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Photoexcited Bacterial Bioluminescence. Identity and Properties of the Photoexcitable Luciferase†

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ABSTRACT: Properties of photoexcitable luciferase are compared with those of luciferase, both isolated from the bacterium *Beneckea harveyi*. The proteins have the same molecular weight, are similarly charged at pH 8, and can be inactivated, with comparable efficiencies, by antibodies against either pure luciferase (a heterodimeric protein) or individual subunits thereof. Compared with luciferase, photoexcitable luciferase has a broader pH range for optimal activity, is more stable under acidic conditions, is less stable under alkaline conditions, and is more resistant at neutral pH to inactivation by heat, urea, and trypsin. A flavine-like chromophore, designated B, can be isolated from photoexcitable luciferase. The binding of B to luciferase restores all the properties characteristic of photoexcitable luciferase. Moreover, photoexcitable luciferases from mutants selected to have heat labile luciferases are also thermally unstable. It is concluded that photoexcitable luciferase actually consists of a luciferase-B complex which is conformationally distinct from luciferase under certain conditions.

Bacterial luciferase (L)[1] catalyzes the bioluminescent oxidation of $FMNH_2$ and a long chain aldehyde by molecular oxygen (Hastings, 1968; Eberhard and Hastings, 1972). This enzyme (mol wt 79,000) consists of two nonidentical subunits, α and β, with the active center located on the α subunit (Meighen et al., 1971a,b; Cline and Hastings, 1972). Luciferase catalyzes the bioluminescent reaction by reacting sequentially with $FMNH_2$, O_2, and aldehyde to form a series of enzyme intermediates (Hastings and Gibson, 1963; Hastings et al., 1973). The enzyme-bound $FMNH_2$ and aldehyde are believed to be oxidized by the oxygen to generate an excited enzyme intermediate (enzyme-flavine*), and ultimately the products: a long chain carboxylic acid, FMN, H_2O, and light (Eberhard and Hastings, 1972; Shimomura et al., 1972; Dunn et al., 1973).

An interesting feature of bacterial bioluminescence is the existence of two types of in vitro luminescence activity attributable to two distinct protein species. The normal activity is initiated by adding $FMNH_2$ to L in the presence of aldehyde and oxygen. Light emission can also be induced by flash irradiation of photoexcitable luciferase (PL) in the absence of FMN or $FMNH_2$ (Gibson et al., 1965; Mitchell and Hastings, 1970). Both reactions are oxygen- and aldehyde-dependent and utilize these substrates in the same sequence. Both have the same rates for the decay of light when tested with aldehydes of different chain length and emit at the same wavelengths. In contrast to the similarities observed in the two luminescence reactions, the two proteins differ in that PL migrates slightly faster than L on DEAE columns at pH 6.25 and pH 7 and is more resistant to sulfhydryl group modification and heat denaturation than L. The PL contains a flavine-like chromophore designated B while L can be isolated chromophore free.

The flavine-like chromophore B could be dissociated from PL by treating the latter with urea or guanidine. The addition of B to L restored the PL activity, with a concomitant loss of L activity, and changed the chromatographic behavior of L on DEAE-cellulose to a pattern similar to PL. Based on these observations, Mitchell and Hastings (1970) proposed that PL is actually the bacterial luciferase-B complex. Nevertheless, the identity of PL still remained un-

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[1] Abbreviations used are: L, bacterial luciferase; PL, photoexcitable luciferase; LB, reconstituted luciferase-B complex; TSL-mutant, mutant with temperature-sensitive luciferase.

clear, especially because reconstituted PL activity (luciferase-B) lacked the fluorescence (λ_{max} near 490 nm) of the original material. We therefore examined the properties of the three protein species (L, PL, and LB) in detail, and have found that the protein moiety of PL is actually L; and that PL and reconstituted LB are indeed identical in every property examined. The earlier failure to observe the restoration of the characteristic fluorescence in LB is demonstrated in the accompanying paper (Tu and Hastings, 1975) to be the result of contamination by FMN whose fluorescence masks that of LB.

