after reduction to 2-hydroxybutyrate with lactate dehydrogenase. These experiments and additional experiments on the stereospecificity of pyruvate kinase with different PEP analogues will be published separately.

Acknowledgments

We gratefully acknowledge the help of Klaas Dijkstra with the NMR spectrometer, and we thank Tom Duffy for preparing pure *E* and *Z* isomers of PEB.

Registry No. *Z*-PEB, 31302-64-4; *E*-PEB, 31302-89-3; PEP, 138-08-9; PEP–sugar phosphotransferase, 56941-29-8; PEP–protein phosphotransferase, 37278-17-4.

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Reversible Inhibition of the Bacterial Luciferase Catalyzed Bioluminescence Reaction by Aldehyde Substrate: Kinetic Mechanism and Ligand Effects†

Thomas F. Holzman and Thomas O. Baldwin*

ABSTRACT: The bioluminescence reaction catalyzed by bacterial luciferase from the luminous marine bacterium *Vibrio harveyi* was found to be subject to reversible, chain length dependent inhibition by aldehyde substrate. The stoichiometry of aldehyde to luciferase in the bioluminescence reaction was 1:1; the kinetics of substrate inhibition were consistent with the binding of a second molecule of aldehyde to luciferase to form an enzymatically inactive complex. These findings indicated that aldehyde interactions with bacterial luciferase from *V. harveyi* could not be adequately described by simple Michaelis–Menten kinetics. The binding of *n*-decanal to luciferase and the bioluminescence reaction velocity were dependent on buffer composition and concentration. Phosphate binding to luciferase reduced enzyme affinity for binding a second molecule of aldehyde; the reciprocal effect was also observed. The existence of a reversible complex between the aldehyde substrate and luciferase suggested that the reaction in vitro did not require ordered binding of $FMNH_2$ and aldehyde, in contrast to the commonly depicted kinetic model implying ordered substrate binding. The data presented here, along with recent observations [Holzman, T. F., & Baldwin, T. O. (1981) *Biochemistry 20*, 5524–5528; Holzman, T. F., & Baldwin, T. O. (1982) *Biochemistry 21*, 6194–6201], suggest a kinetic model for the luciferase-catalyzed reaction in which the order of substrate binding is random.

Bacterial luciferase is a heterodimeric flavin monooxygenase which catalyzes the oxidation of reduced riboflavin 5′-phosphate ($FMNH_2$)[1] and long-chain aldehydes to yield blue-green light [see Ziegler & Baldwin (1981) for a recent review]. The conversion of the aldehyde to acid in the reaction in vitro has been demonstrated (Shimomura et al., 1972; Dunn et al., 1973; McCapra & Hysert, 1973; Vigny & Michelson, 1974). There is evidence for the participation of tetradecanal in the reaction in vivo (Ulitzur & Hastings, 1978, 1979). The aldehyde alkyl chain lengths for maximal activity in vitro have been determined for luciferases from a variety of bacterial species [see

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; BSA, bovine serum albumin; DPDA, 2-(2,4-dichloro-6-phenylphenoxy)-*N*,*N*-diethylamine; DPEA, 2-(2,4-dichloro-6-phenylphenoxy)ethylamine; DϕPA, 2,2-diphenylpropylamine; DϕPA–Sepharose, 2,2-diphenylpropylamine–bis(oxirane)–Sepharose; DTE, dithioerythritol; FMN, riboflavin 5′-phosphate; $FMNH_2$, reduced FMN; LU, light units; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TLU, total light units; $NaDodSO_4$, sodium dodecyl sulfate.

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Ziegler & Baldwin (1981)]. The inhibitory effects of high concentrations of aldehyde on bioluminescence have been evident in previous studies (Hastings et al., 1969; Cline, 1973; Meighen & MacKenzie, 1973; Becvar & Wu, 1981). Similarly, long-chain aliphatic acids and alcohols have been shown to inhibit the bioluminescence reaction in vitro (Spudich, 1963; Spudich & Hastings, 1963; Hastings et al., 1966; Baumstark et al., 1979; Tu, 1979), as have inhibitors of other flavin monooxygenases (Nealson & Hastings, 1972; Makemson & Hastings, 1979). We have recently shown that some *n,n*-diphenyl alkyl acids and amines are also capable of inhibiting the bioluminescence reaction in vitro (Holzman & Baldwin, 1981a,b). However, since the identification of the aldehyde as a substrate for the bioluminescence reaction in vitro, very little has been learned about interactions between luciferase and the aldehyde. The studies presented here describe the nature of luciferase inhibition in vitro by aldehyde substrate and certain consequences of this inhibition.

During the investigation, it was found that the inhibition of luciferase activity was fully reversible upon dilution of inhibited enzyme, indicating that the inhibitory effects of aldehyde did not result from irreversible denaturation of luciferase. Kinetic analysis revealed the inhibition to be consistent with the formation of an inactive $E-S_2$ complex, making possible the unequivocal determination of the stoichiometry of aldehyde substrate to enzyme in the bioluminescence reaction. Kinetic analysis also indicated that the binding of *n*-decanal, both as a substrate and as an inhibitor, was altered by the binding of other ligands. In particular, the phosphate anion altered *n*-decanal binding in a manner consistent with previous observations of the effects of reduced flavin mononucleotide on enzyme interactions with the inhibitor 2,2-diphenylpropylamine (Holzman & Baldwin, 1981b). Finally, the effect of aldehyde chain length on binding and inhibition was examined. The aldehyde binding constants, calculated through the use of the substrate inhibition equations, showed that the affinity of *Vibrio harveyi* luciferase for both the first and second molecules of aldehyde increased with aldehyde chain length.

Experimental Procedures

FMN was a gift from Sigma, and *n*-decanal was purchased from Sigma; *n*-octanal and *n*-dodecanal were from Aldrich; all were used without further purification. Examination of the reagent *n*-decanal by proton nuclear magnetic resonance spectroscopy gave a typical alkyl aldehyde proton spectrum. No acidic or hydroxylic proton resonances were observed. Analysis of all the aldehydes by Fourier-transform infrared spectroscopy confirmed the absence of both acidic and hydroxylic functionalities. All other chemicals and solvents were of reagent quality. Bacterial luciferase from *Vibrio harveyi* (strain 392; Baumann et al., 1980) was purified by chromatography on a new affinity resin (Holzman & Baldwin, 1981a,b, 1982a). Samples of enzyme purified by this method were judged to be >95% pure on the basis of $NaDodSO_4$–polyacrylamide gel electrophoresis (Laemmli, 1970) and were utilized without further purification. Luciferase activity was measured by using a photomultiplier photometer (Hastings et al., 1978). With the light standard of Hastings & Weber (1963), 1 LU was $\sim 1 \times 10^{10}$ quanta/s. The maximum bioluminescence rate is termed I_0 (Hastings et al., 1978). All assays were performed at room temperature, 22–25 °C. The aldehyde substrate, *n*-decanal, was prepared as a sonicated suspension in water at 0.01% v/v (equivalent to 6.40 mM) and was added to the assay mixture immediately before use to minimize oxidation. The molar concentrations of the aldehydes were calculated on the basis of their respective molecular weights and densities and are quoted in terms of the total amount of aldehyde added. The concentrations specified are in a final sample volume of 1.0 mL, prior to injection of reduced flavin. However, since the aldehydes are sonicated into distilled water, it is not clear that a true solution is formed (Baumstark et al., 1979). Stock solutions of the aldehydes were freshly prepared every few hours. The aldehydes used for determination of the stoichiometry of aldehyde binding to luciferase and the chain length effects on binding were sonicated into nitrogen-saturated distilled water and were maintained under nitrogen throughout the experiment.

