Substrate and Substrate Analogue Binding Properties of *Renilla* Luciferase†

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ABSTRACT: Luciferase from the anthozoan coelenterate *Renilla reniformis* catalyzes the oxidative decarboxylation of luciferin consuming 1 mol of O_2 per mol of luciferin oxidized and producing 1 mol of CO_2, 1 mol of oxyluciferin, and light (λ_B, 480 nm) with a 5.5% quantum yield. In this work we have examined the binding characteristics of luciferin, luciferin analogues, and competitive inhibitors of the luciferin-luciferase reaction. The results show that luciferin binding and orientation in the single luciferin binding site of luciferase are highly specific for and dependent upon the three group substituents of the luciferin molecule while the imidazolone-pyrazine nucleus of luciferin is not directly involved in binding. Anaerobic luciferin binding promotes a rapid concentration-dependent aggregation of luciferase which results in irreversible inactivation of the enzyme. This aggregation phenomenon is not observed upon binding of oxyluciferin, luciferyl sulfate, or luciferin analogues in which the substituent at the 2 position of the imidazolone-pyrazine ring has been substantially altered.

Recent work from this laboratory has shown that *Renilla* luciferase (*Renilla* luciferin:oxygen 2-oxidoreductase (decarboxylating), EC 1.13.12.5) is active as a single polypeptide chain monomer of 35 000 daltons and that it produces luminescence by converting *Renilla* luciferin (I, Figure 2), in the presence of O_2, to a protein-bound electronic excited state of oxyluciferin (Matthews et al., 1977). This oxidation process is illustrated in Figure 1. The enzyme was also found to contain three free sulfhydryl groups, no disulfide linkages, and a relatively high proportion of hydrophobic amino acid residues such that it has an average hydrophobicity (Bigelow, 1967) of 1200 cal residue^{-1}.

A fully active synthetic analogue of luciferin has been prepared (III, Figure 2) which consists of a fused imidazolone-pyrazine ring substituted at positions 2 and 8 with benzyl groups and at position 6 with a phenolic group. In this report we examine the binding of this synthetic luciferin to luciferase and the various factors affecting this binding. For simplicity we will refer to this synthetic analogue (III, Figure 2) as luciferin. It differs from native luciferin (I, in Figure 2) only in its substituent at the 2 position. Using luciferin binding and competitive inhibitor analyses, we have demonstrated that the group substituents on the imidazolone-pyrazine ring are absolute requirements for binding to the single luciferin binding site on luciferase.

Experimental Procedure

Materials. All buffers and solutions were prepared using deionized water, or water twice distilled in glass, having a maximum conductivity of 1.2×10^{-6} Ω^{-1}. Methanol was spectrophotometric or equivalent grade, and all other commercially available chemicals were of reagent grade or the finest quality available. *Renilla* luciferase, and the various luciferin analogues, and their derivatives which were used in this study were prepared as previously described (Hori and Cormier, 1973; Hori et al., 1973, 1975; Matthews et al., 1977). Benzyl luciferyl sulfate (Figure 5) was prepared in this laboratory by K. Hori using a method as yet unpublished.

Determination of the Luciferase-Luciferin Dissociation Constant. The K_d for the binding of luciferin (III in Figure 2) to luciferase was determined using a modification of the flow-through dialysis technique of Colowick and Womack (1969). Radioactive tracers were unnecessary due to the extremely high sensitivity of the luminescence assay. In order to prevent luciferase from catalyzing luciferin oxidation during the experiment, the dialysis apparatus and buffer reservoir were maintained under strict anaerobic conditions in an argon atmosphere. The buffer (0.1 M potassium phosphate, 0.5 M NaCl, 1.0 mM Na_2EDTA,[1] 0.6 mM NaN_3, pH 7.5) was boiled followed by bubbling with argon for 1 h, at which point sodium dithionite was added to a final concentration of 1 mM. The 0.3-mL capacity upper dialysis chamber was filled with 1×10^{-7} M anaerobic luciferase and allowed to equilibrate with the 0.6-mL capacity lower flow-through dialysis chamber for 3 h at a flow rate of 0.1 mL per min at 25 °C. Then, at 30-min intervals, the luciferin concentration in the upper chamber was increased by 1.5×10^{-8} M increments via injection of 1.0-μL aliquots of a 4.5×10^{-6} M stock solution in anaerobic 1 M HCl in methanol. At 5-min intervals, luciferin in the dialysate effluent was quantitated by assaying aliquots for total light (Matthews et al., 1977). A control experiment was performed with no luciferase present to determine the rate of dialysis of a known concentration of unbound luciferin. The K_d and number of binding sites were determined from a Scatchard plot of the data (Scatchard, 1949).