Experimental Procedures

The bacterium *Beneckea harveyi* (Reichelt and Baumann, 1973), previously designated as strain MAV (Hastings et al., 1969), was used for the preparation of both luciferase and PL. Luciferase was isolated according to the method of Gunsalus-Miguel et al. (1972) with modifications (Baldwin et al., 1975). The purified luciferase was at least 95% pure but contained a small amount of PL. Luciferase was also purified from an aldehyde-requiring mutant, which contains normal levels of luciferase but is dark in vivo due to the lack of endogenous aldehyde factor(s) (Cline, 1973). This mutant does not have photoexcited luminescence activity and the luciferase isolated from the mutant is free from PL contamination.

In the luciferase and PL purification, PL eluted slightly ahead of L during the DEAE-Sephadex chromatography step (Gunsalus-Miguel et al., 1972). These PL active fractions were pooled, concentrated by ammonium sulfate precipitation (75% saturation), and dialyzed into 0.25 *M* phosphate (pH 7) at 4°. The concentrated sample was then applied to a second DEAE-Sephadex column preequilibrated with 0.35 *M* phosphate (pH 7) and eluted with the same buffer at 4°. The PL obtained was approximately 30–50% pure, as estimated by disc gel electrophoresis at pH 8 and 9.5, and usually contained 10–15% L. Since activities of PL and L are initiated by different means, the presence of one does not interfere with the assay of the other. The PL sample was finally subjected to a series of dialyses to remove oxidized flavine contaminations (Tu and Hastings, 1975).

To reconstitute the protein species responsible for photoexcitable bioluminescence activity, B isolated from PL (Tu and Hastings, 1975) was added to L isolated from an aldehyde-requiring mutant, resulting in a concomitant loss of L activity. The protein species so reconstituted is referred to as LB, which is hypothesized (and evidence is presented to support the hypothesis) to be identical with native PL. The degree of saturation of L with B is defined as the percent inhibition of the initial luciferase activity. All the reconstituted LB samples were subjected to the dialysis treatment referred to above.

FMN and aldehydes were gifts from Sigma and Aldrich, respectively. Ultrapure urea was obtained from Schwarz/Mann. DEAE-Sephadex (Sigma) was prepared as described previously (Gunsalus-Miguel et al., 1972). Trypsin (230 units mg^{-1}) was purchased from Worthington.

The initial maximum intensity, expressed in quanta (q) sec^{-1}, of in vitro luminescent activity was measured at 23° with a photometer (Mitchell and Hastings, 1971) calibrated with the light standard of Hastings and Weber (1963). The luciferase activity was measured by injecting 1 ml of 5×10^{-5} *M* $FMNH_2$ (reduced with H_2 over platinum) into 1 ml of 0.02 *M* phosphate, (pH 7) containing 0.01 ml of a 0.1% (v/v) sonicated suspension of decanal in water, 2 mg of bovine serum albumin, and the enzyme. This is referred to as the standard assay. The purified luciferase had a specific activity of 1.4×10^{14} q sec^{-1} mg^{-1} as determined by the standard assay with decanal at 23°. The PL activity was measured with the apparatus consisting of a flash light source (~0.15 msec and 400 J/flash), a photometer, and a recorder (Mitchell and Hastings, 1970). A Vycor syringe containing PL in 1.2 ml of 0.02 *M* phosphate (pH 7) was flash irradiated and rapidly injected into a vial containing 1 ml of the same buffer and 0.025 ml of 0.1% (v/v) decanal, and the luminescent intensity was measured with the photometer placed beneath the vial. In this assay, the partially purified PL sample had a specific activity of 1.4×10^{11} q sec^{-1} mg^{-1} at 23°. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

