Renilla reniformis Bioluminescence: Luciferase-Catalyzed Production of Nonradiating Excited States from Luciferin Analogues and Elucidation of the Excited State Species Involved in Energy Transfer to *Renilla* Green Fluorescent Protein†

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ABSTRACT: A number of coelenterate-type luciferin analogues with structural changes in the *p*-hydroxyphenyl and *p*-hydroxybenzyl substituents have been synthesized. During chemiluminescence, each of the analogues produces a blue emission arising from the singlet excited state of the corresponding oxyluciferin monoanion. During bioluminescence two emissions are observed with coelenterate-type luciferin and some of its analogues. One of these arises from the amide monoanion ($\lambda_{max} \simeq 480$ nm) and the other arises from the neutral species of oxyluciferin ($\lambda_{max} \simeq 395$ nm). Certain analogues produce both emissions, while others produce only the near-ultraviolet emission. Structural changes in the *p*-hydroxyphenyl substituent result in complete or nearly complete elimination of emission from the monoanion, resulting in over a 100-fold reduction in bioluminescence quantum yield. Structural changes in the *p*-hydroxybenzyl substituent do not have a significant effect on the emission spectrum but decrease the luciferase turnover number approximately 25-fold. The large decrease in the bioluminescence quantum yield observed with some of the analogues can be overcome by addition of green fluorescent protein (GFP). GFP forms a rapid equilibrium complex with luciferase and is known to function in this system as an efficient energy-transfer acceptor [Ward, W. W., & Cormier, M. J. (1978) *Photochem. Photobiol. 27*, 389–396]. Spectral analyses have shown that radiationless energy transfer occurs from the singlet excited state of the oxyluciferin monoanion and not from the neutral excited species. The energy-transfer data suggests that the luciferase-bound monoanion singlet excited state can be quenched by solvent and/or protein functional groups. Energy transfer to GFP can apparently favorably compete with this quenching process. Lifetime measurements have shown that the rate of energy transfer must be at least 3×10^8 s^{-1}.

Three proteins have been implicated in the in vivo bioluminescence of the anthozoan coelenterate, *Renilla reniformis*. All three have been recently purified and characterized. One of these is a calcium and luciferin binding protein (mol wt 18 500) which apparently couples nerve excitation to bioluminescence through calcium (Charbonneau & Cormier, 1979; Cormier & Charbonneau, 1977; Anderson et al., 1974). In the presence of calcium ions, this protein transfers its luciferin to a second protein, *Renilla* luciferase (*Renilla* luciferin:oxygen 2-oxidoreductase, decarboxylating, EC 1.13.12.5), which catalyzes the bioluminescent oxidation of coelenterate-type luciferin, via a dioxetanone pathway, to yield oxyluciferin, CO_2, and light (Matthews et al., 1977a; Hori et al., 1973; Hart et al., 1978). The excited state intermediates formed during this oxidation have been identified as mixtures of the electronic excited singlet states of the monoanion and the neutral species of luciferase-bound oxyluciferin (Hori et al., 1973; Matthews et al., 1977a). The monoanion is apparently the predominant excited state species, judging from the bioluminescence emission spectra. Both in vitro and in vivo, the bulk of this excitation energy must be transferred by a nonradiative process to a third protein, *Renilla* green fluorescent protein (GFP). GFP[1] is a dimer of identical subunits (mol wt 27 000), each containing a chromophore of unknown structure and number responsible for the characteristically structured green fluorescence of the protein (Ward & Cormier, 1979; Wampler et al., 1971). Luciferase interacts rapidly with GFP in a highly specific manner to form an equilibrium complex which is believed to be responsible for the efficient energy-transfer phenomenon (Ward & Cormier, 1978).

The calcium-triggered luciferin binding protein can be replaced in vitro by synthetic luciferin and the reaction of luciferin and luciferase plus oxygen will generate blue light (λ_{max} 483 nm). Binding studies with luciferin analogues and synthetic inhibitors of luciferase (Matthews et al., 1977a) have shown that each of the R group substituents of luciferin (see Figure 1) is involved in binding to the enzyme.

We have recently synthesized a number of luciferin analogues with structural changes in the R_1 and R_3 substituents of luciferin (Figure 1). In this report, we show that certain of these analogues, in the presence of luciferase and oxygen, produce bioluminescence only in the near-ultraviolet. We have been able to show that the reaction rate is dependent on the nature of the R_3 group, while the distribution of monoanion and neutral species excited states is independently governed by the nature of the R_1 group. Using energy transfer as a probe, we also provide evidence that luciferase may generate nonradiating singlet excited states which are not normally observed due to quenching. We have also been able to identify the excited state donor which transfers energy to GFP.

Materials and Methods

Materials. All reagents used for synthesis and purification were the best commercial grades available.

Luciferase was prepared as previously described (Matthews et al., 1977a) and was used in assay buffer (0.5 M NaCl, 0.1 M potassium phosphate, 1.0 mM Na_2EDTA, 0.02% w/v BSA, 0.6 mM NaN_3, pH 7.6) for quantum yield, turnover number, or inhibition constant measurements. For measurements involving energy transfer, luciferase and GFP were dissolved in "energy-transfer buffer" (1 mM Tris, 0.1 mM Na_2EDTA, pH 8.0 (Ward & Cormier, 1978)).