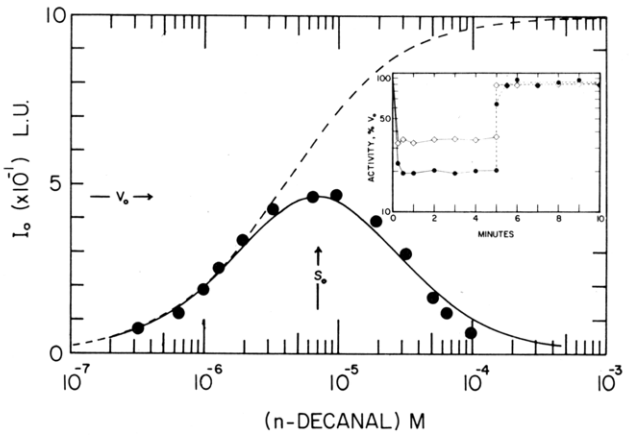


FIGURE 1: Activity profiles for *V. harveyi* luciferase in the standard injection assay in 20 mM Bis-Tris (pH 7.0) (●) after incubation for 30 s at the *n*-decanal concentration specified. The best-fit curve (solid line) was generated for chosen values of [S] by assuming substrate inhibition and by using methods described in the text and the kinetic constants listed in Table II. The dashed curve was generated for chosen values of [S] by assuming typical Michaelis–Menten behavior and by using V_m determined from a double-reciprocal plot. The values of v_0 and $[S_0]$ were taken to be 46.5 LU and 7.0 μM, respectively. The final luciferase concentration in each assay was 0.10 μM. A similar *v* vs. [S] profile was observed for assays performed in 20 mM Pipes (pH 7.0); v_0 was found to be 51.0 LU and $[S_0]$ was 11 μM. Inset: Determination of the reversibility of *V. harveyi* luciferase inhibition by *n*-decanal. Samples of luciferase at 0.10 μM were incubated with either 64 μM *n*-decanal in 20 mM Bis-Tris (pH 7.1) (◊) or 320 μM *n*-decanal in 20 mM phosphate (pH 7.0)–0.2% BSA (●) for periods of time up to 5 min and then assayed by $FMNH_2$ injection. Incubation at these high aldehyde concentrations produced the inhibition observed during the 5-min incubation period (solid lines). After 5 min of incubation, the samples of luciferase in each buffer with *n*-decanal were diluted 1:11 into the corresponding buffer minus aldehyde. These samples were then assayed by $FMNH_2$ injection at regular intervals with an amount of *n*-decanal added to give a maximal v_0 (see Figure 1). These data, corrected for dilution, are plotted on the 5–10-min recovery interval (dashed line).

Results

Reversible Substrate Inhibition. The effect of aldehyde concentration on luciferase activity in the standard $FMNH_2$ injection assay with Bis-Tris buffer is shown in Figure 1. Several general features were evident: the profile of I_0 vs. *n*-decanal concentration was bell-shaped and was symmetric about a substrate concentration, $[S_0]$, which produced a maximum observed reaction velocity (v_0). At high concentrations of *n*-decanal, reduced levels of luciferase activity were observed. The K_m and apparent K_i values for *n*-decanal were found at half-maximal velocity on the left and right sides, respectively, of the bell-shaped velocity vs. substrate concentration curves (Table I).

The bell-shaped curve was similar to the velocity vs. substrate concentration curves observed for enzymes which are reversibly inhibited by one or more substrate molecules (Webb, 1963). An examination of the reversible nature of the in-

Table I: Apparent Values of K_m and K_i for *n*-Decanal from Plots of v vs. [S][a]

buffer	$K_{m,app}$ (μM)	$K_{i,app}$ (μM)	v_0 (LU)	$[S_0]$ (μM)
20 mM Bis-Tris	1.2	30	46.5	7.0
20 mM Pipes[b]	1.8	40	51.0	11
20 mM phosphate	1.5	15	80.0	4.3
20 mM phosphate–0.2% BSA	9.0	200	134	41
200 mM phosphate	1.5	50	138	9.8

[a] See Figures 1 and 4. [b] Plot of v vs. [S] not shown; it was nearly identical with Figure 1.

hibition of bacterial luciferase by *n*-decanal is presented in Figure 1 (inset). Simple dilution of the inhibited enzyme resulted in recovery of 100% of the initial activity, within experimental error, indicating that the inhibition seen at high aldehyde concentrations (Figure 1) was fully reversible.

The aliphatic aldehydes utilized in the bioluminescence reaction in vivo are both hydrophobic and capable of forming Schiff bases with protein amino groups. It could be assumed that inhibition by aldehyde substrate would occur as a result of distortion of the native enzyme conformation brought about by aldehyde alkyl chains intercalating into the interior of the enzyme. In such a model, the aldehyde molecules would be acting as a denaturant and would presumably result in nonspecific irreversible denaturation. However, as indicated by the data in Figure 1, the inhibition was reversible even when the enzyme was incubated with high concentrations of aldehyde for time periods much longer than those required to perform an activity assay.

The reversibility of the aldehyde substrate inhibition indicated that aldehyde binding could not be accurately described by a simple Michaelis–Menten kinetic mechanism; a double-reciprocal plot or a Hoffstee plot should exhibit characteristic curvature (Webb, 1963). Such curvature has been observed during the examination of 2,2-diphenylpropylamine inhibition of aldehyde binding to luciferase (Holzman & Baldwin, 1981b; Holzman, 1982). Similar kinetic behavior has been reported previously but has not been discussed in detail (Hastings et al., 1969; Nealson & Hastings, 1972; Cline, 1973; Meighen & MacKenzie, 1973).

