The Inhibition of Bacterial Luciferase by Mixed Function Oxidase Inhibitors

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SUMMARY

On the hypothesis that bacterial luciferase may be classed as a mixed function oxidase, compounds reported to be specific inhibitors for such enzymes were tested. Inhibitors designated as SKF (2-diethylaminoethyl-2,2-diphenyl valerate), DPDA (N,N-diethyl-2,4-dichloro(6-phenylphenoxy)ethylamine), and DPEA (2,3-dichloro(6-phenylphenoxy)ethylamine) were effective in blocking the in vitro reaction of pure luciferase at inhibitor concentrations between $10^{-5}$ and $10^{-4}$ M. The mode of action of the two different types of compounds has been found to be distinctly different. One (SKF) interferes with the reaction of one of the substrates, namely FMNH$_2$, while the other (DPEA) blocks the site for aldehyde, the second substrate. Both compounds are effective inhibitors of bacterial growth and luminescence, affecting both functions in similar although not identical degrees.

Most, possibly all, bioluminescent reactions require molecular oxygen as a substrate (1). In the reactions of both the firefly (2, 3) and the crustacean Cypridina (4), the stoichiometry involves the utilization of 1 molecule of oxygen per molecule of substrate and the incorporation of both atoms of oxygen. In both cases the luciferin molecule is split, yielding ground state CO$_2$ as one product and an aromatic species in the excited state as the other. In the formal sense these luciferases may be classed as oxygenases (5, 6). In the firefly one of the oxygen atoms appears in the aromatic product and the second in water (7), while in Cypridina one of the oxygen atoms has been reported to occur in the CO$_2$ (8).

In the bioluminescent reaction catalyzed by bacterial luciferase the reaction may be more strictly classed as a monoxygenase, or mixed function oxidase (1). In addition to oxygen and FMNH$_2$, the reaction requires a long chain aldehyde (>8 carbon atoms), the over-all enzymatic reaction having been postulated to occur as shown, where FMN$^+$ is oxidized flavin in an electronically excited state.

$$
\text{FMNH}_2 + \text{RCHO} + O_2 \xrightarrow{\text{luciferase}} \text{FMN}^+ + \text{RCOOH} + \text{H}_2\text{O}
$$

This scheme is only a postulate, neither the stoichiometry nor the products having been experimentally established. Nevertheless, the availability of compounds reported to be specific inhibitors for reactions of the mixed function oxidase type was of interest, especially with regard to establishing the intermediate steps in the luminescent reaction and possibly more general enzymatic features of mixed function oxidases. Compounds designated as SKF 525-A, DPDA, and DPEA (Fig. 1) have been reported to be effective inhibitors of the P-450 mixed function oxidase system from liver microsomes (9).

In the present study we describe an inhibitory effect of these compounds on bacterial bioluminescence, both in vivo and in vitro. From an analysis of their inhibitory effects in the in vitro system, with pure luciferase, the mode of action of the two different types of compounds has been found to be distinctly different. One (SKF) interferes with the binding (or reaction, or both) of reduced flavin mononucleotide (FMNH$_2$), while the other (DPEA) apparently reacts with the luciferase at the binding site for aldehyde.

MATERIALS AND METHODS

Chemical reagents were of analytical quality where available. FMN was a gift of Sigma Chemical Co., and was chemically reduced by bubbling hydrogen in the presence of platinumized asbestos (R. H. Sargent & Co.). Long chain aldehydes (octanal, decanal, and dodecanal) were a gift of the Aldrich Chemical Co., and DPDA and DPEA of the Lilly Laboratories, Indianapolis, Ind. SKF 525-A was kindly supplied by Smith, Kline & French Laboratories, Philadelphia, Pa. Stock solutions (12.5 mg per ml) of these inhibitors were prepared in dimethylsulfoxide and used in all experiments. These solutions correspond to the following molarities: DPEA, 0.045 M; DPDA, 0.042 M; SKF, 0.035 M.

