Role of a Luciferin-binding Protein in the Circadian Bioluminescent Reaction of *Gonyaulax polyedra*

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A luciferin-binding protein (LBP), which binds and protects from autooxidation the substrate of the circadian bioluminescent reaction of *Gonyaulax polyedra*, has been purified to near homogeneity. The purified protein is a dimer with twodesident 72-kDa subunits, and an isoelectric point of 6.7. LBP is a major component of the cells, comprising about 1% of the total protein during the night phase, but drops to only about 0.1% during the day. The luciferin is protected from autooxidation by binding to LBP, and one luciferin is bound per dimer at alkaline pH ($K_a = 5 \times 10^{-7}$ M$^{-1}$). The protein undergoes a conformational change with release of luciferin at pH values below 7, concurrent with an activation of *Gonyaulax* luciferase. LBP thus has a dual role in the circadian bioluminescent system.

Among unicellular marine algae that contribute significantly to the phosphorescence of the ocean, the dinoflagellate *Gonyaulax polyedra* has been widely studied. In *Gonyaulax* extracts, three components have been shown to be involved in luminescence (Hastings and Dunlap, 1986): the enzyme luciferase, the substrate luciferin, and a luciferin-binding protein (LBP). Bioluminescence *in vivo* is under circadian control which ensures that luminescence occurs primarily during the night phase (Johnson and Hastings, 1986).

During night phase, light is emitted as brief (~100 ms) flashes from tiny organelles termed scintillons, which can be observed in the living cells by their bioluminescence or by the fluorescence of the substrate luciferin (Johnson et al., 1985). The scintillons have been shown to contain luciferase (Morse et al., 1989a). Scintillons are extremely fragile so that most of the bioluminescent system components appear in the soluble fraction after extraction. They may be isolated in low yield as intact vesicles by extraction of cells at pH 8 and purified by isopycnic density gradient centrifugation. These vesicles will emit flashes of light, in vitro, when the pH is rapidly lowered to 6 (Fogel et al., 1972). In *Gonyaulax*, the velocity of the luciferase-catalyzed reaction at pH 6 is at least 100 times greater than at pH 8 (Krieger and Hastings, 1968). The binding of luciferin by LBP also shows a strong pH dependence and is far weaker at pH 6 than at pH 8 (Fogel and Hastings, 1971; Krieger, 1972). These complementary pH profiles have led to the postulate that luciferase and LBP act synergistically in the *in vivo* control of light emission (Hastings and Dunlap, 1986).

In this paper we describe the isolation of highly purified luciferin-binding protein and some of its properties. In particular, we have been interested in the effect of pH on the binding of luciferin. Our results suggest that LBP undergoes a reversible conformational change at pH values below neutrality to a form that no longer binds luciferin. The role of LBP in the control over the bioluminescent reaction will be discussed in relation to flashing and the circadian changes in the bioluminescent system.

**MATERIALS AND METHODS**

**Growth, Harvest, and Extraction of Cells**—Cultures of the bioluminescent dinoflagellates *G. polyedra* (for the isolation of luciferase and luciferin-binding protein) and *Pyrocystis lunula* (from which luciferin was extracted) were grown in 1/2 medium under alternating periods of 12 h light (cool white fluorescent lamps; 150 microeinsteins/m$^2$/s) and 12 h dark (Dunlap and Hastings, 1981a) to a cell density of about 10$^7$ cells/liter. *Gonyaulax* cells were harvested in the middle of a dark period by filtration on Whatman 541 filter paper after a 1-h exposure to bright fluorescent light; this inhibits the mechanically stimulated flashing which occurs during filtration so that less luciferin is lost during harvesting (Bode et al., 1963). Higher luciferin levels facilitate the purification of LBP. *P. lunula* cells could be harvested at any time, since the luciferin level (~100 times that of *G. polyedra*) does not vary with time of day. Harvests were routinely done during the day phase in order to ensure photoinhibition of flashing.

**The Luciferase Assay for Luciferin-binding Protein**—The luciferase assay involves rapid mixing of luciferase and luciferin in 1 ml of assay buffer (0.2 M phosphate, pH 6.3, containing 0.25 mM EDTA and 0.1 mg/ml bovine serum albumin) at room temperature in a scintillation vial. Light emission in quanta/s was measured with a photomultiplier tube calibrated with the standard of Hastings and Weber (1963) and corrected to the human standard by multiplying by 2.8. A unit of luciferase was defined as that amount which will produce an initial light intensity of 10$^6$ quanta/s at a luciferin concentration of 0.2 M.

During the *in vitro* luminescent reaction, light intensity rises rapidly to an initial peak ($I_0$) and then decays (Fig. 1). Although the decay is not strictly exponential, the total number of quanta emitted was approximated by $I_0 \times t_0/2$, where $t_0$ is the time needed for the light intensity to decay to one-half $I_0$. Moles of luciferin were calculated by dividing the total quanta by the quantum yield (0.22) and Avogadro's number (Dunlap and Hastings, 1981a).

The luciferase assay was used to measure the amount of luciferin released from LBP by a drop in pH from 8.5 to 6.3. The amount of luciferin released was taken to equal the amount of LBP when, prior to the measurement, the LBP had been saturated by a 10-min incubation with an excess of luciferin at pH 8.5 and the free ligand removed by gel filtration (Sulzman et al., 1978).