Stoichiometry of Aldehyde Binding. The stoichiometry of *n*-decanal binding to luciferase was determined at low aldehyde and high phosphate concentrations by the method of continuous variations (Job, 1928), as shown in Figure 2. These equilibrium binding measurements showed that, for the luciferase-catalyzed bioluminescence reaction, only one molecule of *n*-decanal was required per molecule of luciferase. Furthermore, there appeared to be only one catalytically productive binding site for *n*-decanal on bacterial luciferase.

Kinetics of Substrate Inhibition. The simplest mechanism describing the occurrence of substrate inhibition is the binding of a second molecule of aldehyde by luciferase to form an enzymatically nonproductive complex.[2] If substrate inhibition

[2] The formulation presented here was given originally by Haldane (1930). It assumes that a second substrate molecule binds to (active) ES to form (inactive) ES_2. This analysis ignores the possibility that inhibition could occur by *partial* attachment of substrate to the substrate binding site on the enzyme and assumes the first molecule of substrate always binds in a catalytically productive fashion [see Webb (1963)]; the possible existence of an *inactive* ES complex is ignored. The aldehyde binding scheme presented here is therefore a phenomenological description of the kinetic data. Under this constraint, and for purposes of comparing kinetic data, the constant α represents the effect the formation of (active) ES has on the formation of (inactive) ES_2. In this analysis, α is formally the interaction or coupling constant.

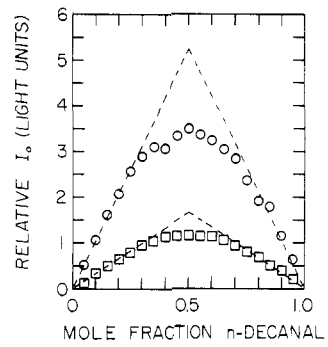


FIGURE 2: Analysis of the stoichiometry of *n*-decanal binding to bacterial luciferase by the method of continuous variations. The effect of phosphate on the apparent K_i values and the reversibility of inhibition suggested that the stoichiometry of aldehyde binding to luciferase could be determined experimentally by using a buffer composition and *n*-decanal concentration at which the amount of inhibited enzyme was relatively low. Therefore, enzyme samples were assayed in a 200 mM phosphate buffer (pH 7.1) by using the standard $FMNH_2$ injection assay. The sum of the luciferase and *n*-decanal concentrations was maintained at either 3.0 (○) or 1.5 μM (□). These concentrations were, respectively, 10–20-fold less than the calculated value for K_i (see Table II).

Table II: Calculated Kinetic and Equilibrium Constants for *n*-Decanal from the Proposed Mechanism for Substrate Inhibition[a]

buffer	V_m (LU)	α	K_d (μM)	K_i (μM)
20 mM Bis-Tris	103	2.7	4.2	12
20 mM Pipes	106	3.5	5.9	21
20 mM phosphate[b]	240	1.0	4.3	4.3
20 mM phosphate–0.2% BSA	260	4.5	19	87
200 mM phosphate	260	5.1	4.3	22

[a] Calculated as described in the text. [b] Values were calculated by assuming $\alpha = 1.0$.

were not occurring, the kinetics should obey a simple Michaelis–Menten rate law, as depicted theoretically for luciferase assayed in Bis-Tris buffer (Figure 1). However, in the event of substrate inhibition, caused by binding of a second aldehyde molecule, the relevant equilibrium and velocity equations are

$$E(RCHO) + RCHO \rightleftharpoons E(RCHO)_2$$

$$v = \frac{V_m}{1 + K_d/[S] + [S]/(\alpha K_d)} \quad (1)$$

where v is the observed rate (in LU), V_m is the maximal rate that would be attained in the *absence* of substrate inhibition, K_d is the dissociation constant of the E(RCHO) complex, and α is the interaction constant ($1 < \alpha < \infty$) indicating the effect the first bound substrate molecule has on the second[2] (Webb, 1963). It can also be shown for the observables v_0 and $[S_0]$ (see Figure 1) that at $v = v_0$, $K_d = [S_0]/\alpha^{1/2}$, and at $[S] = [S_0]$, $\alpha = 4[v_0/(V_m - v_0)]$ (Webb, 1963). These relations may be combined with the velocity equation to yield a solution for V_m:

$$V_m = \frac{2v[S_0][S] - v([S_0]^2 + [S]^2)}{2v_0[S_0][S] - v([S_0]^2 + [S]^2)} \quad (2)$$

For analysis of experimental data over n values of v and [S], the average value of V_m, given by the minimized solution of the least-squares function, may be written

$$\bar{V}_m = \frac{1}{n}\sum_{i=1}^{n} \frac{2v_i[S_0][S_i] - v_i([S_0]^2 + [S_i]^2)}{2v_0[S_0][S_i] - v_i([S_0]^2 + [S_i]^2)} \quad (3)$$

The value of $\bar{V}_m$ calculated in this fashion may then be used to calculate values for α and K_d and subsequently to calculate

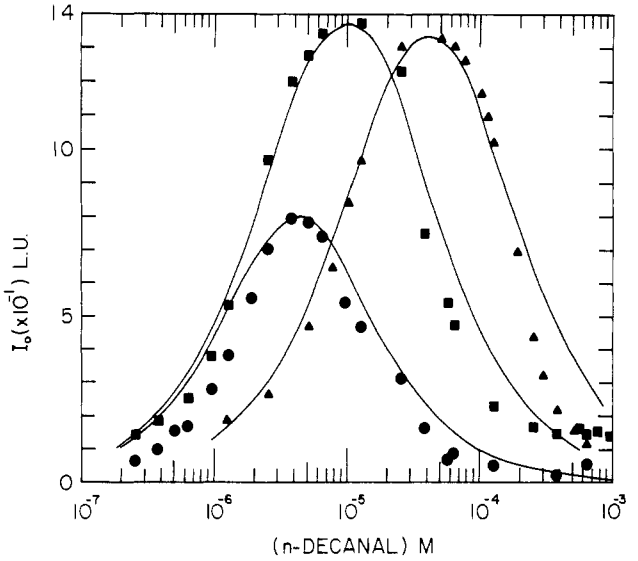


FIGURE 3: Activity profiles for *V. harveyi* luciferase in the standard injection assay after incubation for 30 s at the *n*-decanal concentration specified. The final luciferase concentration in each assay was 0.10 μM. The best-fit curves for the analyses in 200 mM phosphate (pH 7.1) (■) and 20 mM phosphate (pH 7.0)–0.2% BSA (▲) were calculated as described in the text; the resulting kinetic constants are listed in Table II. The curve for 20 mM phosphate (pH 7.0) (●) was calculated by using the equations described in the text and the lowest permissible value for the interaction constant ($\alpha = 1$). For assays in 200 mM phosphate, v_0 was taken to be 138 LU, and [S_0] was 9.8 μM. For assays in 20 mM phosphate–0.2% BSA, v_0 was 134 LU, and [S_0] was 41 μM.

predicted values of v for chosen [S] values with the use of eq 1.