For electrophoresis at pH 8, the method of Gabriel (1971) was followed using ammonium persulfate instead of riboflavine for the polymerization of the stacking gel. The concentration of the separating gel was varied from 4 to 12% with a *N,N'*-methylenebisacrylamide/acrylamide ratio at 1:30 in order to examine both charge and size factors by the method of Hedrick and Smith (1968). Approximately 0.1 ml of sample solution containing 50 μg of protein and 0.001% Bromophenol Blue was applied to each gel and electrophoresis was carried out at 4°. Gels were stained for protein with Coomassie Brilliant Blue R250. Alternatively, gels were also sliced into 1-mm discs for elution to locate luciferase and PL activities. The mobility of a given protein band was defined as (distance of protein migration/distance of Bromophenol Blue migration).

Antisera (designated anti-α, anti-β, and anti-$\alpha\beta$) were produced in three sets of rabbits using preparations of the α subunit, β subunit, and $\alpha\beta$ dimer of *B. harveyi* luciferase, respectively, as antigens. Subunits were isolated in 5 *M* urea (Gunsalus-Miguel et al., 1972), dialyzed into 0.05 *M* Tris-citrate (pH 7.2) containing 0.5 m*M* dithiothreitol and 1 m*M* EDTA, and concentrated by vacuum dialysis. Approximately 2 mg of antigen in 2 ml of buffer was emulsified into an equal volume of complete Freund's adjuvant and injected intramuscularly. Injections were repeated four times over a period of 9 days. One month later, a booster injection was administered using 10 mg of $\alpha\beta$ dimer or 2 mg of the appropriate subunit. Rabbits were bled 1 week after the booster injection and the sera were used without dilution for Ouchterlony tests and enzyme inactivation experiments.

Results

The size and charge of PL and L were compared by disc gel electrophoresis. Using this method, the log of protein mobility is a linear function of gel concentration; the slope is related to molecular weight while the intercept at 0% gel concentration is a function of the net charge of the protein. We have found that the mobilities of PL and L at pH 8 are indistinguishable, within 1-mm resolution, with gel concentrations varying from 4% (mobility 0.92) to 12% (mobility 0.27). Thus, PL and L are similarly charged at pH 8, and have the same molecular weight.

The pH-activity profiles of L, PL, and LB are shown in Figure 1. Unlike L, which has optimal activity near pH 6 in 0.05 *M* phosphate buffer, both PL and LB show an optimal activity plateau over the range from pH 6 to 7.5.

The stabilities of L and PL were compared under both acidic and alkaline conditions at 23° (Figure 2). At pH 4.5,

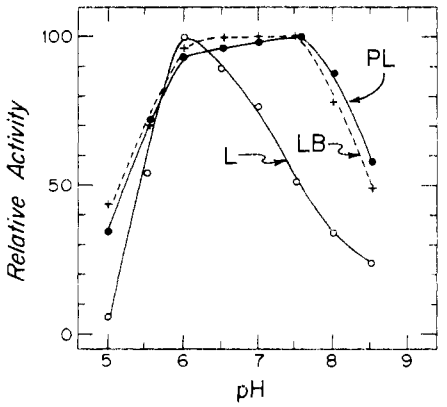

FIGURE 1: The pH dependence of the in vitro bioluminescent activities of L, PL, and LB. The initial luminescent intensities of both $FMNH_2$-initiated and photoexcited activity were measured at 23° in 0.05 *M* phosphate at the different pH values as indicated. For each assay, 10 μg of luciferase (○), 20 μg of PL (○), and 6 μg of LB (63% saturated with B) (+) were used. The curves were normalized at the point of highest activity of each.