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[1] Abbreviations used: GFP, green fluorescent protein; DMF, dimethylformamide.

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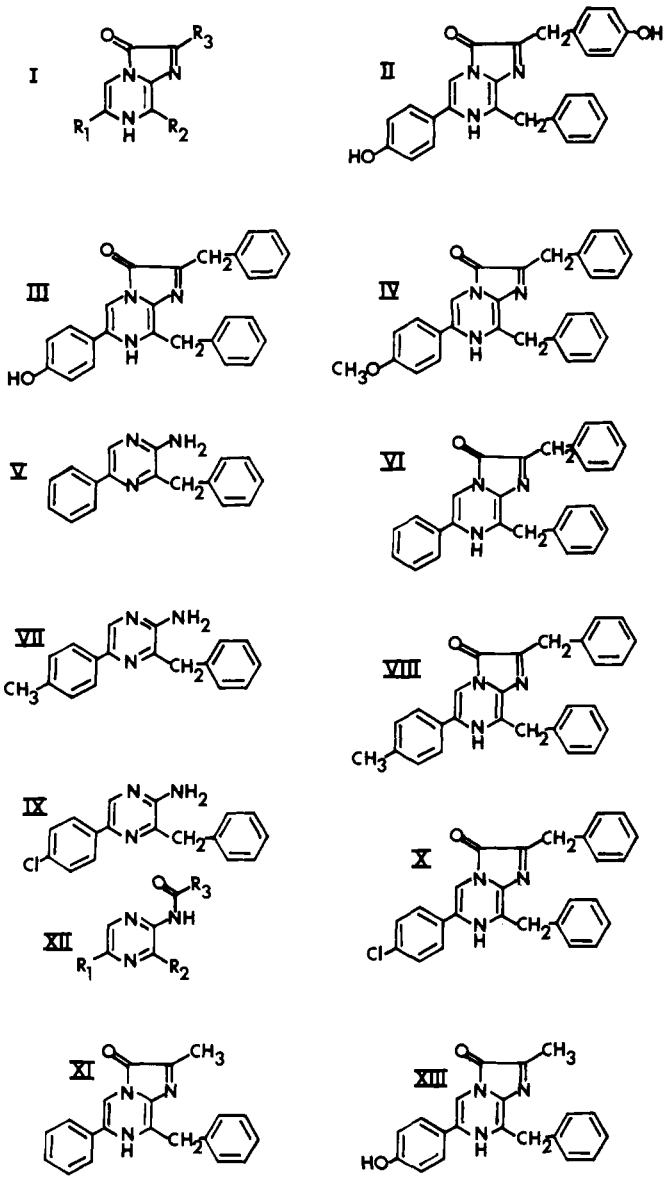

FIGURE 1: Structure of coelenterate-type luciferin, its analogues, and oxyluciferin.

Renilla green-fluorescent protein (GFP) was purified by a procedure already described (Ward & Cormier, 1978, 1979) and was the kind gift of Dr. William W. Ward, Department of Biochemistry & Microbiology, Rutgers University, New Brunswick, NJ 08903. Energy-transfer assays were carried out in a manner similar to the published assay (Ward & Cormier, 1978) by injecting 1 mL of a solution containing luciferase and GFP in energy-transfer buffer into 5–10 μL of the luciferin dissolved in methanol.

Luminescence Assays. Assays were performed on a portable photometer designed and constructed by the University of Georgia Bioluminescence Laboratory (Anderson et al., 1978b). The instrument and its 1P21 photomultiplier tube (Hamamatsu TV Co. Ltd.) were calibrated by using the luminol reaction in both aqueous and Me_2SO solution (Lee et al., 1966). The output of the photomultiplier tube was corrected for its spectral sensitivity by using the manufacturers' published photocathode radiant sensitivity vs. wavelength curves for each of the bioluminescent and chemiluminescent emissions.

Chemiluminescence, bioluminescence, and fluorescence spectra were obtained and corrected by using an on-line spectrophotofluorimeter system previously described (Wampler et al., 1971; Wampler & DeSa, 1971). Fluorescence quantum yields were determined in dimethylformamide (DMF) solutions of the component. Quinine sulfate was used as a standard dissolved in 1 M aqueous H_2SO_4 solution with a value for its fluorescence quantum yield being taken as 0.55 (Parker, 1968). Fluorescence lifetime measurements were made with an Ortec photon counting system by using pyrene in ethanol as a standard (Parker & Hatchard, 1961; Birks et al., 1963).

Luciferin and Etioluciferin Synthesis. Coelenterate-type luciferin (II) was synthesized as has been described (Inoue et al., 1975). The material was purified by chromatography on Sephadex LH-20 in methanol:water (8:2), made 0.1% with concentrated hydrochloric acid.

Benzylluciferin (III) and its methyl ether (IV) were synthesized by a previously described method (Anderson et al., 1978a,b) and purified on LH-20 in the solvent system described above. The etioluciferins V, VII, and IX were synthesized by a previously described method (Kishi et al., 1972) by using acetophenone, *p*-methylacetophenone and *p*-chloroacetophenone, respectively, as primary starting materials. The physical properties of these compounds are listed below.