The best-fit values of α and K_d calculated by this method for luciferase assays in Bis-Tris are shown in Table II. These values and $\bar{V}_m$ were used to calculate the best-fit theoretical curve for substrate inhibition (Figure 1) to the experimental data. It was evident that this kinetic analysis closely approximated the experimental data. Similarly, this analysis demonstrated the error introduced by assuming that the kinetics of luciferase interaction with aldehyde could be described by a simple Michaelis–Menten mechanism. Analysis of the kinetic data as described here provided a reliable method for estimating the binding constants describing the interaction of luciferase with its aldehyde substrate.

Ligand Effects on Substrate Inhibition. The phosphate anion has been shown to competitively inhibit $FMNH_2$ binding and to enhance the quantum yield with neutral side-chain flavins (Meighen & MacKenzie, 1973). In addition, phosphate has been shown to stabilize luciferases from divergent species of luminous marine bacteria to inactivation by urea, by heat, and by proteolysis (Baldwin & Riley, 1980; Holzman & Baldwin, 1980a,b). In developing an affinity purification procedure for luciferase (Holzman & Baldwin, 1981a), it has recently been shown that phosphate, like $FMNH_2$ (Holzman & Baldwin, 1981a,b), can modulate enzyme binding to an immobilized competitive inhibitor of the aldehyde substrate, DϕPA–Sepharose. It is likely that both $FMNH_2$ and phosphate modulate enzyme binding to DϕPA–Sepharose by altering the conformation of the enzyme–aldehyde binding site (Holzman & Baldwin, 1982a). The effects of phosphate on substrate inhibition were examined (Figure 3). The values of [S_0] and v_0 were sensitive to buffer composition and concentration and, as has been noted previously (Hastings & Gibson, 1963a,b), to the presence of other protein species in solution. The highest values of v_0 and [S_0] were observed in 200 mM phosphate (no BSA) and 20 mM phosphate–0.2% BSA (the standard assay buffer). Comparison with the data of Figure 1 showed that the lowest values of v_0 were observed in the cationic organic buffers Bis-Tris and Pipes while the lowest [S_0] value was seen in 20 mM phosphate. BSA appeared to have several effects on enzyme activity. Addition of BSA to 20 mM phosphate increased the enzyme v_0 and the values of K_m and the apparent K_i. Since BSA is known to bind long-chain fatty acids, the alteration of aldehyde binding probably reflects an equilibrium between aldehyde, BSA, and luciferase (Hastings & Gibson, 1963a,b). The effect of BSA on v_0 was less easily explained. It may be a reflection of the binding of aldehyde to BSA, a physical association between luciferase and BSA, or both.

Values of $\bar{V}_m$, α, and K_d in each buffer were calculated by the methods discussed above (Table II, Figure 3) and were used to prepare predicted velocity vs. substrate concentration curves for each buffer (Figure 3). The analyses in 200 mM phosphate and in 20 mM phosphate–0.2% BSA were found to be closely approximated by the predicted curves. However, the substrate inhibition analysis in 20 mM phosphate revealed that the predicted curve fit the data less well, indicating a deviation from the proposed inhibition mechanism. To differing extents for each analysis, at very high aldehyde concentrations there were slight deviations from the predicted curves. These deviations may indicate that more than two molecules of aldehyde bind to luciferase at these concentrations.

Phosphate Effect. The data presented in Figure 3 indicated that phosphate altered the interaction between luciferase and aldehyde. While phosphate had little effect on the K_m for aldehyde ($\sim$1.5 μM), the apparent K_i was increased from $\sim$15 μM in 20 mM phosphate to $\sim$50 μM in 200 mM phosphate (Table I). Therefore, at the higher phosphate concentration, the enzyme was less susceptible to inhibition by *n*-decanal and exhibited a substantial ($\sim$2-fold) increase in activity. The same pattern was observed for the best-fit constants calculated from the proposed substrate inhibition mechanism (Table II).

Although the bell-shaped curves in Figures 1 and 3 resulted from the simultaneous occurrence of two aldehyde binding equilibria, the effects of phosphate were more easily examined when the equilibria were visualized independently. Under these conditions, the "theoretical" curve for the binding of the first molecule of aldehyde was like that presented in Figure 1 (dashed curve), while binding of the second molecule of aldehyde resulted in an inhibition curve equivalent to one obtained by rotation of the theoretical binding curve of Figure 1 about a vertical axis of symmetry at the point [S_0] on the abscissa. When aldehyde binding was viewed from this standpoint, the effects of phosphate on binding were more easily understood; it was evident that all the effects of phosphate on the activity profile were related. That is, phosphate protection from the inhibition caused by the second molecule of aldehyde may be viewed as a shift of the aldehyde inhibition curve to higher substrate concentrations, relative to the aldehyde binding curve. Since phosphate showed little effect on the theoretical aldehyde binding curve, the net result in the experimentally observable bell-shaped curve was an increase in v_0, [S_0], and the apparent K_i.

The data presented in Figure 3 showed that, at high phosphate, luciferase was less susceptible to inhibition by aldehyde than in low phosphate or in Bis-Tris. This observation suggested that the apparent K_d for phosphate, under conditions of substrate inhibition, could be estimated from a plot of luciferase activity vs. phosphate concentration, obtained at a high aldehyde concentration. Such a plot is shown in

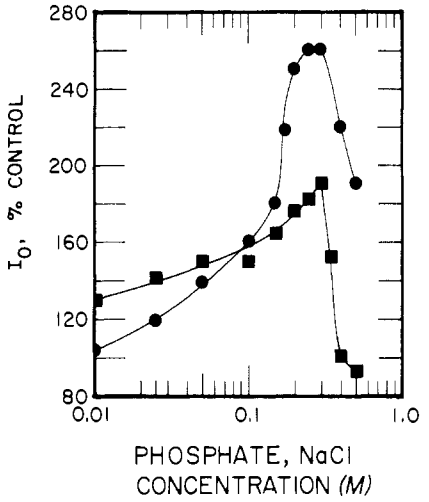
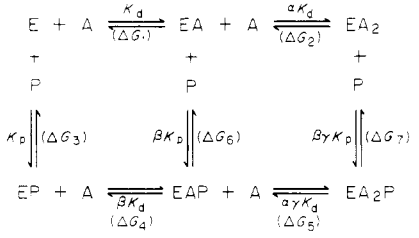


FIGURE 4: Examination of the effects of phosphate and NaCl on I_0 for *V. harveyi* luciferase in the standard $FMNH_2$ injection assay. I_0 values are expressed as percentages of the control I_0 value for luciferase assayed in 20 mM Bis-Tris (pH 7.0). Assays were performed with 0.11 μM luciferase and 64 μM aldehyde in phosphate buffer (●) or 20 mM Bis-Tris with NaCl (■) at the concentrations specified.