Luciferases from two strains of luminous bacteria, designated as Pf and MAV (11) were purified as previously described (12). A stock solution of each (8.5 mg per ml, in 0.01 M phosphate buffer, pH 7.0, and $10^{-4}$ M DTT) was prepared for use in all experiments. These solutions were used for the in vivo experiments. These strains are

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The abbreviations used are: SKF, 2-diethylaminoethyl-2,2-diphenyl valerate; DPDA, N,N-diethyl-2,4-dichloro(6-phenylphenoxy)ethylamine; DPEA, 2,3-dichloro(6-phenylphenoxy)ethylamine; DTT, dithiothreitol; BSA, bovine serum albumin.
from diverse origins; they include both symbiotic and free living forms. The strain numbers used here are those which will be used in a later publication describing the origins and properties of these strains.

In the medium used for growth the NaCl concentration was changed from 3 to 1% in some experiments. The medium consisted of 30 g (or 10 g) of NaCl, 7.0 g of Na₂HPO₄·7H₂O, 1 g of KH₂PO₄, 0.5 g of (NH₄)₂HPO₄, 0.1 g of MgSO₄, 3 ml of glycerol, 0.5 g of Difco yeast extract, and 5 g of Difco Bacto Tryptone per liter of distilled water.

For the "flavin" assay, stock 0.1% v/v aldehyde solutions were prepared as suspensions by sonication of 10 µl of aldehyde in 1 ml of distilled water. Although molarities after dilution in the reaction mixtures were calculated assuming that all of the aldehyde was in solution, this is probably not so. Thus Km values given may not be exactly correct, but the relative values obtained in the presence and absence of inhibitors were reproducible and are considered to be significant. Aldehydes prepared as saturated aqueous solutions might have been used instead, but these are difficult to prepare and suffer from capricious autoxidation (13).

Light-measuring apparatus consisted of a light-tight chamber, designed to hold a standard scintillation vial exposed by means of a shutter mechanism to a photomultiplier tube (RCA - 1P21). After appropriate amplification the output was monitored on an Esterline Angus Speed Servo recorder and expressed in quanta sec⁻¹, established by the standards of Hastings and Weber (14).

Reduced flavin-initiated in vitro assays (flavin assays) were initiated by the injection via a syringe of 1 ml of 5 × 10⁻⁴ M reduced FMNH₂ into the sample vial containing the luciferase in 1 ml of assay buffer (0.01 M phosphate buffer, pH 7.0, 0.1% BSA) plus 50 µl of the appropriate stock aldehyde (except decanal with MAV, which was 10 µl).

Oxygen-initiated in vitro assays (15), referred to as dithionite assays, were accomplished by the injection of 1 ml of an air-saturated dodecanal solution (5 ml of stock aldehyde in 100 ml of distilled water) into a reaction vial containing 10 µl of luciferase in 1 ml of assay buffer, 1 ml of 5 × 10⁻⁴ M FMN, and about 5 mg of solid dithionite (Na₂S₂O₄), adequate to deplete the oxygen and fully reduce the flavin.

Light-initiated bioluminescence was measured by the method of Hastings and Gibson (16). Pure light-induced protein, 10 µl (17, 18), was mixed with 2 ml of 0.05 M phosphate buffer in a Vycor syringe and irradiated by flash discharge (19). The irradiated sample was then injected into a sample vial positioned above the phototube containing 90 µl of dodecanal stock in 1 ml of 0.05 M phosphate buffer. In all assays, the activity was measured by recording the initial maximum light intensity (Io). In both the flavin and dithionite assays the initial intensities and kinetics are identical when identical amounts of luciferase are used.

Flavin-binding experiments were done with the dithionite assay, because at low FMNH₂ concentrations (<10⁻⁷ M), the amount of autoxidation in the syringe is so significant that the injection method of the flavin assay is neither accurate nor reproducible (15). Dilutions of the stock flavin solution were used and the total volume before aldehyde addition was adjusted to 2 ml with distilled water.