2-Amino-3-benzyl-5-(*p*-methylphenyl)pyrazine (V): buff-colored needles; mp 138–139 °C; λ_{max} (methanol) 341 (log ϵ 4.01) and 276 nm (log ϵ 4.23); MS *m/e* 261 (M^+), 245, and 233.

2-Amino-3-benzyl-5-(*p*-methylphenyl)pyrazine (VII): yellow needles; mp 147 °C; λ_{max} (methanol) 345 (log ϵ 3.91) and 277 nm (log ϵ 4.18); MS *m/e* 275 (M^+), 259, and 248.

2-Amino-3-benzyl-5-(*p*-chlorophenyl)pyrazine (IX): buff-colored needles; mp 133–135 °C; λ_{max} (methanol) 342 (log ϵ 4.07) and 285 nm (log ϵ 4.31); MS *m/e* 295 (M^+), 279 and 267.

R_1-Phenylluciferin (VI) was prepared by treating the 2-aminopyrazine derivative (V) with the ethyl acetal of benzylglyoxal at 120 °C for 2 h in 1:1 methanol:6 M HCl under argon. The material was treated as described (Anderson et al., 1978a,b) and chromatographed on LH-20 in the acidic aqueous methanol solvent described above. The yellow fluorescent band was pooled, evaporated under argon, and rechromatographed on LH-20 until the absorption ratio 435/350 nm remained constant. The luciferin (VI) was a yellow solid: mp 96–97 °C; λ_{max} (methanol) 430 (log ϵ 3.93), 360 (log ϵ 3.80), and 250 nm (log ϵ 4.38); MS *m/e* 391 (M^+), 389, and 361; NMR (Me_2SO-d_6) τ 5.68 (2 H, s), 5.40 (2 H, s), 2.6 (10 H, complex), 2.0 (5 H, s), 1.32 (1 H, s).

R_1-*p*-Methylphenylluciferin (VIII) was prepared by a similar procedure from VII and the acetal of benzylglyoxal. The luciferin was purified in a similar manner to yield the luciferin as yellow-brown crystals: mp 104–106 °C; λ_{max} (methanol) 435 (log ϵ 3.93), 355 (log ϵ 3.70), and 248 nm (log ϵ 4.33); MS *m/e* 405 (M^+), 403, and 375.

R_1-Chlorophenylluciferin (X) was prepared from IX and the acetal of benzylglyoxal and purified on LH-20 in a similar way: orange solid; mp 108–110 °C; λ_{max} (methanol) 426 (log ϵ 3.89), 368 (log ϵ 3.79), 256 nm (log ϵ 4.38); MS *m/e* 425 (M^+), 423 and 395.

R_1-Phenyl-R_3-methylluciferin (XI) was prepared from 2-amino-3-benzyl-5-phenylpyrazine and methylglyoxal by using a previously described method (Hori & Cormier, 1973). The product was purified on LH-20: yellow crystals; mp 148–149 °C; λ_{max} (methanol) 425 (log ϵ 3.88), 351 (log ϵ 3.75), and 247 nm (log ϵ 4.21); MS *m/e* 315 (M^+), 287, and 272.

Results

Spectral Properties of Synthetic Luciferins. The synthetic luciferins all show similar spectral properties with visible absorption bands between 425 and 435 nm. Strongly elec-

Table I

luciferin	visible ϵ	ϕ_C[a] (%)	chemiluminescence λ_{max}	ϕ_B[b] (%)	ϕ_B^{GFP}[c] (%)	ϕ_B^{GFP}/ϕ_B	turnover no. (min^{-1})
II	9100	ND	ND	6.9 ± 0.7	13 ± 1.3	1.9	ND
III	8300	0.07	464	4.0 ± 0.4	12 ± 1.2	3	111
IV	8300	0.083	462	$(4.2 \pm 0.4) \times 10^{-2}$	4.1 ± 0.4	97	59
VI	8200	0.13	456	$(1.2 \pm 0.1) \times 10^{-2}$	2.3 ± 0.2	192	93
VIII	8200	0.071	460	$(1.6 \pm 0.2) \times 10^{-2}$	2.0 ± 0.2	125	48
X	7500	0.092	465	$(3.3 \pm 0.3) \times 10^{-3}$	0.7 ± 0.07	212	ND
XI	8000	0.096	461	$(2.6 \pm 0.3) \times 10^{-3}$	0.4 ± 0.04	154	4
XIII	9000	0.076	472	0.3 ± 0.03	7.4 ± 0.7	25	1.2

[a] Chemiluminescence quantum yield, photons emitted per molecule of luciferin reacted. [b] Bioluminescence quantum yield, photons emitted per molecule of luciferin reacted. [c] Bioluminescence quantum yield, in the presence of GFP, photons emitted per molecule of luciferin reacted. ND, not determined.