Figure 4. The reaction velocity (I_0) was plotted vs. either phosphate or NaCl concentration. The K_d for phosphate estimated from this experiment was ~0.1 M. It was evident that although both NaCl and phosphate increased I_0, the effect of phosphate was substantially greater than that of NaCl. The differential effects of the two salts suggested that the increases in bioluminescence could not be attributed entirely to nonspecific solution ionic strength effects on enzyme conformation but rather appeared to be due to the effects of phosphate binding to luciferase.

The observed increase in activity (I_0) in high concentrations of phosphate could have resulted from an increased decay rate or from an increased quantum yield. The velocity, decay rate, and quantum yield were examined for assays performed in 20 mM phosphate (pH 7.0) and in 200 mM phosphate (pH 7.0). The luciferase and *n*-decanal concentrations were, respectively, 0.11 and 64 μM. Data revealed that the value of I_0 in high phosphate was over twice that in low phosphate, while the decay rates were identical. Thus, the observed increase in I_0 in high phosphate was correlated with an increase in the quantum yield of the bioluminescence reaction. This observation suggested that the inhibition of bioluminescence resulted from a decrease in the concentration of the emitting species rather than a change in the nature of the emitting species and was consistent with the kinetic description for substrate inhibition presented above.

Energetics of Phosphate Binding. Based on these data, it was possible to examine the network of linked binding equilibria between wild-type *V. harveyi* luciferase (E), its aldehyde substrate *n*-decanal (A), and phosphate (P). The equilibria were defined as follows:



The experimentally determined binding constants in this network were K_d and αK_d (=K_i), determined in Bis-Tris (Table II), βK_d (=K_d in high phosphate), $\alpha\gamma K_d$ (=K_i in high phosphate), and $\beta\gamma K_p$ (from Figure 4). Since $K_d \simeq \beta K_d$ (Table II), then $\beta \simeq 1$, so that $\Delta G_1 \simeq \Delta G_4$ and $\Delta G_3 \simeq \Delta G_6$. Based on the linked equilibria, it was evident that $\alpha\gamma K_d/(\alpha K_d) = 1.8$. These interaction constants made possible the calculation of K_p; $\beta\gamma K_p \simeq 0.1$ M or $K_p \simeq 0.1$ M$/(\beta\gamma) \simeq 50$ mM.

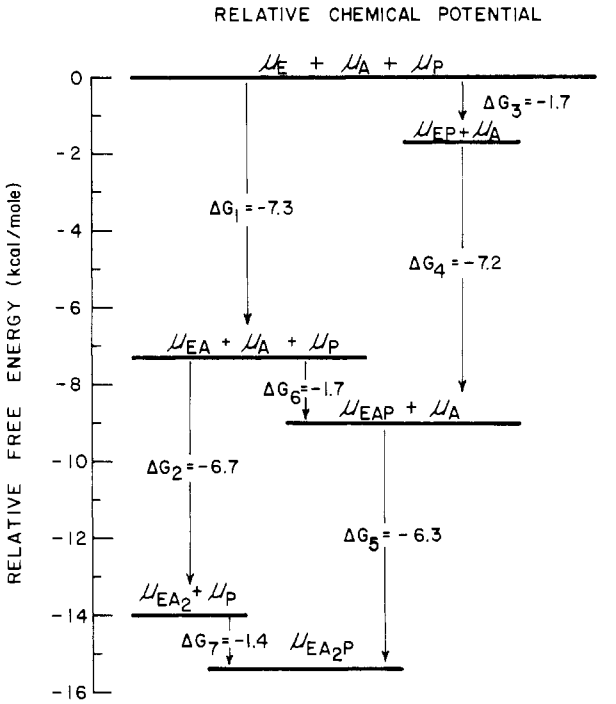


FIGURE 5: Free-energy diagram for the binding of phosphate to bacterial luciferase in the presence of substrate inhibition. Analyses are based on the method of Weber (1975).

From these values, the free-energy change for each binding interaction was calculated, and a free-energy diagram was constructed (Figure 5). The binding equilibria and the free-energy diagram demonstrate several points about phosphate and aldehyde binding to luciferase. It was evident that the formation of EAP by either route proceeded in an independent fashion. However, binding of phosphate to the enzyme–aldehyde complex decreased the free energy of binding of the second molecule of aldehyde and vice versa. Similarly, the effect of the first molecule of aldehyde on the second was reflected in the differences in binding free energies. Thus, for example, the coupling free energy for the phosphate–aldehyde couple was $\Delta G_7 - \Delta G_6 = +0.3$ kcal/mol (for $EA_2 \rightarrow EA_2P$ vs. EA → EAP); for the aldehyde–aldehyde couple, the coupling free energy was $\Delta G_5 - \Delta G_2 = +0.4$ kcal/mol (for EAP → EA_2 vs. EA → EA_2P); both of the couples were within the range of values reported for other systems (Weber, 1975). The positive free-energy changes in both of these couples showed that the binding of phosphate and aldehyde do not proceed independently; the interaction between these ligands is antagonistic (Weber, 1975).

Effect of Aldehyde Chain Length on Inhibition. The effect of aldehyde chain length on the shape and position of the substrate inhibition profile in Bis-Tris buffer is presented in Figure 6. The apparent and calculated kinetic constants for the curves are presented in Table III. Application of eq 1 and 3 to the experimental data gave calculated curves which fit well. The data indicated that the strength of binding of both the first and second molecules of aldehyde increased with increasing chain length, but for each aldehyde, there were substantial differences between the apparent binding and inhibition constants and the constants calculated from kinetic