Inhibitor Studies. Several luciferin analogues, their corresponding oxidation products, and molecules structurally similar to portions of the luciferin molecule were screened for their ability to inhibit the luciferin-luciferase reaction. Those molecules found to inhibit luciferase were than analyzed kinetically to determine their mode of inhibition. Each inhibitor, at concentrations which produced approximately 50% and

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[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; BSA, bovine serum albumin; K_d, dissociation constant; K_i, inhibition constant; K_m, Michaelis constant; λ_B, bioluminescent emission maximum.

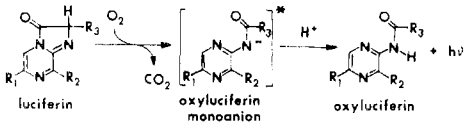

FIGURE 1: Bioluminescent and chemiluminescent oxidation pathway for luciferins of the fused imidazolone-pyrazine type.

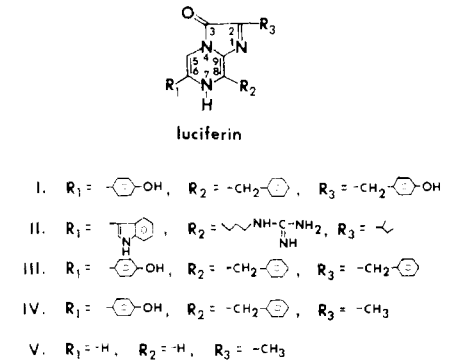

FIGURE 2: Luciferin analogues. Data on the structure of R_3, compound I, has not been published.

25% inhibition, was combined with luciferase (5×10^{-9} M) in assay buffer (0.1 M potassium phosphate, 0.5 M NaCl, 1.0 mM Na_2EDTA, 0.6 mM NaN_3, 0.02% w/v BSA, pH 7.6, 25 °C). Luminescence assays were performed, as previously described (Matthews et al., 1977), by mixing 1.0 mL of luciferase in buffer, with or without inhibitor, with 10 μL of benzyl luciferin in 1 M HCl in methanol. Duplicate assays were performed at final luciferin concentrations of 2.00×10^{-8} M, 2.50×10^{-8} M, 3.33×10^{-8} M, 5.00×10^{-8} M, and 1.00×10^{-7} M. Peak intensities were taken as measures of initial rate. The inhibitors which did not affect the rate of the reaction when extrapolated to infinite luciferin concentration were taken to be competitive inhibitors. A Lineweaver-Burk plot of data taken from a typical experiment is presented in Figure 3. The K_i values, representing the ratio of the dissociation and association rate constants for the competitive inhibitor molecule with luciferase, which we assume to be equivalent to the K_d, were determined from Hofstee plots of the unweighted data (Hofstee, 1959). The K_i values determined from these experiments were consistent for the two inhibitor concentrations tested.

Inhibition constants for benzyl oxyluciferin and native luciferyl sulfate (I, Figure 5) were determined as a function of pH as described above. The buffer (0.1 M potassium phosphate, 0.1 M sodium borate, 0.3 M NaCl, 1.0 mM Na_2EDTA, 0.6 mM NaN_3, 0.02% w/v BSA) was adjusted with concentrated HCl or 10 M NaOH to yield pH values in the range from 6 to 10 at approximately 0.2 pH unit intervals.