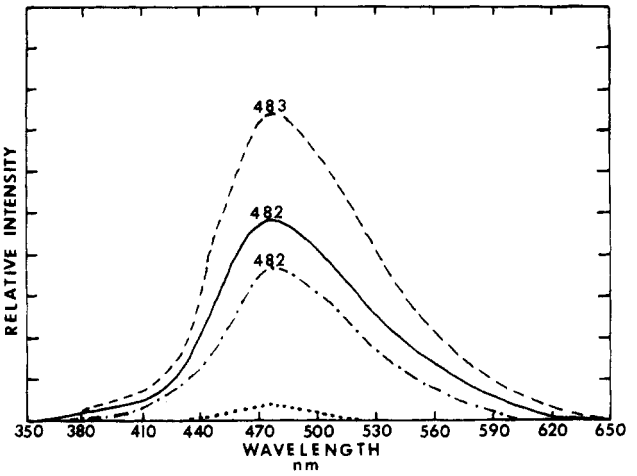

FIGURE 2: Effect of structural modifications of the R_3 substituent of luciferin on the corrected bioluminescence emission spectra. II (---); III (—); XIII (···); emission intensity from XIII amplified tenfold (-·-). See Figure 1 for structure assignments. Emission maxima are indicated on the figure.

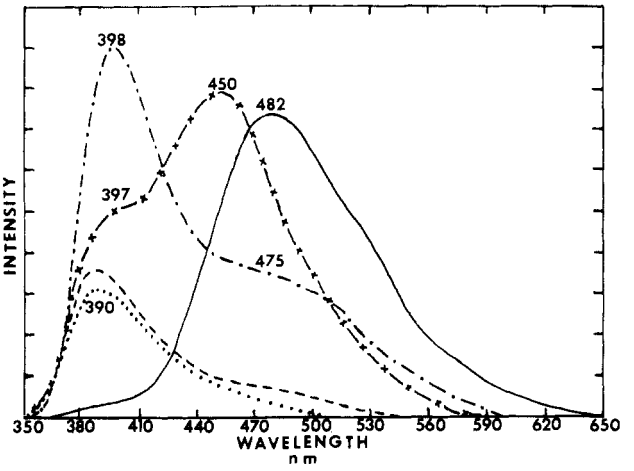

FIGURE 3: Effect of structural modifications of the R_1 substituent of luciferin on the corrected bioluminescene emission spectra. III (—); IV (---); VI (···); VIII (-·-); X (X—X). See Figure 1 for structure assignments. Emission maxima are indicated on the figure.

tron-withdrawing substituents at the 4 position of the R_1 group (Figure 1) tend to blue shift this absorption band as does methyl substitution at the R_3 position (Hori & Cormier, 1973). In methanol solution, all display yellow fluorescence ($\lambda_{max} \simeq$ 520 nm) upon excitation with 350-nm light. The mass spectra of the luciferins all display a parent ion, with the characteristic loss of 2 mass units when the R_3 substituent is benzyl or *p*-hydroxybenzyl. This is in line with the known propensity of these types of structures to oxidize upon heating (Hori et al., 1977).

The extinction coefficient for the benzylluciferin analogue (III, Figure 1) has been reinvestigated and the values found were slightly different from those previously reported (Hori et al., 1973). The molar extinction coefficients for absorption at 435, 340, and 262 nm have been revised to 8300, 5100, and 23 000, respectively. The previous value (Hori et al., 1973) of 9700 for the molar extinction coefficient at 435 nm was probably in error due to the relatively small amounts of material available for accurate weighing. The molecular weight of the luciferin has been revised due to findings of one water of crystallization for benzylluciferin (III, Figure 1), in line with a similar finding for II (Inoue et al., 1975). These revised values have been used in calculating the bioluminescence and chemiluminescence quantum yields reported here.

Chemiluminescence of Luciferin Analogues. The chemiluminescence emission spectra of the luciferin analogues were obtained by dissolving a small amount of the sample in DMF. Bright blue luminescence occurred spontaneously in all cases,

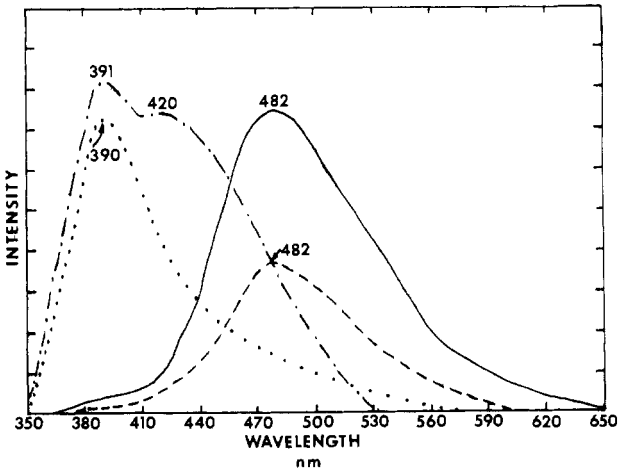

FIGURE 4: A comparison of the effects of structural modifications of either the R_1 or R_3 (or both) substituents of luciferin on the corrected bioluminescence emission spectra. III (—); XIII (---); VI (···); XI (-·-). See Figure 1 for structure assignments. Emission maxima are indicated on the figure.

and the various corrected emission maxima are shown in Table I. In all cases, the emission was a single broad band peaking in the range 456–472 nm, and this emission has been shown to be due to the monoanion amide of the oxyluciferin product XII, Figure 1 (Hori et al., 1973).