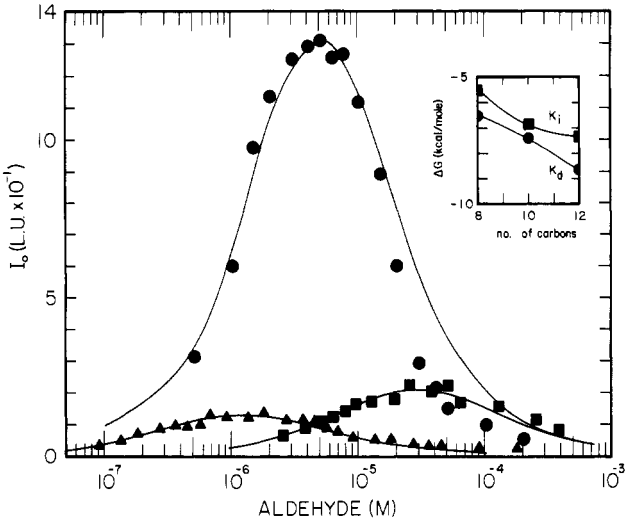


FIGURE 6: Activity profiles for *V. harveyi* luciferase in the standard injection assay as a function of aldehyde chain length: (■) *n*-octanal; (●) *n*-decanal; (▲) *n*-dodecanal. Assays were performed in 20 mM Bis-Tris (pH 7.1) after enzyme, at 0.24 μM, was incubated at the specified aldehyde concentration for ~15 s. The best-fit curves for each aldehyde (solid lines) were calculated as described in the text. The apparent and calculated kinetic and binding constants for each aldehyde are listed in Table III. Inset: Free energy of binding as a function of aldehyde chain length. The apparent kinetic and equilibrium constants estimated from the data were as follows: *n*-octanal, K_m = 5.0 μM, $K_{i,app}$ = 250 μM, v_0 = 21 LU, [S_0] = 30 μM; *n*-decanal, K_m = 1.1 μM, $K_{i,app}$ = 20 μM, v_0 = 130 LU, [S_0] = 5.3 μM; *n*-dodecanal, K_m = 0.2 μM, $K_{i,app}$ = 8.0 μM, v_0 = 13 LU, [S_0] = 1.3 μM.

Table III: Calculated Kinetic and Equilibrium Constants for *n*-Octanal, *n*-Decanal, and *n*-Dodecanal[a]

	V_m (LU)	α	K_d (μM)	αK_d (K_i) (μM)
n-octanal	40	4.8	14	66
n-decanal	300	2.5	3.3	8.2
n-dodecanal	21	9.3	0.41	3.8

[a] Calculated as described in the text; also see Figure 6.

analysis (Table III). For example, the apparent binding constants were 2–3 times less than the calculated values, while the apparent inhibition constants were 2–3 times greater than the calculated values. These differences arise, as discussed above, because substrate binding and inhibition are occurring simultaneously. Using the calculated values of K_d and K_i (Table III) for each aldehyde, we calculated the free energies of binding of the first and second molecules of aldehyde to luciferase. In Figure 6 (inset), these free-energy values are plotted vs. the number of carbon atoms in the aldehyde alkyl chain. These data demonstrated that as aldehyde chain length increased so did the negative free energy of binding of both the first and second molecules of aldehyde to luciferase.

Discussion

Ligand Binding. While it is apparent from these and other studies that phosphate has dramatic effects on the activity of luciferase, circular dichroism studies suggest that phosphate binding does not cause any gross change in enzyme secondary structure (Holzman & Baldwin, 1980a). Other observations suggest that phosphate binding to luciferase at the site normally reserved for the $FMNH_2$ phosphoryl moiety gives rise to subtle changes in enzyme structure. These observations indicate that conformational alterations in enzyme structure are responsible for (1) enhanced enzyme activity with flavin substrate analogues (Meighen & MacKenzie, 1973), (2) stabilization of luciferase to inactivation by heat, proteinases, and denaturants (Baldwin & Riley, 1980; Holzman & Baldwin, 1980a), and (3) enhanced enzyme affinity for DφPA–Sepharose (Holzman & Baldwin, 1981a,b, 1982a).

The present data suggest that phosphate binding has little effect on the enzyme's K_m and K_d for aldehyde but rather shifts the inhibition curve (binding of the second molecule of aldehyde) to higher substrate concentrations. This shift results in the expected increases in v_0, [S_0], $K_{i,app}$, and K_i for the observed bell-shaped velocity vs. substrate concentration profile. If the phosphate anion is bound at the $FMNH_2$ phosphate site, then the binding of aldehyde in the presence of phosphate and the binding of aldehyde to enzyme subsequent to $FMNH_2$ binding could result in similar effects. That is, the luciferase–phosphate or luciferase–$FMNH_2$ complexes should be less easily inhibited by high aldehyde concentrations and should have greater initial rates of bioluminescent emission and greater quantum yields. For the luciferase–$FMNH_2$ complex, all three of these effects have been observed (Meighen & MacKenzie, 1973; Meighen & Bartlet, 1980). In fact, the luciferase–$FMNH_2$ complex has been shown to be completely insensitive to inhibition by *n*-decanal concentrations as high as 0.64 mM (Meighen & MacKenzie, 1973). The observations presented here show the following: (1) in the absence of aldehyde, luciferase bound phosphate with a K_d of approximately 50 mM, a value close to that previously reported by Baldwin & Riley (1980); (2) the EAP complex had a decreased affinity for binding a second (inhibitory) molecule of aldehyde, as indicated by increases in both the apparent and calculated K_i values; (3) the EA_2 complex had reduced affinity for phosphate; and (4) as expected from the observed decrease in binding affinity for the second molecule of aldehyde, the quantum yield of the bioluminescence reaction in high phosphate was increased. A simple interpretation of the phosphate effect is that phosphate binding alters the conformation of the aldehyde binding site so as to partially (or completely) preclude substrate inhibition. Conversely, binding of a second molecule of aldehyde alters phosphate binding, perhaps reflecting the effects of the second aldehyde molecule on $FMNH_2$ binding and therefore on inhibition of activity. These observations suggest that at high phosphate concentrations, luciferase binds the first molecule of aldehyde in a normal fashion, but the aldehyde binding site conformation is altered so as to reduce the likelihood of binding a second (inhibitory) molecule of aldehyde.

The effects of aldehyde chain length on the calculated K_d and K_i values provide another view of the aldehyde binding site. The enzyme affinity for binding the first molecule of aldehyde increased with chain length to a greater degree than did the enzyme affinity for the second molecule of aldehyde. That is, the K_i appeared to approach a limiting value as a function of aldehyde chain length, suggesting a size limit to the dimensions of the portion(s) of the aldehyde site which may be involved in substrate inhibition.