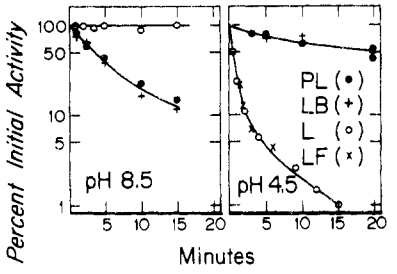

FIGURE 2: The effect of pH on the stability of different forms of luciferase and photoexcitable luciferase. Twenty micrograms of L (○), LF (L in 10 m*M* FMN) (X), PL (●), or LB (60% saturated with B) (+) in 20 μl of 0.02 *M* phosphate (pH 7) were each diluted 100-fold into 0.05 *M* sodium acetate buffer (pH 4.5) or pyrophosphate buffer (pH 8.5) at 23°. After different times, the remaining $FMNH_2$-initiated and photoexcited activities were determined at each pH using 1 and 1.2 ml of sample solution, respectively.

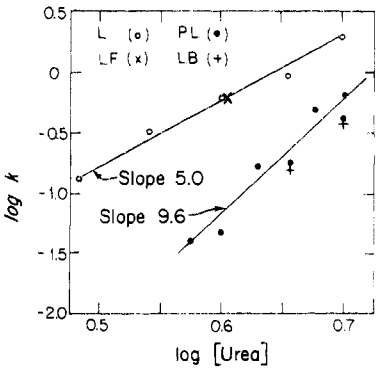

FIGURE 3: Effect of urea concentration on the apparent first-order rates of inactivation by urea of different forms of luciferase and photoexcitable luciferase at 23°. One milligram each of L(○), LF (L supplemented with 1 m*M* FMN) (X), PL (●), and LB (70% saturated with B) (+) in 1 ml of 0.05 *M* phosphate buffer (pH 7) containing 3–5 *M* urea was incubated for 5–10 min. At various times, 10- and 20-μl aliquots were withdrawn for determinations of $FMNH_2$-initiated and photoexcited activity, respectively, at 23°. Initial activities were obtained by extrapolation to zero time. The apparent first-order rates (in min^{-1}) of inactivation were determined from semilogarithmic plots of remaining activity vs. time. Least-squares analysis was used for all data analysis.

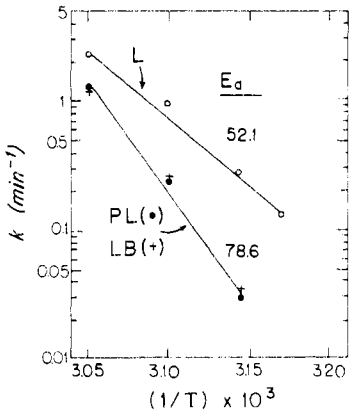

FIGURE 4: Arrhenius plot of the dependence of apparent first-order thermal inactivation rates on the reciprocal of the absolute temperature for L (○), PL (●), and LB (60% saturated with B) (+). Solutions containing approximately 0.5 mg of protein in 0.5 ml of 0.02 *M* phosphate buffer (pH 7) were incubated at the different temperatures between 42.5 and 55° for 15 min. At various times, 10- and 20-μl aliquots were withdrawn for determinations of $FMNH_2$-initiated and photoexcited activities, respectively, at 23°. The apparent first-order rates of inactivation were determined as in Figure 3.

L was quite labile and PL was only slowly inactivated, while at pH 8.5 PL was inactivated much faster than L. The presence of 0.1 m*M* FMN did not improve the luciferase stability at pH 4.5, and at both pH's the stability of reconstituted LB was identical with that of PL.

Inactivation of L and PL in the presence of 3–5 *M* urea at 23° showed pseudo-first-order kinetics for the initial 3–10 min. At any given urea concentration, L was inactivated more rapidly than was PL. In Figure 3, the log of the rate constant for inactivation was plotted against the log of urea molar concentration according to the equation of Tanford (1961)

