Polgar, L. (1979) *Eur. J. Biochem. 98*, 369.
Shinitzky, M., & Goldman, R. (1967) *Eur. J. Biochem. 3*, 139.
Sluyterman, L. A. E., & De Graaf, M. J. M. (1970) *Biochim. Biophys. Acta 200*, 595.
Sluyterman, L. A. E., & Wijdenes, J. (1970) *Biochim. Biophys. Acta 200*, 593.
Thompson, R. C., & Bauer, C. A. (1979) *Biochemistry 18*, 1553.
Tombs, M. P., Souter, F., & Maclagan, N. F. (1959) *Biochem. J. 73*, 167.
Westerik, J. O., & Wolfenden, R. (1972) *J. Biol. Chem. 247*, 8195.

Binding of 2,2-Diphenylpropylamine at the Aldehyde Site of Bacterial Luciferase Increases the Affinity of the Reduced Riboflavin 5′-Phosphate Site†

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ABSTRACT: We have found a new class of inhibitors of the bacterial bioluminescence reaction, the *N*,*N*-diphenylalkylamines and acids. We have studied the action of one of these compounds, 2,2-diphenylpropylamine. The amine was competitive with the long-chain aliphatic aldehyde substrate ($K_i \simeq 0.1$ mM) but caused an increase in the affinity of the enzyme for reduced riboflavin 5′-phosphate ($FMNH_2$). The inhibitor was attached to Sepharose 6B by a bis(oxirane) spacer, and the interactions of bacterial luciferase with the immobilized ligand were analyzed. The binding of luciferase to the immobilized inhibitor was enhanced by $FMNH_2$ and was decreased by decanal. The results of these studies showed that the 2,2-diphenylpropylamine–luciferase complex has an increased affinity for $FMNH_2$. Likewise, the $FMNH_2$–luciferase complex has an increased affinity for 2,2-diphenylpropylamine. The inhibitor also binds to the enzyme–4a-peroxydihydroflavin complex to block the binding of the aldehyde substrate, while binding of the aldehyde substrate to either the free enzyme or the enzyme–4a-peroxydihydroflavin complex blocks binding of 2,2-diphenylpropylamine.

Investigation of the inhibition of enzymes by the reversible binding of small molecules has been used extensively in the analysis of enzyme structure and function. Inhibitors that have been used include biologically important allosteric modifiers and synthetic substrate analogues, as well as molecules that bear little structural similarity to either substrate(s) or allosteric ligands. The substrate specificity of the bacterial luciferase reaction has been studied by using analogues of the flavin substrate (Mitchell & Hastings, 1969; Meighen & MacKenzie, 1973; Watanabe et al., 1980; Tu et al., 1977) and analogues of the aldehyde substrate (Spudich & Hastings, 1963; Hastings et al., 1966); compounds that bear little structural similarity to either the flavin or the aldehyde have also been used in competitive binding studies (Tu & Hastings, 1975; Makemson & Hastings, 1979).

The enzyme bacterial luciferase is an $\alpha\beta$ dimer that catalyzes the oxidation by O_2 of $FMNH_2$[1] and a long-chain saturated aldehyde to yield FMN, the carboxylic acid, and blue-green light (Figure 1). The enzyme has a single active center located primarily, if not exclusively, on the α subunit (Meighen et al., 1971a,b; Cline & Hastings, 1972). A single flavin is required per $\alpha\beta$ during the light-emitting reaction (Becvar & Hastings, 1975). The stoichiometry of the aldehyde substrate with respect to enzyme is generally assumed to be one as well, but it has not been determined.