An Alternative Kinetic Scheme for the Luciferase-Catalyzed Reaction. The chemical and kinetic mechanisms for the bacterial bioluminescence reaction have, since the observations of Hastings & Gibson (1963a,b), been refined several times over the last 2 decades [see the review by Ziegler & Baldwin (1981)]. The original observations of Hastings & Gibson (1963a,b) were interpreted to suggest the existence of a chemically reduced form of luciferase produced by a chemical reaction between enzyme and $FMNH_2$ (Hastings & Gibson, 1963a,b; Hastings et al., 1963, 1964, 1965; Spudich &

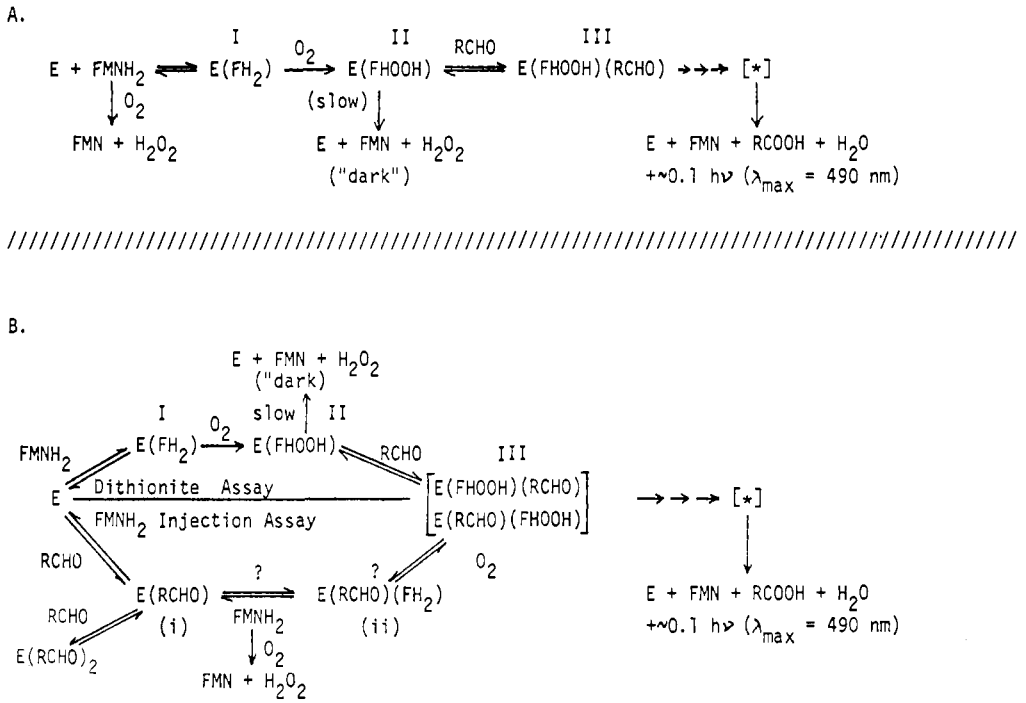


FIGURE 7: Bioluminescence reaction pathways in vitro. (A) Standard scheme depicting the reaction pathway [see Ziegler & Baldwin (1981)] based on the model of Hastings & Gibson (1963a,b). (B) Revised scheme based on evidence presented here. It should be noted that the standard scheme shows steps involving binding reactions and chemical reactions between enzyme-bound intermediates. Both schemes A and B represent arbitrary binding reaction sequences into which are incorporated the known enzyme-bound chemical intermediates (see Discussion).

Hastings, 1963). This model was subsequently abandoned when (1) it became evident that the enzyme contained no disulfide linkages (Hastings, 1968; Tu et al., 1977) and (2) the original observation that $FMNH_2$ was oxidized to FMN in the absence of oxygen proved irreproducible (Gibson et al., 1966; Hastings & Gibson, 1967). Later work demonstrated a single $FMNH_2$ binding site per molecule of enzyme (Meighen & Hastings, 1971) and invalidated (Becvar & Hastings, 1975) the hypothesis that two flavins participated in the bioluminescence reaction. However, since the abandonment of the "reduced enzyme" hypothesis, the reaction pathway has been presented and discussed in the literature as an implicit kinetic reaction sequence into which the features of the known enzyme-bound intermediates have been incorporated [for example, see Hastings & Gibson (1967), Hastings (1968), Meighen & Hastings (1971), and Cline & Hastings (1972)]. The most recent form of the pathway (Hastings & Nealson, 1977; Ziegler & Baldwin, 1981) is seen in Figure 7A. Although this pathway has evolved from a series of observations of enzyme–substrate interactions, the manner in which it is drawn conveys the impression that $FMNH_2$ binds to the enzyme prior to aldehyde binding. From the standpoint of substrate binding, the pathway presents an arbitrary kinetic mechanism, since there is no evidence as to the order of substrate binding prior to participation in the bioluminescence reaction.

Several investigators have presented experimental results which suggest that the kinetic reaction pathway in vitro (Figure 7A) does not require ordered $FMNH_2$ and aldehyde binding, in contrast to the kinetic mechanism implied by Figure 7A. These observations include the data reported here and by Holzman & Baldwin (1981a,b), the phosphate enhancement of enzyme binding to immobilized inhibitor (Holzman & Baldwin, 1982a), the enhancement of aldehyde binding by $FMNH_2$ ribityl side-chain hydroxyls (Meighen & MacKenzie, 1973), and the activity differences seen as a function of order of substrate addition (Meighen & Bartlet, 1980). In addition, the present data provide explanations for previous observations. For example, in the dithionite assay (Meighen & Hastings, 1971), where luciferase is first mixed with $FMNH_2$, the enzyme is insensitive to aldehyde inhibition (Meighen & MacKenzie, 1973), and the I_0 and quantum yield are increased (Meighen & Bartlet, 1980), whereas in the standard $FMNH_2$ injection assay, where luciferase is first mixed with aldehyde, inhibition reduces I_0 and the quantum yield (see Figure 7).

It has been shown that in the standard $FMNH_2$ injection assay with *V. harveyi* luciferase, the compound 2,2-diphenylpropylamine (DϕPA) competitively inhibits *n*-decanal binding to luciferase, and in the dithionite assay DϕPA actually enhances $FMNH_2$ binding to enzyme (Holzman & Baldwin, 1982a,b). In a similar fashion, luciferase affinity for DϕPA–Sepharose is enhanced by $FMNH_2$ and phosphate and is reduced by *n*-decanal (Holzman & Baldwin, 1981b, 1982a). If the interactions of luciferase with DϕPA–Sepharose parallel those of the enzyme with *n*-decanal, then either *n*-decanal or $FMNH_2$ must be able to bind first to luciferase. Indeed, the binding of aldehyde to enzyme alone can be inferred from previous observations (Nicoli et al., 1974). If the bioluminescence reaction can proceed with either substrate bound first, then it occurs by a random kinetic mechanism. In such a mechanism, depending on the experimentally chosen order of substrate addition, a binary complex may form between either substrate and the enzyme. On the basis of the previous inhibitor studies and the data presented here, the formation of an aldehyde–enzyme complex, (i) in Figure 7B, in the standard $FMNH_2$ injection assay may preclude the formation of intermediate I (Figure 7A,B), leading instead to an enzyme–aldehyde–$FMNH_2$ complex (ii), which in the presence of O_2 forms intermediate III (Figure 7A,B). Although this revised pathway accounts for evidence presented here, the prediction of the existence of (ii) in the standard injection assay will have to be tested by further experimentation. This is an important point to note since it may be that (ii) does not exist. Should this be the case, then the species EA and EA_2 are

transitory dead-end complexes, and aldehyde would be obliged to enter the bioluminescence reaction after $FMNH_2$.