Results

Interactions of Luciferase with Luciferin. The results of the luciferin binding and stoichiometry determination are presented in Figure 4. Luciferase was found to have a single binding site for luciferin (III in Figure 2) per 35 000 daltons with a K_d of 3×10^{-8} M. When luciferin and luciferase are combined anaerobically, a concentration-dependent aggregation of the luciferin-luciferase complex occurs which is accompanied by irreversible inactivation of the enzyme. Since the K_d for the luciferin-luciferase complex is small, the luciferin binding and stoichiometry determinations were necessarily carried out at low concentrations of both luciferin and luciferase and at these low concentrations no problem with

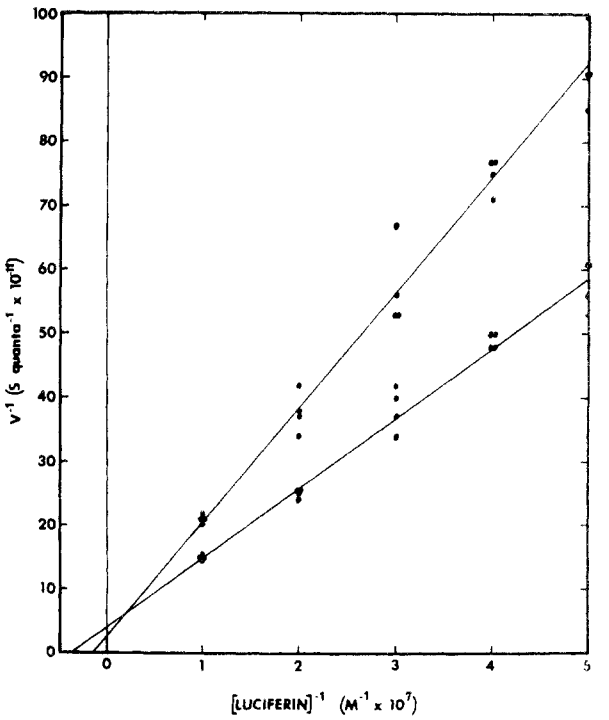

FIGURE 3: Lineweaver-Burk plot (Lineweaver and Burk, 1934) of benzyl luciferyl sulfate (see Figure 5) inhibition data. These data are typical of the many inhibition experiments performed. The solid lines are linear least-squares fits of the data. The upper line (1.22×10^{-7} M benzyl luciferyl sulfate) has a slope of 17.9 ± 0.7 with a $1/v$ intercept at 3 ± 2 and a linear correlation coefficient of 0.988. The lower line (no added inhibitor) has a slope of 1.9 ± 0.4 with a $1/v$ intercept at 4 ± 1 and a linear correlation coefficient of 0.989. The details of the assay are described in the Experimental Procedure section. An inhibitor was taken to be competitive when the intercepts at the $1/v$ axis were the same.

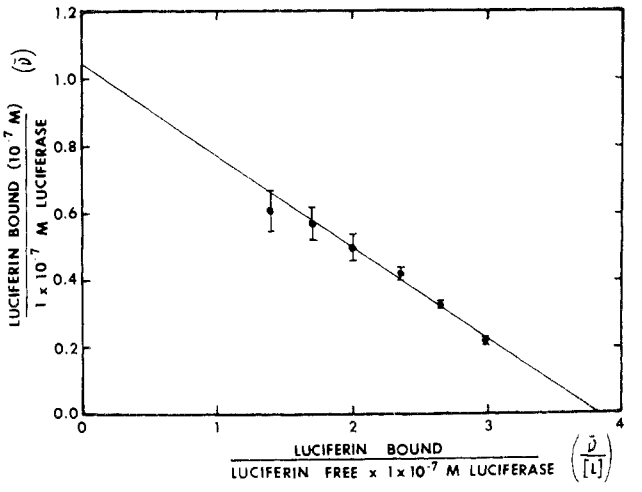

FIGURE 4: Scatchard plot of luciferin-luciferase binding data determined via flow-through dialysis experiments. See experimental section for details.