Nealson & Hastings (1972) demonstrated that several compounds known to be inhibitors of the P-450 mixed-function oxidase system from liver microsomes (McMahon et al., 1969) are also inhibitors of luciferase. The compounds used by Nealson and Hastings were 2-(2,3-dichloro-6-phenylphenoxy)ethylamine (DPEA), 2-(2,3-dichloro-6-phenylphenoxy)-*N*,*N*-diethylamine (DPDA), and 2-(*N*,*N*-diethylamino)ethyl 2,2-diphenyl-*n*-pentanoate (SKF 525A) (see Figure 2). Both DPEA and DPDA are competitive with aldehyde (Nealson & Hastings, 1972). SKF 525A, on the other hand, blocks the formation of intermediate II, probably by competing with $FMNH_2$ for binding to the enzyme. The fluorescent dye 8-anilino-1-naphthalenesulfonate (Ans) is an inhibitor of luciferase competitive with $FMNH_2$ but has little effect on the binding of FMN (Tu & Hastings, 1975). Makemson & Hastings (1979) showed that *N*-benzyl-*N*-methyl-2-propynylamine (pargyline), known to inhibit monoamine oxidase by the formation of a covalent adduct with the flavin (Chuang et al., 1974), is a reversible inhibitor of luciferase, apparently competitive with aldehyde. Structural analogues of the aldehyde substrate also inhibit the bioluminescence reaction. Long-chain aliphatic alcohols and acids as well as long-chain aldehydes with an unsaturated bond close to the aldehyde functional group are inhibitors of the luciferase reaction (Spudich & Hastings, 1963; Hastings et al., 1966).

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[1] Abbreviations used: Ph_2PA, 2,2-diphenylpropylamine (DϕPA in figures); Ph_2PA–Sepharose, 2,2-diphenylpropylamine–bis(oxirane)–Sepharose; FMN, riboflavin 5′-phosphate; $FMNH_2$, reduced FMN; DTE, dithioerythritol; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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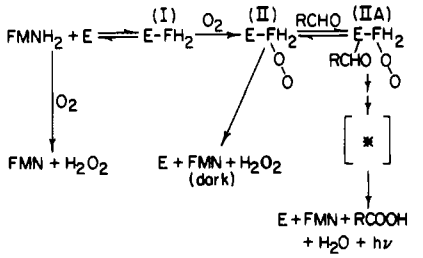

FIGURE 1: Postulated bioluminescence reaction pathway in vitro.

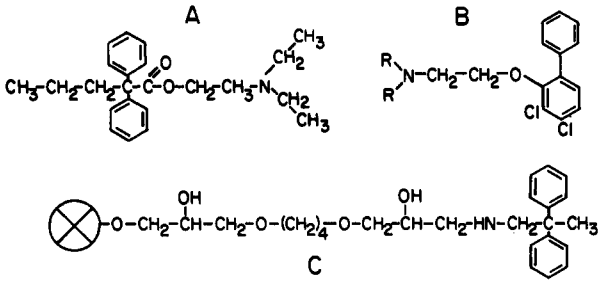

FIGURE 2: Structures of several inhibitors of bacterial luciferase. Compound A is 2-(*N,N*-diethylamino)ethyl 2,2-diphenyl-*n*-pentanoate (SKF 525A); compound B is 2-(2,3-dichloro-6-phenylphenoxy)-ethylamine (DPEA) for R = H and 2-(2,3-dichloro-6-phenylphenoxy)*N,N*-diethylamine (DPDA) for R = C_2H_5. Compound C is the most probable structure for Ph_2PA–Sepharose prepared as described under Materials and Methods. An alternative structure would result if the epoxide were to open in the opposite direction to yield the primary alcohol with the ligand attached to the penultimate carbon.

The binding of $FMNH_2$ by luciferase has been studied both by kinetic methods (Meighen & Hastings, 1971; Watanabe et al., 1974) and by equilibrium methods (Becvar et al., 1976; Becvar & Hastings, 1975) to determine the affinity and the stoichiometry of the interaction. Aldehyde binding, on the other hand, has been studied only by kinetic techniques which rely on the activity of the enzyme (Cline & Hastings, 1972; Nealson & Hastings, 1972; Watanabe & Nakamura, 1972). While it has been possible to determine the effects of chemical modification reactions that cause a loss of activity upon the binding of $FMNH_2$ and FMN (Meighen et al., 1971b; Nicoli et al., 1974, 1976; Welches & Baldwin, 1981), the binding of aldehyde to chemically modified inactive enzyme has not been investigated.

The experiments reported here were directed initially toward the development of an affinity system for purification of bacterial luciferase. The ligands used were selected because they were commercially available, had structural similarities with known inhibitors of bacterial luciferase, and could be readily attached to chromatographic media (Figure 2). During our investigation of the interaction of 2,2-diphenylpropylamine (Ph_2PA)[1] with bacterial luciferase, we found that the ligand bound more tightly to the enzyme–$FMNH_2$ complex than to the enzyme alone and that the ligand was competitive with aldehyde. This paper describes these experiments and presents data that suggest that Ph_2PA–Sepharose might be very useful for studying structural perturbations in the aldehyde binding region of the active center of bacterial luciferase.