$$-d[N]/dt = k[N] = k'[N]c^m$$

where [N] is the native protein concentration, k is the apparent first-order rate constant of inactivation, c is the molar concentration of urea, k' is an experimental constant independent of c, and m is the apparent order of reaction or the difference between the average number of urea molecules complexed with the denatured and the native protein. Over a limited range of urea concentration, log k is close to a linear function of log c, and the value of m can then be calculated from the slope of the log-log plot. Results in Figure 3 indicate that, in addition to the average number of urea molecules complexed with the native protein, the binding of 10 and 5 urea molecules are required for the inactivation of PL and L, respectively. Thus, PL is more stable to urea denaturation than is L. It is also shown in Figure 3 that the presence of 1 m*M* FMN does not stabilize L against unfolding by 4 *M* urea. Moreover, the more stable conformation of PL can be reconstituted by the binding of B to L. Thermal inactivation experiments (Figure 4) show that PL and LB behave identically and are both more stable than L with regard to heat denaturation over the range of 42.5–55°. The E_a of thermal inactivation was calculated to be 52.1 ± 4.1 kcal mol^{-1} for L and 78.6 ± 3.6 kcal mol^{-1} for PL and LB, respectively.

Cline and Hastings (1971, 1972) isolated temperature-sensitive mutants (designated class TSL) which produce luciferases with lower thermal stabilities due to lesions in one of the subunits. Cline (1973) observed that both L and PL from the β-defective TSL-9 mutant are more thermolabile

Table I: Thermal Stability of L and PL from Temperature-Sensitive Mutants.

Mutant	Subunit Location of Lesions[a]	k_1 of Thermal Inactivation[b] (min^{-1}) L Activity (35°)	k_1 of Thermal Inactivation[b] (min^{-1}) PL Activity (35°)
Wild type		0.01	0.01
TSL-18	α	0.24	0.30
TSL-1	β	0.64	0.16
TSL-3	β	0.01	0.01
TSL-20	β	0.50	0.44

[a] Characterizations were detailed previously (Cline and Hastings, 1972). [b] Wild type and mutants of *B. harveyi* were each cultured, ar 22°, in 40 ml of the complete minimal medium supplemented with glycerol (Nealson et al., 1972), and were harvested when the in vivo bioluminescence reached the peak intensity. Cells from 3 ml of culture medium were suspended in 1 ml of H_2O containing 1 m*M* EDTA and 0.1 m*M* dithiothreitol for lysis overnight at 4°. The supernatant of lysates was incubated at 20 and 35° for 15 min, and L and PL activities were measured after different times to determine the k_1 of thermal inactivation as described in Figure 3. Both PL and L were stable at 20°.

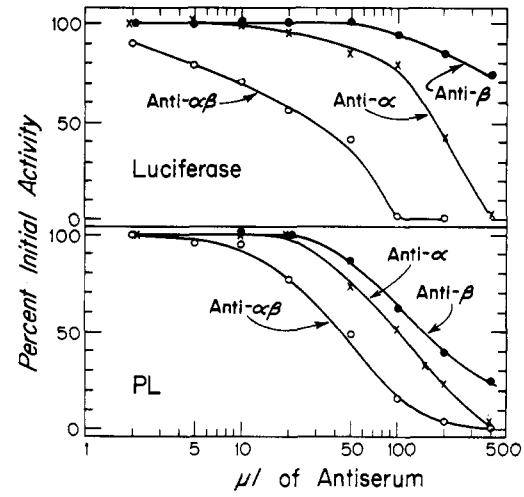

FIGURE 5: Inactivation of L and PL activities by antiluciferases. Various amounts of anti-α (X), anti-β (●), or anti-$\alpha\beta$ (○) were each added to a sample solution containing 38 μg of the partially purified PL in 0.02 *M* phosphate (pH 7) to a final volume of 0.5 ml. After an incubation of 4 hr at 4°, 200 and 10 μl of each sample was used to determine the remaining activity of PL and L, respectively. Controls were treated under the same conditions without added antisera.

than the corresponding protein species from wild type *B. harveyi*. We have examined the thermal stabilities of L and PL from one α-defective and three other β-defective TSL-mutants (Table I). L and PL from all four TSL-mutants are stable at the permissive temperature, 20°. At 35°, L and PL from the mutants TSL-1 -18, and -20, which are highly temperature sensitive, were rapidly inactivated. L and PL from the mutant TSL-3, which is inactivated only above 40°, were not inactivated at 35°.