aggregation was encountered. However, when luciferase and luciferin were combined anaerobically at higher concentrations, i.e., in the 10^{-5} M range, the solution became turbid within 30 s. Over a 5-min period this resulted in the accumulation of an inactive yellow aggregate at the bottom of the vessel. The yellow color of the aggregate is due to bound luciferin. This phenomenon of aggregation was accompanied by a loss of over 99.9% of the initial luciferase activity. Precipitation and activity loss do not occur when 10^{-5} M luciferin and luciferase are mixed aerobically and allowed to react, or when

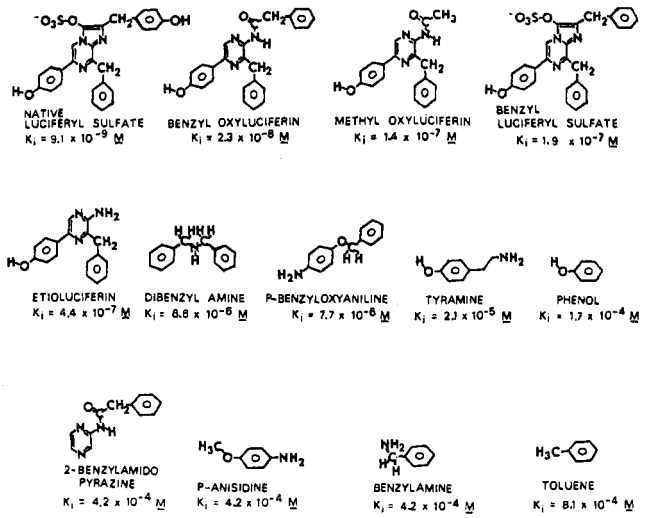

FIGURE 5: Competitive inhibitors of the luciferin–luciferase reaction. The K_i values were kinetically determined and represent the ratio of the dissociation and association rate constants for the inhibitor with luciferase, i.e., the K_d.

10^{-5} M luciferase is combined aerobically or anaerobically with excess benzyl oxyluciferin or native luciferyl sulfate (Figure 5).

Of interest here is the observation that methyl luciferin (IV, Figure 2), at final concentrations of 10^{-5} to 10^{-4} M, does not cause significant activity loss or observable aggregation when it complexes anaerobically with 10^{-5} M luciferase. This luciferin analogue will, however, react with luciferase to produce light under aerobic conditions (Hori and Cormier, 1973), but the turnover number with this analogue is approximately 1 μmol min^{-1} μmol^{-1} enzyme (unpublished data) as compared with a corresponding value of 111 determined with luciferin (III, Figure 2) (Matthews et al., 1977).

Competitive Inhibitor Studies. No spectral change or light production occurs over a period of several hours when compound V (Figure 2), at 10^{-5} M, is incubated aerobically with stoichiometric amounts of luciferase demonstrating that this luciferin analogue is not oxidized by the enzyme. Furthermore, analogue V, and substances which are structurally similar to it such as imidazole, pyrazine, 2-methylpyrazine, 2-aminopyrazine, 2-hydroxypyrazine, and 2-acetamidopyrazine, are not inhibitors of luciferase. In addition, compounds such as short chain aliphatic alcohols, aldehydes, amines, and ketones are not competitive inhibitors of luciferase and these compounds show little or no inhibition at concentrations of 0.1 M. On the other hand, as shown in Figure 5, competitive inhibitors of luciferase are invariably molecules containing one or more phenyl groups. Those inhibitors listed in Figure 4 that contain a single phenyl group have K_i values in the 10^{-4} to 10^{-5} M range and can be viewed as simulating the binding of a single group substituent of *Renilla* luciferin. Competitive inhibitors of luciferase containing two phenyl groups have K_i values in the 10^{-6} to 10^{-7} M range whereas inhibitors containing three phenyl groups have K_i values in the 10^{-7} to 10^{-9} M range. This latter, and most effective, group of competitive inhibitors are luciferin derivatives such as luciferyl sulfate or oxyluciferin in which the group substituents are identical with those found in native or benzyl luciferin (I, III in Figure 2). Combining competitive inhibitors of luciferase, such as phenol or toluene with luciferin analogue V (Figure 2), does not result in an enhancement of inhibition nor do such combinations result in the oxidation of analogue V by luciferase.