Materials and Methods

Chemicals. Trizma base, Bis-Tris, Pipes, bovine serum albumin, and FMN were products of Sigma Chemical Co. Aldehydes, 1,4-diglycidyl ether (95% purity), and 2,2-diphenylpropylamine were purchased from Aldrich Chemical Co. Sepharose 6B was a product of Pharmacia Fine Chemicals. All other chemicals were of reagent quality.

Bacterial Cultures. Bacterial luciferase from *Vibrio harveyi* (strain 392; formerly *Beneckea harveyi*) was purified by affinity chromatography using Ph_2PA–Sepharose.[2] The classification of these bacteria was done by Baumann and his collaborators (Reichelt & Baumann, 1973; Baumann et al., 1980).

Analysis of Ligand Binding. Ligand binding from solution was measured by the effect of the ligand on the velocity of the reaction. Ligand binding with the Ph_2PA–Sepharose was measured by loss of luciferase activity from solution. The binding of ligand from solution was measured by using three different assay methods: the $FMNH_2$ injection assay (Hastings et al., 1978), the double injection assay (Spudich & Hastings, 1963), and the dithionite assay (Meighen & Hastings, 1971; Tu & Hastings, 1975). All three methods rely on the order of binding of substrates (see Figure 1) and allow determination of different kinetic parameters. In the $FMNH_2$ injection assay, $FMNH_2$ is injected into a vial containing enzyme, aldehyde, O_2, and the ligand under study. Any flavin not bound to the enzyme is removed within 300 ms by the autoxidation pathway (Gibson & Hastings, 1962; Hastings & Gibson, 1963). The enzyme-bound flavin (intermediate I) reacts with O_2 to form intermediate II, the 4a-peroxydihydroflavin (Hastings et al., 1973). Intermediate II reversibly binds aldehyde to form intermediate IIA, which decays in the rate-limiting step to form, ultimately, enzyme, FMN, the carboxylic acid, and blue-green light (Ziegler & Baldwin, 1981). Added ligands could interfere with flavin binding or aldehyde binding, or both.

In the double injection assay, the reaction is initiated by injection of $FMNH_2$ into a vial containing enzyme, O_2, and any ligand under study. The reaction proceeds to the formation of intermediate II, but no further due to the lack of aldehyde. Intermediate II (or the complex of intermediate II with ligand under study) decays to form enzyme, FMN, and H_2O_2, but no light (Hastings & Balny, 1975). A secondary injection of aldehyde will convert any intermediate II to intermediate IIA and ultimately to products, with light emission. The initial intensity of the luminescence is proportional to the amount of intermediate II present at the time of aldehyde injection (Spudich & Hastings, 1963). By varying the time delay between the injection of flavin and the injection of aldehyde, it is possible to determine readily the decay rate of intermediate II.

The dithionite assay is useful at low concentrations of $FMNH_2$ where autoxidation of catalytically reduced flavin would cause technical difficulties. In this method, the reaction is initiated by injecting aldehyde and O_2 into a vial containing enzyme, $FMNH_2$, and a slight excess of sodium dithionite. Before injection of aldehyde and O_2, the enzyme binds $FMNH_2$ in accord with a stoichiometry of 1:1 (Becvar & Hastings, 1975), the equilibrium constant, and the concentrations of enzyme and flavin. Upon injection of O_2 and aldehyde, the enzyme-bound flavin is converted to intermediate II and any free flavin is removed by the autoxidation pathway (Figure 1). With saturating aldehyde, the initial intensity of bioluminescence following reaction with aldehyde is proportional to the initial concentration of intermediate I. Therefore, variation of the concentration of $FMNH_2$ allows a direct determination of the affinity of the enzyme for $FMNH_2$ (Meighen & Hastings, 1971).

[2] The use of Ph_2PA–Sepharose to purify luciferases from *V. harveyi*, *V. fischeri*, and *Photobacterium phosphoreum* was described at the 25th Annual Meeting of the Biophysical Society (Holzman & Baldwin, 1981). A future publication will describe these procedures.