Cell growth and in vivo luminescence were monitored as previously described (20). The immediate effect of inhibitors on

![Fig. 1. Structural formulae for DPDA, DPEA, and SKF.](image-url)
mediate II. has properties similar to the analogous chemically generated intermediate, and designated by Roman numerals. Chemical generation of the two types of inhibitors. Intermediates are enzyme-bound, as shown in Fig. 1, and the values recorded at 3 min. For explanation of strain origins, see "Materials and Methods."

### Table I

| Strain | Origin | DPEA | DPDA | SKF |
|--------|--------|------|------|-----|
| MAV    | Local isolate | 80 100 | 50  80 | 20 100 |
| Pf     | A.T.C.C. | 80 100 | 100 100 | 100 100 |
| PP     | A.T.C.C. | 95 100 | 95  100 | 95 100 |
| Ei-4   | Free living | 80 100 | 80  100 | 0   45 |
| PR-1   | Free living | 75 100 | 65  100 | 55  90 |
| BG-1   | Free living | 2   25 | 90  100 | 0   75 |
| W-9S   | Free living | 75 100 | 55  90 | 15  80 |
| ND-2   | Free living | 100 100 | 100 100 | 75 100 |
| P-2    | Free living | 90  100 | 95  100 | 0   90 |
| SA-2   | Symbiotic | 70  100 | 95  100 | 0   80 |
| 22     | Symbiotic | 70  100 | 65  100 | 0   80 |
| L-V    | Symbiotica | 0  35 | 85  100 | 100 100 |
| CJ     | Symbiotica | 25  75 | 75  100 | 100 100 |
| PJ     | Symbiotica | 50  100 | 100 100 | 45 100 |
| L-4    | Symbiotica | 100 100 | 90  100 | 0   20 |
| SQ-1   | Symbiotica | 50  90 | 95  100 | 65 100 |
| PP     | Symbiotica | 50  95 | 95  100 | 90 100 |

**Fig. 3.** Scheme depicting the hypothesized intermediates and pathways in bacterial bioluminescence and the sites of action by the two types of inhibitors. Intermediates are enzyme-bound, and designated by Roman numerals. Chemical generation of the intermediates occurs via FMNH₂ reaction with luciferase; the light-initiated pathway involves the interactions of photon(s) with a luciferase-related protein (LIP = light inducible protein) to produce an intermediate Iₐ, which after reaction with oxygen has properties similar to the analogous chemically generated intermediate II.

**Fig. 4.** Effect of inhibitors on flavin-initiated in vitro bioluminescence. One microliter of the stock solution of MAV luciferase (see "Materials and Methods") was added in each experiment; 5 μl of SKF-525A (Δ—Δ) or DPEA (O—O) were added to the reaction mixture prior to the injection of FMNH₂. The inset shows the integrated curves for similar reactions, indicating that DPEA (O—O) does not lower the final light yield while SKF (Δ—Δ) does. Ordinate, light intensity. One unit = 10⁹ quanta sec⁻¹.
FIG. 5. Effect of inhibitors upon the in vitro bioluminescence using the dithionite assay, plotted on a logarithmic scale. Each assay included 10 μl of stock luciferase, and, when used, 5 μl of inhibitor. *Ordinate*, light intensity. One unit = 2 x 10¹⁰ quanta sec⁻¹. FMNH₂ concentrations given are before mixing; final concentrations are one-half of these absence. The experiments of Fig. 8, which will be presented in a later section, may be referred to in order to clarify this point.

The difference between the inhibitory character of the two compounds may be seen in Fig. 4. With DPEA the initial intensity is less, but the rate of decay is also slower, so that the total amount of light obtained is similar. In fact, the yield is, within error, the same for the two (see inset, Fig. 4). Referring to the reaction scheme (Fig. 3), we can conclude that with DPEA the same amount of Intermediate II must have been formed in the first instance, and that while it was discharged over a longer time period, the relative amount reacting via the aldehyde (high quantum yield) pathway was unchanged.