Effect of pH on the K_i Values for Oxyluciferin and Luciferyl Sulfate. The inhibition constants for benzyl oxyluciferin and native luciferyl sulfate (Figure 5) and the apparent K_m for luciferin (III in Figure 2) vary by over an order of magnitude within the pH range from 6 to 10 as shown in Figure 6. The pH dependency profiles for the K_i values of benzyl oxyluciferin and native luciferin sulfate and the K_m of luciferin show similar inflection points at approximately pH 7.9, 8.2, and 8.0, respectively, and all three profiles show minimums at approximately pH 6.5. For comparison purposes luciferase activity as a function of pH is also illustrated in Figure 6.

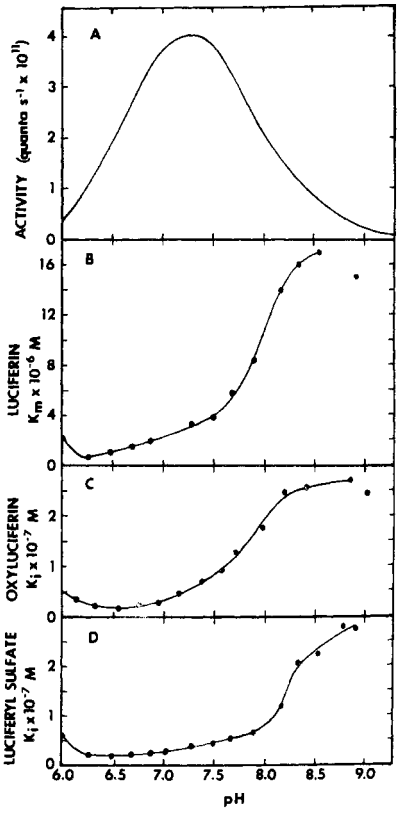

FIGURE 6: Effect of pH on (A) the activity of luciferase; (B) the apparent K_m for luciferin; (C and D) the K_i values for benzyl oxyluciferin and native luciferyl sulfate.

Discussion

The luciferase catalyzed oxidation of luciferin is an oxygenase type reaction which results in oxidative decarboxylation of the imidazolone–pyrazine ring (see Figure 1). Simple derivatives of this fused ring structure such as V (Figure 2) will undergo a luminescent oxidation when dissolved in aprotic solvents (Goto et al., 1968) and the chemistry of this oxidative reaction is analogous to the luciferase catalyzed oxidations of *Renilla* and *Cypridina* luciferins (Goto et al., 1968; Hori et al., 1973). Analogue V, however, will not react with *Renilla* luciferase to produce light. Thus the group substituents on luciferin, i.e., R_1, R_2, and R_3 (Figure 2), function as highly specific enzyme recognition sites which are required for binding to the enzyme and for proper orientation of the imidazolone–pyrazine ring in the catalytic site. The fact that *Cypridina* luciferin (II, Figure 2) will not produce significant amounts of light with *Renilla* luciferase, or vice versa, is an illustration of the specificity imparted by the R groups of these luciferins.

It is evident, from examining competitive inhibitors of *Renilla* luciferase, that only molecules which simulate one or more of the group substituents on the imidazolone–pyrazine ring of luciferin bind to the enzyme and that the phenyl por-

tions of these group substituents are primary features of the binding interaction. Furthermore, since the binding strength increases, by approximately 3 orders of magnitude as the number of phenyl groups contained in a competitive inhibitor increases from 1 to 3, we can conclude that these group substituents function cooperatively in the binding of luciferin to luciferase. As a corollary to this observation, it is apparent that molecules structurally similar to fragments of the fused imidazolone-pyrazine portion of luciferin, as well as luciferin analogue V (Figure 2), do not bind at the luciferin binding site of luciferase. This demonstrates that the fused imidazolone-pyrazine nucleus of the luciferin molecule is not directly involved in the binding interaction. This point is further supported by the observation that the dissociation constants for benzyl luciferin (III, Figure 2) and benzyl oxyluciferin (Figure 5) are essentially the same, showing that disruption of the fused imidazolone-pyrazine ring of luciferin has little effect on the binding strength.