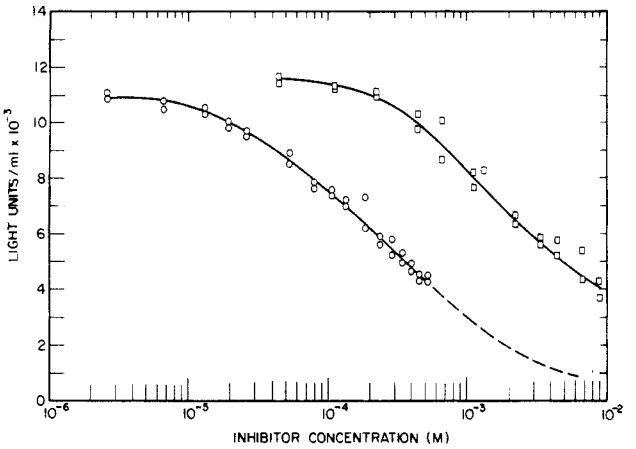

FIGURE 3: Inhibition of luciferase activity by 2,2-diphenylpropylamine and 2,2-diphenylpropionic acid. Luciferase activity was measured by the standard $FMNH_2$ injection technique in the presence of the inhibitors 2,2-diphenylpropylamine (○) and 2,2-diphenylpropionic acid (□). Prior to injection of $FMNH_2$, each assay (23 °C) contained 7.8 μg of luciferase, 20 mM Bis-Tris, pH 7.1, 10 μM decanal, and the indicated concentration of inhibitor.

Synthesis of Ph_2PA–Sepharose. Epoxy-activated Sepharose was prepared essentially as described by Sunderberg & Porath (1974). To 50 mL (settled volume) of Sepharose 6B were added 50 mL of 1,4-diglycidyl ether, 50 mL of 0.6 N NaOH, and 100 mg of sodium borohydride. The slurry was mixed by rotation in a round-bottomed flask at room temperature for ~8 h. The epoxy-activated Sepharose was collected on a sintered-glass funnel, washed to neutrality with distilled water, and suction dried.

The epoxy-activated Sepharose was then allowed to react with 2,2-diphenylpropylamine. The alkylamine was dissolved at 10 mg/mL in dioxane/0.2 M sodium carbonate, pH 10.5 (1:1 v:v), and 1 mL added to each gram of epoxy-activated Sepharose. The slurry was mixed by rotation in a round-bottomed flask at 60 °C for 24 h. The pH of the slurry was checked and adjusted at 3-h intervals during the first 12 h and again at 18 h. After the 24-h reaction, the affinity resin was collected on a coarse-pore sintered-glass funnel and washed sequentially with the following solutions (volumes per gram of suction-dried Sepharose used initially): 6 mL of 1:1 dioxane/water, 6 mL of 1:1 dioxane/0.20 M phosphate, pH 7.0, and 12 mL of 95% ethanol. The affinity resin was then either used directly or stored in 50% ethanol/water at 4 °C. Elemental analysis for nitrogen in lyophilized samples of modified and unmodified Sepharose indicated that the modified Sepharose contained ~0.5% nitrogen by weight and that the unmodified Sepharose contained insignificant amounts of nitrogen. The hydrated affinity resin contained ~10 μmol of 2,2-diphenylpropylamine/mL. The modified Sepharose is referred to as Ph_2PA–Sepharose.

Results

Effects of 2,2-Diphenylpropylamine and 2,2-Diphenylpropionic Acid on Aldehyde Binding. When either 2,2-diphenylpropylamine or 2,2-diphenylpropionic acid was added to a vial containing luciferase, aldehyde, and air-equilibrated buffer, the luciferase activity measured by the flavin injection assay was dramatically inhibited (Figure 3). It is apparent from this plot that both the amine and the acid inhibit the bioluminescence reaction. The apparent dissociation constant of the 2,2-diphenylpropylamine was ~0.2 mM and that of the 2,2-diphenylpropionic acid was ~2 mM. An analysis of this inhibition by 2,2-diphenylpropylamine is shown in Figure 4. The inhibitor appeared to be competitive with the aldehyde substrate; that is, at high concentrations of aldehyde, the effect of the inhibitor was lost. From these data, the inhibition constant of the luciferase–2,2-diphenylpropylamine complex (K_i) was calculated by the method of Hunter & Downs (1945) to be ~0.1 mM. There was definite curvature of the data in Figure 4, suggestive of aldehyde substrate inhibition (see Discussion).