On the other hand, when the luminescent reaction is inhibited with SKF, the rate of decay is not significantly different, so that a lower yield accompanies an inhibited reaction. This can be explained by assuming that the compound inhibits the actual formation of intermediate, possibly from the very outset, but that the intermediate which is formed possesses essentially unaltered kinetic properties. The inhibitor thus might act by blocking at the initial stage of reaction between FMNH₂ and luciferase.

Similar experiments are illustrated in Fig. 5, plotted on a logarithmic scale to facilitate comparison of the rates of decay of luminescence in the different cases. In this figure we have included as well experiments carried out at a 100-fold lower concentration of FMNH₂ which indicate that SKF, but not DPEA, is competitive with FMNH₂.

The experiments shown in Fig. 6 provide additional evidence for the competitive nature of the interaction of SKF at the flavin site for luciferase. Without inhibitor present the apparent Michaelis constant for the binding of reduced flavin is 7 x 10⁻⁷ M; in the presence of 1.7 x 10⁻⁴ M SKF the Kₘ is 4 times greater (2.7 x 10⁻⁴ M). In the case of reactions inhibited by 1.1 x 10⁻⁴ M DPEA the Kₘ for FMNH₂ is not greatly altered.

DPEA is, however, competitive with aldehyde, while SKF is not (Fig. 7). The value obtained for the uninhibited enzyme

FIG. 6. Reciprocal plot of the relationship between FMNH₂ concentration and initial light intensity, in the presence and absence of inhibitors. The data for the control (×) and with SKF (○) are plotted on a linear scale in the inset. Reaction mixtures included 10 μl of stock luciferase and, when used, 5 μl of inhibitor. In order to illustrate the lack of effect of DPEA upon the Kₘ, the data was normalized to the Vₘₐₓ obtained in the control and SKF-inhibited reactions. *Ordinate*, reciprocal of velocity of reaction, calculated in intensity units, where 1 unit = 2 x 10¹⁰ quanta sec⁻¹.

FIG. 7. Reciprocal plot of the relationship between dodecanal concentration and initial light intensity in the presence and absence of inhibitor. The data for the control (×) and with DPEA (○) are plotted on a linear scale in the inset. Reaction mixtures and ordinates as given in legend to Fig. 6. Vₘₐₓ is not reached with the inhibitor competitive with aldehyde because the reaction with MAV luciferase is inhibited at high aldehyde concentrations (II), as is evident from the control. The Vₘₐₓ of the control was normalised to the control Vₘₐₓ as described in Fig. 6.
was $6.5 \times 10^{-5}$ m for a sonicated preparation of dodecanal; in the presence of $10^{-4}$ m DPEA the $K_m$ for aldehyde was 4 times greater ($2.6 \times 10^{-4}$ m). DPEA and DPDA were found to be competitive with all aldehydes tested (octanal, decanal, and dodecanal), and with the luciferases from both strains (MAV and Pf). Table II presents a list of enzymes, aldehydes tested, and the different values obtained for $K_m$.

An additional and very significant feature of the inhibition by DPEA is illustrated in Fig. 8. In the earlier experiments it was shown that the lifetime of Intermediate II is considerably extended in the presence of the inhibitor, while at the same time its quantum yield is unaffected. The question as to whether or not the presence of aldehyde is required for DPEA to have this effect on the lifetime of the intermediate was examined in the experiments of Fig. 8. Reactions were initiated without aldehyde but with inhibitor in one series (A), and without aldehyde but with inhibitor in the second group (B). Aldehyde was then added secondarily at several later times. The partial time course for each of these reactions is shown. By connecting the initial maximum intensity for each of these (dotted line), one can deduce the lifetime of the intermediate. The time course for a reaction in which both aldehyde and inhibitor were present from the beginning is also shown, illustrating that the lifetime of the intermediate in the presence of inhibitor is extended in a similar way, both with and without aldehyde.

The action of the inhibitors when added secondarily (after the reaction has been initiated) provides an interesting method for examining the effect of these inhibitors on the previously formed intermediates during the decay phase of the reaction. SKF has no effect on the luminescence when added secondarily. This is expected since its action appears to involve the reduced flavin site, where reaction is essentially complete within 1 sec after FMNH$_2$ addition. Since excess FMNH$_2$ is oxidized nonenzymatically (Fig. 3), no further substrate is available to the luciferase after the first exposure.

