate of (nonhydroxylatable) effector, as well as in the absence of substrate or effector. This latter case is more directly comparable to our study of luciferase because the substrate (aldehyde) which is "hydroxylated" is absent and peroxide is produced.

The slow secondary spectral changes recorded in the *P. fischeri* samples are reminiscent of the continued protection of *B. harveyi* luciferase against sulfhydryl modification which continues long after activity of II has decayed (Nicoli et al., 1974). Also, slow enthalpy increments have been reported to occur after luminescence decay (Mangold & Langerman, 1975). These may result from relatively slow conformational changes in luciferase after intermediate II decay or light emission. Although no slow secondary spectral changes occurred in the *B. harveyi* samples, the FMN and luciferase produced from intermediate II did exhibit an apparently higher binding affinity than that of the exogenously added FMN and apoluciferase. The tighter binding is not explained by the presence of 0.35 M phosphate in this study because variation of phosphate concentration from 0.05 M to 0.8 M had little effect on binding affinity of FMN. One possible explanation is that the release of the *B. harveyi* intermediate decay product, FMN, from its binding site is quite slow; thus a true equilibrium was not achieved and the binding affinity was overestimated. Alternatively, the conformation of apoluciferase may be different from that of luciferase obtained after the intermediate II decay. with the latter having a higher affinity for FMN.

The effect of phosphate concentration and buffer composition on II stability constitutes another effect of anions (principally phosphate) on luciferase. Its thermal inactivation, protease inactivation (Nicoli et al., 1976), and reduced flavin binding (Meighen & MacKenzie, 1973) are also affected. This "anion" effect may explain the early observation (Hastings & Gibson, 1963) that high enzyme concentration apparently stabilized the intermediate; this may have resulted from ammonium sulfate and/or phosphate-buffer. Mitchell (1969) reported that the lifetime of intermediate II could be extended to many days by precipitation and storage in ammonium sulfate. An "anion" effect has also been observed in the *p*-hydroxybenzoate hydroxylase reaction, namely, effects on the conversion rates between the intermediates (Entsch et al., 1974, 1976). In that system monoanions (e.g., Cl^-, N_3^-) have a greater effect than dianions (HPO_4^{2-}, SO_4^{2-}). Monoanions may have a greater effect than phosphate on luciferase II stability (Figure 5) but apparently not on the other properties listed above. High phosphate apparently does not alter the stability of the luciferase intermediate in the presence of aldehyde (Hastings & Gibson, 1963).

With regard to the possibility that there is an active apoprotein species, our results show conclusively that the active intermediate involves a bound flavin. The failure of Murphy et al. (1974) to detect this appreciable level of flavin is difficult to explain, unless, perhaps, their preparation was such that the amount of intermediate which they isolated was too little to assess spectrally. A quantitative reanalysis of their results is precluded because no values were given either for absorbance or for the intensity or total photon yield from their samples. Quantum yields were expressed per flavin estimated by fluorescence, a technique which may be compromised in those samples containing added apoflavodoxin because the FMN-apoflavodoxin complex is nonfluorescent (Mayhew, 1971), and in those containing luciferase because the FMN complex with *B. harveyi* luciferase is also nonfluorescent (Baldwin et al., 1975).

It is possible that the stability properties of the oxygen-containing intermediate in the bacterial reaction are functionally significant in vivo. The spontaneous decomposition rate of II is subject to environmental (buffer) conditions (Figure 5). On this basis one might be led to speculate that the in vivo luciferase conformation is such that II is stable, so that, under conditions where aldehyde (substrate) is limiting, luciferase would not turn over and would therefore not unnecessarily consume reducing equivalents.

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