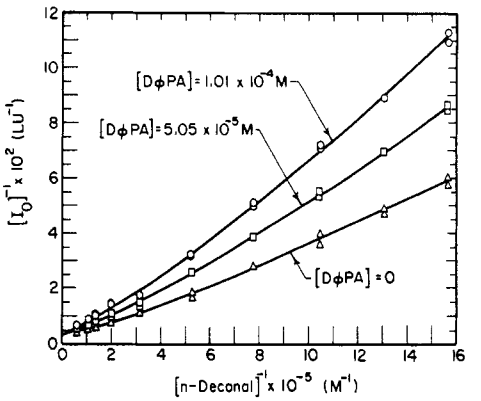

FIGURE 4: Double-reciprocal plot of the relationship between decanal concentration and initial light intensity in the presence and absence of 2,2-diphenylpropylamine. The standard $FMNH_2$ injection assay was used. Prior to injection of $FMNH_2$, each assay (22 °C) contained 25 μg of luciferase, 20 mM Bis-Tris, pH 7.0, and decanal and Ph_2PA at the concentrations indicated in a volume of 1.0 mL.

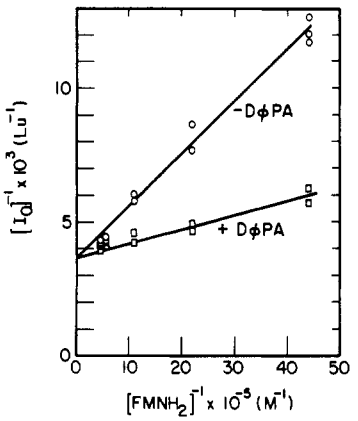

FIGURE 5: Double-reciprocal plot of the relationship between $FMNH_2$ concentration and initial light intensity in the presence and absence of 2,2-diphenylpropylamine. Luciferase activity was measured by the dithionite assay in the presence (□) and absence (○) of 0.4 mM 2,2-diphenylpropylamine. Prior to injection of an air-saturated decanal suspension, each assay (23 °C) contained 7.8 μg of luciferase, 20 mM Bis-Tris, pH 7.1, and $FMNH_2$ as indicated in a volume of 1.0 mL.

Effect of 2,2-Diphenylpropylamine on $FMNH_2$ Binding. The effect of 2,2-diphenylpropylamine on the binding of $FMNH_2$ was determined by using the dithionite assay described under Materials and Methods; the data are shown in Figure 5. The 2,2-diphenylpropylamine, which was competitive with the aldehyde substrate, actually enhanced the affinity of the enzyme for $FMNH_2$ by ~4.3-fold.

It appeared from these data (Figures 3–5) that the inhibitory effect of 2,2-diphenylpropylamine was due specifically to blocking the binding of aldehyde to intermediate II. Furthermore, the effect of 2,2-diphenylpropylamine on the binding of $FMNH_2$ suggested that the molecule bound to luciferase without flavin, forming a species having a higher affinity for $FMNH_2$ than the enzyme alone. This hypothesis was tested in the experiment shown in Figure 6. Addition of 2,2-diphenylpropylamine to a solution of enzyme and subsaturating $FMNH_2$ (in the presence of a slight excess of dithionite) caused an increase in the amount of enzyme-bound $FMNH_2$.

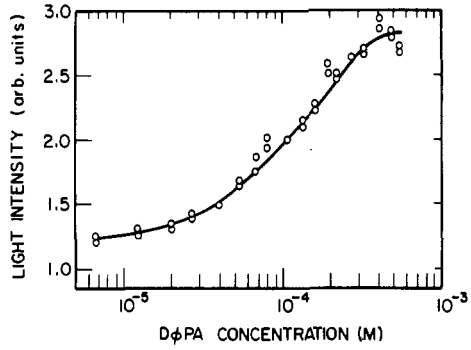

FIGURE 6: Effect of 2,2-diphenylpropylamine on luciferase activity in the presence of $FMNH_2$ at a subsaturating concentration. Luciferase was assayed by the dithionite method. Prior to injection of an air-saturated decanal suspension, each assay (23 °C) contained 16 μg of luciferase, 20 mM Bis-Tris, pH 7.1, and 0.38 μM $FMNH_2$ in a volume of 1.0 mL.