DPEA, on the other hand, exerts a marked effect when added secondarily (Fig. 9). This effect is quite different with different aldehydes, being most marked with hexanal and octanal, but less with decanal and absent with dodecanal. There is also some difference with the other type of luciferase, Pf, for only a slight effect of secondary addition of DPEA occurs with hexanal, and there is no effect when any of the other aldehydes are used. This marked difference between Pf and MAV luciferases regarding the effect of secondary addition of DPEA is the only major difference between the two luciferases found with respect to their interactions with these inhibitors.

An alternative and novel method for initiating in vitro bacterial bioluminescence was reported some years ago by Gibson et al. (19). Luciferase preparations exposed to an intense brief light from a flash discharge lamp were found to subsequently emit a kinetically characteristic aldehyde-stimulatable bioluminescence. The only differences are that this light-induced bioluminescence involves modified luciferase (17) and it neither requires nor is it stimulated by added FMN; the pathway was therefore postulated as shown in Fig. 3, entering at the stage of Intermediate II (16). A strong prediction of the present studies with inhibitors is that the DPEA should inhibit this light induced bioluminescence and SKF should not. This was indeed found to be the case (Table III).

Hammond and White (10) reported that the carotenoid hydroxylation system of Staphylococcus aureus was more sensitive to the inhibitory effect of DPEA, but this was not the case for the bacterial luciferase. The reaction in the presence of inhibitor is extended in a similar way, both with and without aldehyde.

![Fig. 8. Effect of DPEA on the kinetics of the luminescent reaction in the absence of dodecanal, plotted on a logarithmic scale. All reactions (10 ml of luciferase, 5 ml of DPEA) were initiated by injecting FMNH$_2$ at zero time. With aldehyde present from the beginning (C), the intensity and decay rate were decreased from the control (A), as previously described. In reactions where the aldehyde addition was made at a later time (control, A; DPEA, ), the rate at which the intermediate (II) decays can be deduced (dashed line) from the initial intensity after aldehyde addition. Ordinate, light intensity. One unit = $2 \times 10^{13}$ quanta sec$^{-1}$.](http://www.jbc.org/)
to inhibition by DPEA and DPD compared to inhibition by DPEA and DPD.

We examined both classes of inhibitors for a differential effect of this type. The results (Fig. 10) are presented as a plot of light intensity per ml of culture versus cell density in a series of experiments at different inhibitor concentrations. If the luminescent system is more sensitive to the inhibitor than is growth, then the level of luminescence of the control culture should be greater at any given cell density than the level of luminescence in the presence of inhibitor. Clearly no differential effect of this type was seen.

### Table III

| Inhibitor | Percentage inhibition, FMNH₂-initiated | Percentage inhibition, light-initiated |
|-----------|----------------------------------------|----------------------------------------|
| None      | 0%                                     | 0%                                     |
| DPEA      | 78%                                    | 67%                                    |
| DPD       | 73%                                    | 32%                                    |
| SKF       | 73%                                    | 5%                                     |

**Fig. 9.** Effect of secondary addition of 10 µl of DPEA on the luminescent reaction, plotted on a logarithmic scale. The reaction (with 1 µl of luciferase) was initiated by FMNH₂ injection at zero time with octanal as the aldehyde. To reactions initiated without inhibitor (×, Δ, ○), DPEA was added secondarily causing prompt inhibition and an alteration of the decay rate to the lower value exhibited by the reaction in which DPEA was present from the start (○). Ordinate, light intensity. One unit = 2 × 10⁸ quanta sec⁻¹.