As shown in Figure 4, there are significant differences in the K_i values for benzyl oxyluciferin ($K_i = 2.3 \times 10^{-8}$ M) and methyl oxyluciferin ($K_i = 1.4 \times 10^{-7}$ M). There are also large differences between the turnover numbers (TN) for benzyl luciferin (TN = 111) and methyl luciferin (TN = 1). Thus the R_3 substituent (Figure 2) is capable of influencing both the binding strength and the reaction rate. These observations are interesting in view of the facts that native luciferin and benzyl luciferin (I and III in Figure 2), but not methyl luciferin (IV in Figure 2), will induce a rapid aggregation of luciferase under anaerobic conditions. Luciferase, at 10^{-5} M concentrations, will self-associate in the absence of luciferin but this is a slow process requiring several days to convert 20% of the enzyme to its self-associated form (Matthews et al., 1977). Benzyl oxyluciferin does not cause luciferase to aggregate under any conditions and space-filling molecular models show that oxyluciferin exhibits considerably more flexibility than does luciferin with respect to the orientation of its R_3 substituent. Furthermore, we have previously observed that the ground state enzyme-oxyluciferin complex is different from the biochemically produced excited state by virtue of the fact that the ground state complex is not fluorescent (Matthews et al., 1977).

Luciferyl sulfate, like luciferin, is relatively inflexible with respect to the orientation of its R_3 substituent. Therefore, it would be reasonable to expect that binding of luciferyl sulfate would induce luciferase aggregation. However, the K_i of benzyl luciferyl sulfate (Figure 4) is 1.9×10^{-7} M whereas the K_d value for benzyl luciferin (III in Figure 2) is 3×10^{-8} M and the K_i for benzyl oxyluciferin (Figure 4) is 2.3×10^{-8} M. This difference in the binding strength for luciferyl sulfate relative to the binding strengths for luciferin and oxyluciferin indicates that the sulfate group interferes with binding to a significant extent. The decrease in K_i for benzyl luciferyl sulfate could explain why luciferyl sulfate binding does not induce luciferase aggregation.

The effects of pH on the K_m for luciferin, and on the K_i values for benzyl oxyluciferin and native luciferyl sulfate, are similar with inflection points near pH 8.0 (Figure 6). Luciferins of the *Renilla* type have a pK_a value near 8.5 (Goto and Kishi, 1968) but oxyluciferin and luciferyl sulfate do not have a pK_a value near 8. Thus the observed effects of pH on binding of these compounds appear to be at the level of protein functional group ionization.

References

Bigelow, C. C. (1967) *J. Theor. Biol. 16,* 187.

Colowick, S. P., and Womack, F. C. (1969) *J. Biol. Chem. 244,* 774.

Goto, T., Inoue, W., Sugiura, S., Nishikuwa, K., Isobe, M., and Abe, Y. (1968), *Tetrahedron Lett.,* 4035.

Goto, T., and Kishi, Y. (1968), *Angew. Chem. 7,* 407.

Hofstee, B. H. J. (1959), *Nature (London) 184,* 1296.

Hori, K., and Cormier, M. J. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 120.

Hori, K., Wampler, J. E., Matthews, J. C., and Cormier, M. J. (1973), *Biochemistry 12,* 4463.

Hori, K., Anderson, J. M., Ward, W. W., and Cormier, M. J. (1975), *Biochemistry 14,* 2371.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 58,* 658.

Matthews, J. C., Hori, K., and Cormier, M. J. (1977), *Biochemistry, 16,* 85.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.