This was demonstrated by the increase in light emitted upon injection of O_2 with a saturating level of aldehyde. The dissociation constant of 2,2-diphenylpropylamine calculated from these data was ~ 0.1 mM, in excellent agreement with the dissociation constant calculated from the aldehyde competition data from Figures 3 and 4. The results of this experiment support the conclusion that 2,2-diphenylpropylamine enhances the affinity of the enzyme for $FMNH_2$.

Effects of $FMNH_2$ and of Aldehyde on Binding of 2,2-Diphenylpropylamine. The reciprocal effect, that is, the effect of $FMNH_2$ on the affinity of the enzyme for 2,2-diphenylpropylamine, was determined by using Ph_2PA–Sepharose (Figure 7). The affinity resin was mixed with a solution of luciferase under conditions such that ~25% of the enzyme remained in solution. Titration of this suspension with $FMNH_2$ (in the presence of a slight excess of sodium dithionite) resulted in a marked drop in the concentration of free enzyme, in accord with the expected result for a reciprocal effect. That is, the effect of $FMNH_2$ was to increase the affinity of the enzyme for 2,2-diphenylpropylamine. This experiment was repeated using decanal as the titrant (Figure 7). The effect of decanal was to cause a dramatic increase in the solution concentration of luciferase, in accord with the competitive binding suggested by the results of the experiment shown in Figure 4. This experiment showed that aldehyde binds to free enzyme to block interaction with Ph_2PA–Sepharose.

Effect of 2,2-Diphenylpropylamine on the Lifetime of Intermediate II. If 2,2-diphenylpropylamine indeed inhibited the bioluminescence reaction by complexing with intermediate II to block aldehyde binding, the intermediate II–inhibitor complex might have a different stability from intermediate II. The lifetimes of intermediate II and of the intermediate II–inhibitor complex were measured by the double injection assay described under Materials and Methods and were nearly identical.

Discussion

Meighen & MacKenzie (1973) showed that the secondary hydroxyls of the ribityl chain of the reduced flavin substrate have a strong influence on the affinity for the aldehyde substrate. It is almost certain that the flavin site and the aldehyde site on the luciferase α subunit are contiguous such that reaction between the two substrates can occur, so it is not surprising that structural changes in one site can change binding at the other. However, an interaction between binding of aldehyde and binding of flavin has not been demonstrated except by the work of Meighen & MacKenzie (1973).

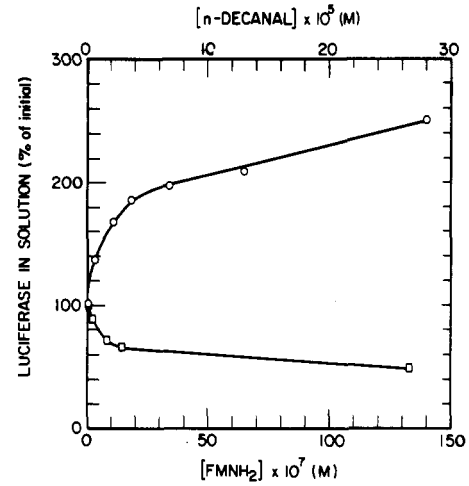

FIGURE 7: Effects of $FMNH_2$ and decanal on the binding of bacterial luciferase to Ph_2PA–Sepharose 6B. Two 100-mg aliquots of suction-dried resin were incubated with separate samples of luciferase at 1 mg/mL in 0.10 M phosphate, pH 6.5, and 1 mM DTE at 4 °C for 30 min. The resin samples were then pelleted by centrifugation, and the supernatants were decanted. Activity measurements of the supernatants by the standard $FMNH_2$ injection assay indicated that each sample of resin had bound ~0.5 mg of luciferase. The effects of $FMNH_2$ on luciferase bound to the resin were determined in the following manner. One of the pelleted resin samples with bound luciferase was resuspended in 4.0 mL of N_2-saturated 0.10 M phosphate, pH 6.5, and 1 mM DTE. The sample was then isolated from the atmosphere with a serum bottle stopper, O_2-free N_2 was gently blown over the surface, the sample was cooled to 4 °C, and 50 μL of a 15 mg/mL sodium dithionite solution was injected. The luciferase in solution (100%) was determined prior to addition of aliquots of FMN. After each aliquot of FMN was added, the anaerobic sample was gently agitated to mix the resin. After the resin had settled (~10 min), at 10-μL aliquot of supernatant was withdrawn and assayed for luciferase activity in the standard $FMNH_2$ injection assay (□). The effects of decanal on luciferase bound to the resin were determined in a similar experiment. The second sample of resin containing bound luciferase was placed in 4.0 mL of 0.10 M phosphate, pH 6.5, and 1 mM DTE, at 4 °C. After the initial concentration of free luciferease (100%) was determined, small aliquots of a 0.10% (v/v) decanal suspension were added. After each aliquot of decanal was added, the resin sample was gently agitated and allowed to settle. Activity measurements of the luciferase in the supernatant were then made by using the standard $FMNH_2$ injection assay (○).