The chemiluminescence quantum yields are also listed in Table I and were obtained as previously described (Hori et al., 1973).

Bioluminescence of Luciferin Analogues. The biolu-

Table II

oxyluciferin[a]	ϕ_F[b] (%), neutral species	ϕ_F[c] (%), monoanion	lifetime[d] (ns) neutral species	lifetime[d] (ns) monoanion	intrinsic lifetime (ns) neutral species	intrinsic lifetime (ns) monoanion
R_1 = *p*-methoxyphenyl, R_3 = benzyl	23.5	18.3	1.3	3.3	5.7	18
R_1 = phenyl, R_3 = benzyl	10.6	11.3	ND	ND	ND	ND
R_1 = phenyl, R_3 = methyl	10.1	13.1	ND	ND	ND	ND
GFP in water	80		5.4		6.7	

[a] R_2 = benzyl. [b] In DMF. [c] In basic DMF. [d] Using pyrene in ethanol as standard (see text).

minescence emission spectra of the various analogues in the presence of luciferase are shown in Figures 2–4. In Figure 2, the effects of changing the R_3 substituent of coelenterate-type luciferin (II, Figure 1) to a benzyl group (III, Figure 1) and a methyl group (XIII, Figure 1) on the emission spectra are shown. In each case, the spectral distribution is unchanged, being a composite of a small contribution from the neutral amide oxyluciferin emission ($\lambda_{max} \simeq 395$ nm) and a large contribution from the monoanion form whose emission maxima lies near 482 nm (Matthews et al., 1977a).

In contrast, dramatic changes in the bioluminescence emission spectra occur upon modification of the R_1 substituent of luciferin. As shown in Figure 3, several analogues were examined in which the para position of the R_1 substituent had been modified. The emission spectrum which uses III (Figure 1) is included for reference. The emission with III is more than 100-fold more intense than any of the others shown in Figure 3. The latter emissions have been amplified so that their spectral details may be observed. It can be seen that para substitution of R_1 with either –H (VI, Figure 1) or $-CH_3$ (VIII, Figure 1) results in a loss of the monoanion emission normally observed at 482 nm. Instead one observes a low-level bioluminescence in the ultraviolet (390 nm) due to emission from the neutral species excited state of the corresponding oxyluciferin. As shown in Table I, the bioluminescence quantum yields for VI and VIII (Figure 1) are approximately 300-fold less than that observed for III (Figure 1). This large quantum yield difference is due to lack of emission from the monoanion. Substitution at the para position of R_1 by either $-OCH_3$ (IV, Figure 1) or –Cl (X, Figure 1) results in a substantial lowering of the monoanion emission at 482 nm as evidenced by a 100–1000-fold drop in the observed bioluminescence quantum yields (Table I). In these cases, low level emissions are observed in both the ultraviolet and visible regions of the spectrum. The visible bioluminescence emission band at 450 nm observed with X (Figure 1) is due to a shift in the monoanion emission to the blue. This was determined by an examination of the fluorescence emissions of the various oxyluciferin monoanion analogues in base-containing DMF.

A comparison of the bioluminescence emission spectra illustrated in Figure 4 shows that changes in the R_1 substituent of luciferin, rather than R_3, have the major effect on the shape of the emission spectra. Change in the R_3 substituent from benzyl (III, Figure 1) to methyl (XIII, Figure 1) reduces the quantum yield approximately 20-fold (Table I) but does not change the spectral shape or emission maxima. In contrast, changes in the R_1 substituent from *p*-hydroxybenzyl (III, Figure 1) to phenyl (IV, Figure 1) result in a total loss of monoanion emission with a resultant several-hundred-fold reduction in quantum yield (Table I).

The turnover numbers are listed for some of the analogues in Table I. It can be seen that, where R_3 is a methyl substituent, the turnover number is reduced approximately 100-fold. The effects of each of the changes in the R groups are apparently independent as shown by analogue XI which has a low turnover number and also emits mainly from the neutral species of the oxyluciferin product.

Fluorescence Quantum Yields and Lifetimes. The relatively low bioluminescence quantum yields observed with some of the luciferin analogues described above are not due to correspondingly low fluorescence quantum yields for the oxyluciferins. These results are shown in Table II. Similarities are observed in the fluorescence quantum yields for three of the oxyluciferin analogues in DMF with and without added base. The neutral species fluorescence quantum yields ranged from approximately 10 to 24%. Addition of base to ionize the amide to the monoanion resulted in values ranging from approximately 13 to 18%. Clearly the differences observed in the bioluminescence quantum yields are not accounted for by the relatively small differences in observed fluorescence quantum yields of the oxyluciferin products obtained in organic solvents.

The fluorescence lifetime for one of the oxyluciferins in neutral and basic DMF is also shown in Table II. The value for *Renilla* GFP in water is also included.