**Fig. 10.** Inhibition of growth and in vivo luminescence by the different inhibitors used at the final concentrations indicated. Ordinate in main graph, light intensity (1 unit = 2 × 10⁸ quanta sec⁻¹) per ml of culture. Ordinate in inset and abscissa in main graph, cell density in arbitrary units, measured at 660 nm. Abscissa in inset, time. In the inset the effect of the inhibitors upon growth is shown, and data from these same experiments is plotted on the main graph. This shows that the level of in vivo luminescence is, with some exceptions, proportional to cell density, independent of whether or not the culture is inhibited.
In fact, with DPEA a small but clear differential effect favoring luminescence was observed. The luminescence of cells at mid-log phase was slightly greater than that of uninhibited cells at the same density (Fig. 10, left). With SKF a pronounced differential inhibition of in vivo bioluminescence occurred, but only at the very early stages of growth (Fig. 10, right), at a time prior to the onset of the synthesis of new luciferase (20). The fact that this does not occur at later stages may be connected with the character of the SKF inhibition of growth, which involves a concentration-dependent lag, during which little growth occurs, followed by growth at a rate very similar to that of the uninhibited control (Fig. 10, right inset). It seems likely that the SKF is detoxified during this lag period, and that the apparent differential inhibition of luminescence may be only a reflection of the inhibition of that luciferase which was present at the time of inoculation. This pattern of growth inhibition is strikingly different from that which occurs with DPEA (Fig. 10, left inset) where the rate of growth is affected by and in proportion to the amount of the inhibitor present.

**DISCUSSION**

Bacterial luciferase provides an unusually rapid and sensitive method for the study of oxidase inhibitors. The instantaneous reaction rate is given directly by the light intensity, eliminating the necessity for measuring the accumulation of a reaction product which in other systems may sometimes be remote from the point of inhibition. Probably the most important advantage of the luciferase system relates to favorable enzymology. The enzyme is available in the pure state, its subunit structure has been elucidated, and the substrates and certain aspects of the intermediate steps are known (11, 22). In addition, the lifetime of the reaction intermediates is extraordinarily long (tens of seconds or minutes), making possible the analysis of the individual steps (21).

From the present study it is already clear that the two types of inhibitors act at different sites on the luciferase, each being competitive with a different one of the substrates. The results are readily interpretable in terms of the intermediates and steps in the reaction previously postulated (Fig. 5). The specific and different effects of the two inhibitors is also of considerable interest in terms of the interpretation of the effect of these inhibitors in other oxidase systems, where such distinctions have not yet been reported. It seems reasonable that the site blocked by SKF, competitive with FMNH₂, might be similar to analogous sites on other mixed function oxidase enzymes, where flavins are involved in many instances (5). With DPEA and DPDEA the more general basis for inhibition is less evident, since long chain aldehydes is not a substrate in most oxidase systems. It is clear that DPEA inhibition does not involve the oxygen site as such, since secondary addition of DPEA inhibits, and it is known that the oxygen step occurs only at the very beginning (21). Indeed after initial reaction with oxygen the luminescence is independent of oxygen and secondary inhibition by DPEA occurs equally well with or without oxygen. The fact that secondary inhibition by DPEA is ineffective with some aldehydes may relate to the relative strengths of binding of aldehyde and inhibitor.

It is also clear that the inhibitor acts on the enzyme and not the aldehyde, since inhibition is effective in the absence of aldehyde (Fig. 8). This experiment also indicates that a stable inhibitor-intermediate complex is formed (II-DPEA, Fig. 3), and that both it and the aldehyde-intermediate complex (II-A) are in equilibrium with free intermediate.

Studies of the effects of these inhibitors on other luciferases such as firefly (7), Cypridina (4), and Renilla (23) might provide valuable contributions to our understanding of the more general aspects of the mechanism in bioluminescent systems, since the reactions in these differ considerably.

A prediction of the model proposed for DPEA inhibition is that the rate of luminescence decay, i.e. the apparent first order constant for the decay, should be related to the ratio of inhibitor-aldehyde present, by virtue of the equilibria shown in Fig. 3 (IIₐ = II = II-DPEA). Decay rates intermediate between those shown in Fig. 5 (with and without DPEA) occur either with less inhibitor or with more aldehyde, independent of absolute concentrations.

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