Long-chain alcohols are known to be inhibitors competitive with aldehyde which bind to and stabilize intermediate II (Hastings et al., 1966; Tu, 1979). However, in these studies there was no indication that *binding* of flavin affected binding of alcohol or vice versa. Even very slight structural changes in the molecule binding in the aldehyde site can have profound and unpredictable effects on the lifetime of intermediate II–IIA. With the *V. harveyi* enzyme at ~20 °C, the apparent first-order rate constant for decay of intermediate II is 0.055 s^{-1}; for intermediate IIA with octanal, decanal, and dodecanal, the rate constants are 0.025, 0.22, and 0.027 s^{-1}, respectively (Cline & Hastings, 1972). That is, the lifetimes of the intermediates IIA with octanal and dodecanal are about the same and ~10 times that of the intermediate IIA with decanal. The slight effect on the lifetime of intermediate II resulting from binding of 2,2-diphenylpropylamine is thus difficult to evaluate.

The results presented in this paper show that binding of 2,2-diphenylpropylamine is competitive with binding of aldehyde but enhances the affinity of the enzyme for $FMNH_2$. Likewise, binding of $FMNH_2$ enhances the affinity of the enzyme for 2,2-diphenylpropylamine. The simplest explanation of these observations is that the 2,2-diphenylpropylamine binds in the aldehyde binding site to stabilize a conformer of the

enzyme having an increased affinity for $FMNH_2$. This interpretation is supported by measurements that show that a mutant luciferase, AK-6, having normal aldehyde binding but deficient $FMNH_2$ binding (Cline & Hastings, 1972), binds to Ph_2PA–Sepharose with the same affinity as the wild-type enzyme, while another mutant luciferase, AK-20, having normal $FMNH_2$ binding but deficient aldehyde binding (Cline & Hastings, 1972), binds to Ph_2PA–Sepharose with lower affinity than does the wild-type luciferase (Holzman & Baldwin, 1981).

The Lineweaver–Burk analysis of the aldehyde–2,2-diphenylpropylamine competition data (Figure 4) showed clear curvature, suggestive of aldehyde inhibition at high concentrations. This is not unexpected; aliphatic aldehydes would be expected to bind to and denature proteins. We have investigated this apparent inhibition and find it to be rapidly reversible and highly dependent upon solution buffer conditions. Our experiments, however, do not yet show conclusively whether this is a bona fide substrate inhibition or a nonspecific structural perturbation.

Another point of interest in these results is the clear demonstration of binding of decanal to luciferase in the absence of flavin in a fashion competitive with 2,2-diphenylpropylamine. Addition of aldehyde to a suspension of Ph_2PA–Sepharose with bound luciferase caused a release of the luciferase. The generally accepted scheme of the bacterial bioluminescence reaction is depicted as a sequential addition of first flavin, then O_2, and finally aldehyde (see Figure 1). This scheme is based upon analysis of the rate of the rise of luminescence using stopped-flow techniques (Hastings & Gibson, 1963). However, in the normal flavin injection assay, the enzyme is added to a buffered solution containing the aldehyde substrate and dissolved O_2, and the bioluminescence reaction is initiated by injection of $FMNH_2$. It is possible that aldehyde can bind either before or after binding of $FMNH_2$, not exclusively to intermediate II. It may be important that the enzyme used in our studies was from *V. harveyi*, while the luciferase used in the stopped-flow experiments was from *Vibrio fischeri*. In any case, the order of binding and the apparent interactions between the flavin site and the aldehyde site certainly warrant further investigation.