Effects of Luciferin Analogues on Energy Transfer to GFP. As seen in Figure 2 the luciferase catalyzed oxidation of coelenterate-type luciferin (II, Figure 1) produces a bluish emission (λ_{max} 483 nm). Upon the addition of micromolar concentrations of *Renilla* GFP, the color of emitted light changes from blue to green (λ_{max} 509 nm) by an efficient and radiationless energy-transfer process (Ward & Cormier, 1978). As shown by Ward & Cormier (1978) this process occurs by formation of a rapid equilibrium protein–protein complex between luciferase and GFP.

With coelenterate-type luciferin (II, Figure 1) and its benzyl analogue (III, Figure 1) energy transfer is accompanied by a two- to threefold increase in photon yield (see Table I and Ward & Cormier, 1978). Energy transfer occurs with all luciferin analogues tested resulting in the emission of green light (λ_{max} 509 nm) characteristic of the fluorescence of GFP. In the presence of GFP, increases in photon yields of approximately 25–200-fold were observed with most of the luciferin analogues tested (Table I). This increase is approximately the same as the decrease in ϕ_B observed between the benzyl analogue of luciferin (III) and the analogue tested.

The spectral details of energy transfer by using luciferin analogues VI and X (Figure 1) provide the basis for an understanding of the large increase in photon yields observed upon addition of GFP. These data are presented in Figure 5. With luciferin analogue X, low level emissions occur from both the neutral species (λ_{max} 397 nm) and the monoanion (λ_{max} 450 nm) in the absence of GFP. In the presence of GFP, emission from the monoanion is eliminated but the neutral

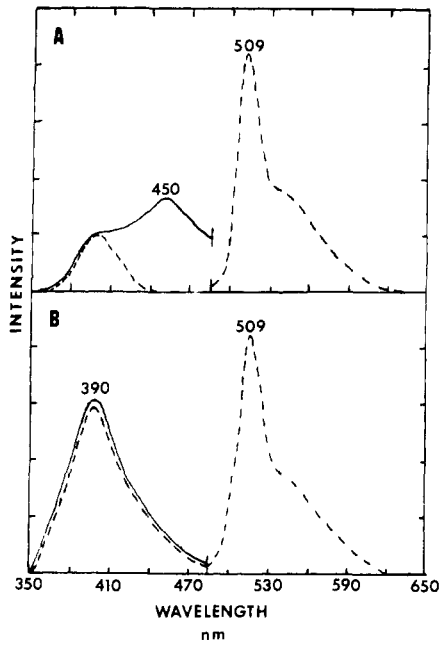

FIGURE 5: (A) Corrected bioluminescence emission spectra of luciferin analogue X with luciferase (—) and with luciferase plus GFP (---). (B) Corrected bioluminescence emission spectra of luciferin analogue VI with luciferase (—) and with luciferase plus GFP (---). In Figures 5A and 5B, the spectral intensity in the region from 350 to 485 nm has been amplified approximately 200-fold for clarity.

species emission remains unchanged (Figure 5A). This is accompanied by a bright emission in the green due to energy transfer with a 212-fold increase in photon yield. With luciferin analogue VI, emission occurs only from the neutral species (λ_{max} 390 nm) in the absence of GFP as shown in Figure 5B. In the presence of GFP, the neutral species emission remains unchanged, while a new emission band occurs characteristic of the fluorescence of *Renilla* GFP. The green emission represents a 192-fold increase in photon yield.

Discussion

Earlier work from this laboratory (Hori et al., 1973), the experiments reported here, and work with model compounds (Goto, 1968; McCapra & Chang, 1967) have shown that, during chemiluminescence of luciferin and its analogues, emission occurs exclusively from the electronic singlet excited state of the oxyluciferin monoanion product. Hart et al. (1977) and Matthews et al. (1977a) showed that this is also true for bioluminescence using luciferins II and III (Figure 1) with one interesting difference. In the luciferase catalyzed reaction, the major emission was also observed to occur from the luciferase-bound oxyluciferin monoanion but a second low level emission near 400 nm was also observed and assigned to the luciferase-bound oxyluciferin (neutral species). As shown here, all of the active analogues of luciferin, which represent structural changes in the R_3 substituent (see Figure 1), produce similar spectral emissions with light being emitted from both the monoanion and neutral excited state species. In these cases, emission from the monoanion always predominates.

In chemiluminescence, structural changes in the R_1 substituent of luciferin do not have an appreciable effect on the emission characteristics. Furthermore, emission occurs exclusively from the oxyluciferin monoanion excited state and the chemiluminescence quantum yield ϕ_C remains essentially the same for all analogues. In the luciferase-catalyzed reactions, structural changes in the R_1 substituent have a pronounced effect on the observed emission characteristics as illustrated by Figures 3 and 4. In these cases, emission from the monoanion is either not observed or reduced over 100-fold. By contrast, emission from the neutral species remains relatively unaltered.

Since energy transfer during *Renilla* bioluminescence is an efficient nonradiative process (Ward & Cormier, 1978) and involves a specific interaction between luciferase and GFP, we decided to use GFP as a probe for nonradiating excited states. As Table I and Figure 5 indicate, the addition of micromolar concentrations of GFP to a luciferase–R_1 luciferin analogue mixture resulted in a 100–200-fold increase in photon yields with emission occurring primarily from the electronic singlet excited state of GFP. Since the fluorescence quantum yield of *Renilla* GFP is 80% (Ward & Cormier, 1978) and the fluorescence quantum yields for the various oxyluciferin analogues (neutral species) in DMF vary from 10 to 24%, one would expect more than an eightfold increase in photon yield for luciferin analogues VI and VIII assuming energy-transfer efficiencies of 100%. The observed 100–200-fold increase in photon yield, with emission characteristic of GFP fluorescence, may be explained by a number of mechanisms.