Implications of Substrate Inhibition. The occurrence of true, reversible, substrate inhibition is a rare enzymatic phenomenon. Its occurrence in the luciferase-catalyzed bioluminescence reaction presents unique opportunities for a variety of studies, but it is also important to note the implications for interpretation of previous studies. Calculations based on enzymatic activity measurements [for example, apparent K_d values for aldehydes (Cline, 1973; Cline & Hastings, 1972) and analysis of inhibition of aldehyde binding by compounds such as 2-decenal (Spudich & Hastings, 1963), 2-propynylamine (pargyline) (Makemson & Hastings, 1979), long-chain acids and alcohols (Hastings et al., 1966; Tu, 1979), and compounds like DPDA and DPEA (Nealson & Hastings, 1972)] may be in error because the nature of substrate inhibition precludes the use of a simple double-reciprocal plot alone for determining substrate and inhibitor binding constants (Webb, 1963). In a like fashion, error may be introduced into thermodynamic calculations of enzyme–aldehyde interactions (Becvar & Wu, 1981) by the reduced values of I_0, and therefore quantum yield (use of v_0 rather than V_m), seen in the standard injection assay.

The protection of the luciferase reactive sulfhydryl by aldehyde from alkylating reagents (Nicoli et al., 1974) also warrants reevaluation in light of the quantitative description of aldehyde binding reported here. If protection is related to aldehyde binding, then it should vary with the fractional saturation of either (or both) the productive or the inhibitory aldehyde sites. On the other hand, if protection is related to the occupied volume of the aldehyde binding site(s), then at equal fractional saturations with aldehyde, different extents of protection should be observed as a function of aldehyde chain length.

Certain conclusions about enzyme–substrate interactions may, in the presence of substrate inhibition, have somewhat different interpretations. For example, data on interspecies luciferase subunit exchange could be interpreted to have different structural and mechanistic implications than originally proposed by Meighen & Bartlet (1980). These authors presented evidence which suggested that the luciferase β subunit was involved in the initial binding of $FMNH_2$ in the bioluminescence reaction and that subsequent steps appeared to involve the α subunit rather than the β subunit. However, as we have recently reported (Holzman & Baldwin, 1982b),[3] certain portions of their data can, given the occurrence of substrate inhibition, be interpreted instead as evidence that the active center of luciferase may be closely associated with the interface between the two subunits, rather than residing exclusively or partially on either the α or the β subunit. This later proposal has received additional support from similar conclusions drawn from examination of the luciferase–aldehyde site by photoaffinity labeling (Tu & Henkin, 1983).

For certain of these previous studies, notably those of Spudich & Hastings (1963) and Hastings et al. (1969), it is worth noting that the extent of inhibition and the concentration ranges over which it occurs are apparently different for different species of luminous marine bacteria. In particular, Hastings observed that the luciferase from *Vibrio fischeri* is not inhibited at the same aldehyde concentrations as the *V. harveyi* enzyme (Hastings et al., 1969). Similarly, Meighen & Bartlet (1980) have noted that the luciferase from *Photobacterium phosphoreum* does not appear to be inhibited by aldehyde. Thus, generalizations about substrate inhibition for luciferases from species other than *V. harveyi* may be premature. In any case, it is evident that the interactions of luciferase with its aldehyde substrate can be studied by relatively simple kinetic techniques and are certainly deserving of further study.

[3] Portions of this work were presented at the 38th Annual Southwest/6th Rocky Mountain Regional Meeting of the American Chemical Society (Holzman & Baldwin, 1982b).

Acknowledgments

We are indebted to Drs. James E. Becvar, Donald W. Pettigrew, Shiao-chun Tu, Richard B. Thompson, and Miriam M. Ziegler for their discussions and critical comments.

Registry No. Luciferase, 9014-00-0; *n*-decanal, 112-31-2; *n*-octanal, 124-13-0; *n*-dodecanal, 112-54-9.

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Low-Temperature Luminescence Characterization of 124-Kilodalton Phytochrome from *Avena sativa*†

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ABSTRACT: Phytochrome which is predominantly of 124-kDa molecular mass has been investigated by low-temperature luminescence and optically detected magnetic resonance (ODMR) spectroscopy. In addition to characteristic phosphorescence spectra of Tyr and Trp, we find a previously unreported phosphorescence spectrum from a short-lived triplet state having λ_{max} 465 nm. This spectrum (chromophore X) apparently originates from covalent modifications of the protein. The intensity of the chromophore X emission decreases with increasing concentration of a sulfhydryl reagent used in the extraction procedure. Small amounts of these UV-absorbing covalent modifications are not as readily detected in the absorption spectrum of in vitro phytochrome although their effect on structural and physical properties of phytochrome may be significant. Triplet-singlet energy transfer to the tetrapyrrole is confirmed to originate from both Trp and chromophore X in the red light absorbing form of phytochrome. This conclusion is based partially on the observation of Trp and chromophore X ODMR signals while monitoring the delayed fluorescence of the tetrapyrrole. Although no delayed fluorescence was observed from the far-red light absorbing form of phytochrome, enhanced triplet-singlet energy transfer is suggested by the observed increase of the phosphorescence decay rates in this form of phytochrome.

Phytochrome, a protein containing a thioether-linked linear tetrapyrrole chromophore, is responsible for a wide variety of photomorphogenic responses in plants. A phenomenon unique to this chromoprotein is its ability to undergo photoreversible transformation between two forms. Upon absorption of red light (660 nm), the physiologically inactive red-absorbing form of phytochrome (P_r)[1] is converted to the physiologically active far-red-absorbing form (P_{fr}). P_{fr} is converted back to P_r upon absorption of far red light (730 nm). Although extensive studies have been made on the phytochrome-mediated phys-

† From the Department of Chemistry (T.A.C. and A.H.M.) and the Department of Biochemistry and Biophysics (J.C.L.), University of California, Davis, California 95616. *Received January 5, 1983.* This work was supported, in part, by Grants CHE 82-07265 (A.H.M.) and PCM 81-08090 (J.C.L.) from the National Science Foundation.

[1] Abbreviations: CD, circular dichroism; EG, ethylene glycol; kDa, kilodalton; ME, 2-mercaptoethanol; ODMR, optical detection of (triplet-state) magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; P_r, red light absorbing form of phytochrome; P_{fr}, far-red light absorbing form of phytochrome; SAR, specific absorbance ratio; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-2846$01.50/0