Recently it has been found that bacterial luciferase can be rapidly inactivated by proteases, such as trypsin (Njus et al., 1974; Baldwin, 1974). To examine the susceptibility of PL to trypsin digestion, 10 μg of trypsin in 10 μl of H_2O was added to 1 ml of the substrate enzyme solution in 0.02 *M* phosphate (pH 7) and incubated at 25°. After different times, aliquots were withdrawn for the determination of remaining PL and L (present as a contaminant in the same sample) activities at 23°. At sample concentrations of 0.275, 0.55, and 1.1 mg of protein/ml, the apparent first-order rates of inactivation of PL were 0.03, 0.04, and 0.03 min^{-1}, respectively, and those of the coexisting L were 0.08, 0.14, and 0.19 min^{-1}, respectively. When LB samples (30% saturated with B) were treated with trypsin the same way, inactivation rates of LB were found to be the same as those of PL at corresponding concentrations. Thus, trypsin inactivates PL and LB with the same efficiency, but less rapidly than it inactivates luciferase.

Antibodies against pure luciferase and its individual subunits were used to characterize PL immunochemically. The antibodies were first characterized by double diffusion analysis on Ouchterlony plates using luciferase and its individual subunits as antigens. Based on the pattern of precipitin lines, it was concluded that (1) anti-α specifically cross-reacts with the α subunit and the $\alpha\beta$ dimer but not the β subunit; (2) anti-β specifically cross-reacts with the β subunit and the $\alpha\beta$ dimer but not the α subunit; (3) anti-$\alpha\beta$ serum contains active species capable of binding both subunits and the $\alpha\beta$ dimer. All three antiluciferases are capable of inactivating both L and PL with comparable efficiencies (Figure 5). It was also found that 1 m*M* FMN does not protect L from inactivation by antiluciferases.

Discussion

Confirming the earlier hypothesis of Mitchell and Hastings (1970), we have shown that the protein moiety of PL is identical with L, based on the following: (1) PL and L have the same molecular weight (disc gel electrophoresis), (2) both PL and L can be inactivated by antiluciferase with comparable efficiencies, (3) TSL-mutants produce both thermolabile luciferases and thermolabile photoexcitable luciferases, (4) native luciferase completely free of PL can be converted to photoexcitable luciferase by the addition of a low molecular weight compound designated B, obtained from purified PL. This product, designated LB, is identical with PL with respect to every property studied.

The present work shows, however, that PL, presumably as a consequence of the binding of B, is conformationally distinct from L under certain conditions. At pH 7, PL exists in a more stable conformation than L, with regard to inactivation by trypsin, urea, and heat, and quantitative differences between PL and L in the degrees of inactivation by antiluciferases have been observed. Under acidic conditions the PL activity is more stable, while L activity is more stable under alkaline conditions.

In the studies with TSL-mutants (Table I), two other α-defective mutants, TSL-10 and TSL-17, were also examined. However, the extremely low PL activities detected in these mutants prevented an accurate measurement of inactivation rates. The aldehyde-requiring mutants do not contain any PL unless the in vivo bioluminescence is induced by the addition of aldehyde (Mitchell and Hastings, 1970). These findings indicate that PL is not an artifact of isolation procedures and that PL is probably formed as a consequence of the bioluminescent reaction. In agreement with this hypothesis, the extremely low PL activities observed with TSL-10 and TSL-17 may be directly related to the fact that both mutants produce only weak in vivo bioluminescence (Cline and Hastings, 1972).

Acknowledgments

We thank Drs. J. E. Becvar, M. Z. Nicoli, S. J. Girsch, and, especially, T. O. Baldwin for their numerous helpful

discussions. We are also grateful to Drs. T. O. Baldwin and V. Gooch for their assistance with the proteolysis experiments and least-squares analyses, respectively, and Patricia Dobson for purifying luciferase from the aldehyde-requiring mutant.

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