A mechanism consistent with the known chemical and biochemical data is described below. In this scheme, both forms of the oxyluciferin singlet excited state are formed as shown in Figure 6, but the monoanion excited states are quenched by interaction with solvent and/or luciferase functional groups. GFP is known to bind specifically to luciferase via a rapid equilibrium protein–protein interaction (Ward & Cormier, 1978) and in this mechanism the rate of energy transfer to GFP would be fast enough to compete with the quenching process. In this regard, measurements with luciferin analogue IV have been informative. The emission of analogue IV with luciferase is due to both forms of the product as shown in Figure 3. With this analogue the lifetime of the monoanion excited state of oxyluciferin can be compared with the rate of the deactivation processes on the enzyme. Using the measured lifetime (3.3 ns; Table II) of the corresponding oxyluciferin monoanion in DMF, one can conclude that the quenching process must occur at a similar rate to be efficient. The rate of energy transfer, therefore, would have to be faster than 3×10^8 s^{-1}.

Recent studies have shown that the fused imidazolone–pyrazine ring system of luciferin is not involved in binding to luciferase (Matthews et al., 1977b). It was also shown that each of the R group substituents on luciferin was involved in binding to the enzyme in an apparently cooperative manner. The three R groups are thought to serve as highly specific enzyme recognition sites which are required not only for binding to the enzyme but also for proper orientation of luciferin in the active center so as to maximize bioluminescence quantum yields and minimize competing quenching processes. Thus structural changes in the R groups might tend to slightly alter the position of the excited state oxyluciferin in the active center. This could result in increased quenching rates of the excited state oxyluciferin monoanion due to enhanced interaction with amino acid functional groups or solvent. Changes in the R_3 substituent of luciferin could alter its position in the active center of luciferase, resulting in the observed large decrease in reaction rate (turnover number) and small to moderate effect on the quenching rate of the excited state product. Energy transfer to GFP results in nearly complete recovery of the photon yields. On the other hand, changes in the R_1 substituent of luciferin could alter its position in the active center of luciferase, resulting in the observed small decrease in reaction rate but large effect on the quenching rate of the excited state product. Thus in these cases one observes

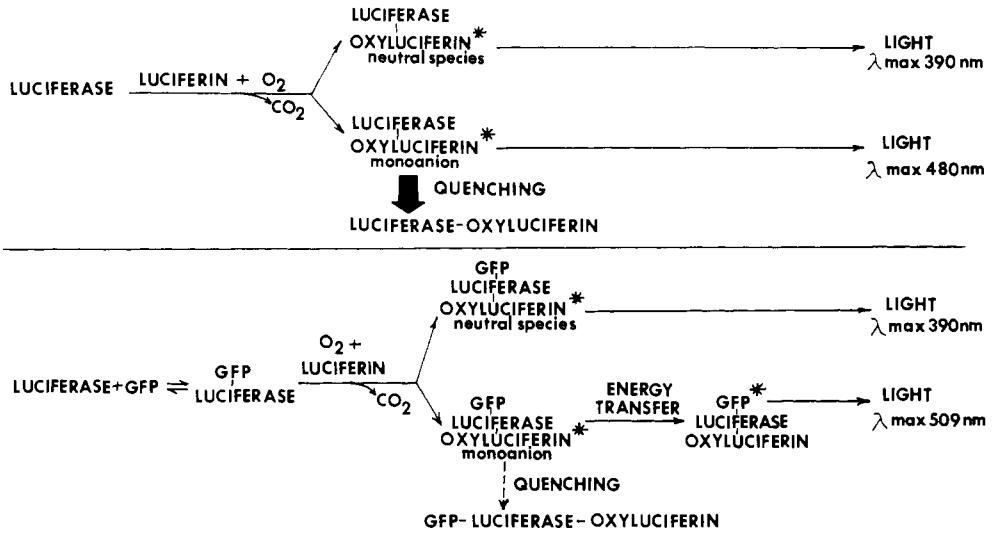

FIGURE 6: Proposed scheme for the luciferase-catalyzed oxidation of coelenterate-type luciferin in the presence and absence of GFP.

complete or nearly complete elimination of emission from the excited state oxyluciferin monoanion depending upon the luciferin analogue used. Energy transfer to GFP again results in high recoveries of the photon yields. The large increase in photon yields observed with GFP suggest that GFP is effectively outcompeting the increased quenching rates observed with luciferin analogues.