References

Baumann, P., Baumann, L., Bang, S. S., & Woolkalis, M. J. (1980) *Curr. Microbiol. 4*, 127–132.

Becvar, J. E., & Hastings, J. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3374–3376.

Becvar, J. E., Baldwin, T. O., Nicoli, M. Z., & Hastings, J. W. (1976) *Flavins Flavoproteins, Proc. Int. Symp., 5th, 1975*, 94–100.

Chuang, H. Y. K., Patek, D. R., & Hellerman, L. (1974) *J. Biol. Chem. 249*, 2381–2384.

Cline, T. W., & Hastings, J. W. (1972) *Biochemistry 11*, 3359–3370.

Gibson, Q. H., & Hastings, J. W. (1962) *Biochem. J. 83*, 368–377.

Hastings, J. W., & Gibson, Q. H. (1963) *J. Biol. Chem. 238*, 2537–2554.

Hastings, J. W., & Balny, C. (1975) *J. Biol. Chem. 250*, 7288–7293.

Hastings, J. W., Gibson, Q. H., Friedland, J., & Spudich, J. (1966) *Biolumin. Prog., Proc. Conf., 1965*, 151–186.

Hastings, J. W., Balny, C., LePeuch, C., & Douzou, P. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3468–3472.

Hastings, J. W., Baldwin, T. O., & Nicoli, M. Z. (1978) *Methods Enzymol. 57*, 135–152.

Holzman, T. F., & Baldwin, T. O. (1981) *Biophys. J. 33*, 255a.

Hunter, A., & Downs, C. E. (1945) *J. Biol. Chem. 157*, 427–446.

Makemson, J. C., & Hastings, J. W. (1979) *J. Bacteriol. 140*, 532–542.

McMahon, R. E., Mills, J., Culp, H. W., Gibson, W. R., Miller, W. M., & Marshall, F. J. (1969) *J. Med. Chem. 12*, 207–211.

Meighen, E. A., & Hastings, J. W. (1971) *J. Biol. Chem. 246*, 7666–7674.

Meighen, E. A., & MacKenzie, R. E. (1973) *Biochemistry 12*, 1482–1491.

Meighen, E. A., Nicoli, M. Z., & Hastings, J. W. (1971a) *Biochemistry 10*, 4062–4068.

Meighen, E. A., Nicoli, M. Z., & Hastings, J. W. (1971b) *Biochemistry 10*, 4069–4073.

Mitchell, G., & Hastings, J. W. (1969) *J. Biol. Chem. 244*, 2572–2576.

Nealson, K. H., & Hastings, J. W. (1972) *J. Biol. Chem. 247*, 888–894.

Nicoli, M. Z., Meighen, E. A., & Hastings, J. W. (1974) *J. Biol. Chem. 249*, 2384–2392.

Nicoli, M. Z., Baldwin, T. O., Becvar, J. E., & Hastings, J. W. (1976) *Flavins Flavoproteins, Proc. Int. Symp., 5th, 1975*, 87–93.

Reichelt, J. L., & Baumann, P. (1973) *Arch. Mikrobiol. 94*, 283–330.

Spudich, J., & Hastings, J. W. (1963) *J. Biol. Chem. 238*, 3106–3108.

Sunderberg, L., & Porath, J. (1974) *J. Chromatogr. 90*, 87–98.

Tu, S.-C. (1979) *Biochemistry 18*, 5940–5945.

Tu, S.-C., & Hastings, J. W. (1975) *Biochemistry 14*, 4310–4316.

Tu, S.-C., Hastings, J. W., & McCormick, D. B. (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 832.

Watanabe, T., & Nakamura, T. (1972) *J. Biochem. (Tokyo) 72*, 647–653.

Watanabe, T., Tomita, G., & Nakamura, T. (1974) *J. Biochem. (Tokyo) 75*, 1249–1255.

Watanabe, T., Nakamura, T., Matsui, K., & Kasai, S. (1980) *Flavins Flavoproteins, Proc. Int. Symp., 6th, 1978*, 125–129.

Welches, W. R., & Baldwin, T. O. (1981) *Biochemistry 20*, 512–517.

Ziegler, M. M., & Baldwin, T. O. (1981) *Curr. Top. Bioenerg.* (in press).