Under energy transfer conditions, emission from the neutral species excited state of oxyluciferin remained unchanged. Furthermore in luciferin analogues IV and X, where emissions from both the neutral and monoanion species are observed, it is only the monoanion emission that is eliminated upon addition of GFP. These results suggest that energy transfer to GFP occurs from the excited state monoanion species of oxyluciferin rather than from the neutral species excited state as illustrated in Figure 6. These results are consistent with the facts that the overlap of the monoanion emission and the absorption of GFP is very good, whereas this is not true for the neutral species emission. We have used the equations of Stickler & Berg (1962) and Birks & Munro (1964) to estimate the extinction coefficient for the chromophore of GFP based on its intrinsic lifetime (see Table II). The calculated value of 54 000 L mol^{-1} cm^{-1} is fivefold lower than that recently obtained by a variety of methods (Ward & Cormier, 1979). This suggests that the protein has between four and six chromophores per 52 000 mol wt dimer. The similarities between this protein and some of the subunits of the photosynthetic pigments of blue-green algae are striking. For example, b-phycoerythrin contains 6 phycoerythrobilin groups per $\alpha\beta$ unit (mol wt 35 000), while the γ subunit of B-phycoerythrin contains 2 phycourobilin and 2 phycoerythrobilin groups per 30 000 mol wt and exhibits an intense absorption band at 498 nm (Glaser & Hixson, 1977).

This report provides the first indication that an enzyme is capable of generating nonradiating singlet excited states produced by a chemical reaction. As in the case of luciferin analogues VI and VIII, the nonradiating excited states are detected only by the use of energy transfer to GFP as a probe. The emissions from the reaction of luciferin analogues VI and VIII with luciferase also are among the shortest wavelength emissions reported for bioluminescence.[2] Extrapolation of these emission spectra on a wavenumber plot to zero gives a minimum wavelength of emission of about 340 nm. This requires that the chemical oxidation reaction produce at least 84 kcal mol^{-1} to populate the excited state responsible for this emission.

Current theories of chemiluminescence in solution have proposed an electron-transfer step for efficient singlet excited state production (McCapra, 1977; Koo & Schuster, 1977). At the present we have no evidence to exclude an electron-transfer process in either energy transfer or dioxetanone decomposition in *Renilla*. We feel that this may be a suitable biological system for testing an electron-transfer mechanism and looking at the similarities between energy-transfer processes in bioluminescence and photosynthesis.

In summary, this report provides the first indication of protein-induced quenching of chemically produced singlet excited states. For certain analogues, we also report bioluminescent emission in the near-ultraviolet and provide evidence for the identification of the excited state species responsible for energy transfer to *Renilla* GFP. At the present time, little is known about the protein structure of luciferase and GFP, but this type of information will be of obvious importance in the understanding of requirements for efficient energy transfer.

[2] A similar short wavelength emission has recently been observed in the in vivo bioluminescence emission from a luminous fish (Wampler, 1978).

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Intracellular pH Measurements in Ehrlich Ascites Tumor Cells Utilizing Spectroscopic Probes Generated in Situ†

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ABSTRACT: The uncharged, colorless molecule fluorescein diacetate diffuses into Ehrlich ascites tumor cells at neutral pH, where intracellular esterases release the chromophore fluorescein. The negatively charged dye is retained by the cell, permitting the intracellular pH to be estimated from the shape of the pH-dependent absorption spectrum. The diacetate derivative of 6-carboxyfluorescein may be used similarly and has the additional advantage of a slower rate of leakage out of the cell but requires incubation at pH 6.2 to facilitate initial entry into the cell. After removal of external dye by centrifugation, 80–92% of the remaining dye is unresponsive to external pH changes. Calibration of the intracellular fluorescein spectra is obtained by equilibration of the internal and external pH with nigericin in K^+ buffers. Results of intracellular pH measurements by this method are in good agreement with those obtained by measuring the distribution ratio of the weak acid 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione, under a variety of metabolic conditions. Besides the accurate estimation of intracellular pH, the method permits the kinetics of intracellular pH changes as small as 0.01 to be followed. Intracellular fluorescein reports pH changes occurring in both the cytoplasmic and the mitochondrial compartments, whereas 6-carboxyfluorescein reports only the cytoplasmic compartment. At equivalent concentrations, nigericin is more effective than valinomycin plus the protonophore 1799 in dissipating plasmalemma pH gradients. Either is effective at lower concentrations in dissipating mitochondrial pH gradients. Addition of glucose to Ehrlich ascites cells results in a transient acidification of the cytoplasm in close correspondence to the intracellular lactate levels. The transient acidification can be explained by the initial rapid rate of glycolysis exceeding the rate of lactate export.

Diacyl derivatives of fluorescein have been used as sensitive indicators for determining the presence of intracellular esterases. The colorless, neutral diacetate compound diffuses into the cell, where intracellular enzymes release the strong chromaphore fluorescein ($E_{490}^{mM} = 90$; quantum yield ≈ 0.9) by hydrolytic removal of the acetate groups. The intracellular retention of fluorescein has been used to monitor the intactness of cell membranes (Rotman & Papermaster, 1966) and to distinguish live from dead cells in cell sorting devices (Bonner et al., 1972). In addition, fluorescence polarization measurements on intracellular fluorescein have been used to determine the viscosity of the cell cytosol in *Euglena* and yeast (Burns, 1969; Cercek & Cercek, 1972).

Since the spectrum of fluorescein is highly dependent on pH (Fothergill, 1964), a natural extension of these previous

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