**Purification of Luciferase**—Freshly harvested cells from 15 liters of culture were resuspended in 40 ml of extraction buffer (100 mM Tris, pH 8.5, containing 10 mM EDTA and 10 mM dithiothreitol) and broken by a single pass through a French press (6,000 p.s.i.). Cell debris was removed by centrifugation at 15,000 × g for 15 min. Extraction buffer saturated with ammonium sulfate and adjusted to
pH 8.5 was added, and the protein precipitating between 30 and 56% saturation was collected. The pellet was resuspended in 4 ml of extraction buffer and desalted by passage through a 2.5 x 25-cm P-10 (Pharmacia LKB Biotechnology Inc.) column equilibrated with column buffer (extraction buffer diluted 1:100 containing 5 mM β-mercaptoethanol and 5 mM NaCl). The brown protein-containing fractions were pooled and diluted with column buffer until the conductivity was less than 0.7 mmho. The pooled fractions were then loaded onto a 2.5 x 15-cm DEAE-Bio-Gel A column equilibrated with column buffer and washed with the same buffer. The washings were discarded and a salt gradient from 3 to 335 mM NaCl in column buffer was used to elute luciferase (peak at 1.2 mmho conductivity) and separate it from LBP (peak at 5 mmho conductance). LBP-free luciferase-containing fractions were pooled and used as a stock solution for luciferin assays. Aliquots of luciferase were stored at -70 °C in column buffer containing 20% glycerol and 150 mM NaCl. The concentration of luciferase in the stocks was estimated to be about 7 μg/ml.

**Purification of Binding Protein**—LBP fractions eluted from the DEAE-Bio-Gel A column were pooled and concentrated by precipitation with 75% saturated ammonium sulfate. The precipitate, resuspended in 4 ml of extraction buffer, was chromatographed on a Sepharose S-300 column (Pharmacia) which had been equilibrated with column buffer containing 100 mM NaCl. Fractions containing LBP activity were pooled and concentrated by adsorption on a small (2.5 x 2 cm) hydroxyapatite column equilibrated in S-300 buffer; the protein was eluted with a gradient from 1 to 500 mM sodium phosphate, pH 7.5, in S-300 buffer. The elution of LBP was followed by ultraviolet light by the blue fluorescence of its bound luciferin.

**Preparation of Luciferin**—The luciferin used in these experiments was isolated from day phase *P. lunula*, a photosynthetic dinoflagellate in which the luciferin content is at least 100 times that of *Gonyaulax* (Seliger et al., 1969). Cells from 15 liters of culture were added to 75 ml of 2 mM K₂HPO₄, pH 8.5, containing 5 mM β-mercaptoethanol at 100 °C. After 20 s the extract was rapidly chilled in an ice bath, sparged with argon, and the pH adjusted to 8 with NaOH. Because free luciferin is extremely sensitive to inactivation by oxygen, all buffers were sparged with pure argon for 30 min before use. The extract was centrifuged at 27,000 g for 30 min, and the supernate diluted with dH₂O to a conductance of less than 0.7 mmho before loading onto a coarse DEAE-cellulose column (Sigma). A gradient from 0 to 500 mM KCl in 2 mM K₂HPO₄ containing β-mercaptoethanol was used to elute luciferin, measured by its absorption at 390 nm. This luciferin was used as a stock solution and stored in 1-ml aliquots at -70 °C under argon, at a typical concentration of 4 μM as determined both by absorption at 390 nm (ε₉₀ = 2.8 x 10⁵ cm⁻¹ M⁻¹) and by the quanta emitted in the luciferase reaction (Dunlap and Hastings 1981a, 1981b).

**Antibody Preparation**—Several milligrams of purified LBP were treated with 1.3 mM glutaraldehyde at 37 °C for 30 min, and the excess glutaraldehyde was removed by overnight dialysis against phosphate-buffered saline, pH 7.2. The sample in 1 ml was emulsified by sonication with 1 volume of complete Freund's adjuvant and by the Coomassie Blue dye binding assay (Bio-Rad). Antibody Preparation—Several milligrams of purified LBP were treated with 1.3 mM glutaraldehyde at 37 °C for 30 min, and the excess glutaraldehyde was removed by overnight dialysis against phosphate-buffered saline, pH 7.2. The sample in 1 ml was emulsified by sonication with 1 volume of complete Freund's adjuvant and injected subcutaneously into rabbits. A booster injection of 1 mg of LBP was given after 1 month, and the animals were bled 2 weeks later. Affinity-purified antibody was eluted with 0.2 M glycine, pH 2.8, from anti-LBP serum-treated nitrocellulose strips to which purified LBP had been transferred after SDS-PAGE (Olmeded, 1981). Before use, it was dialyzed against 10 mM Tris-buffered saline, pH 7.2.

**Protein Measurements**—Protein was measured by reaction either with the Folin reagent of Lowry et al. (1951), the Biuret method, or by the Coomassie Blue dye binding assay (Bio-Rad).

**Electrophoresis and Western Blotting**—SDS-polyacrylamide gel electrophoresis was performed on 8.5% gels according to the method of Laemmli (1970). The gels were either stained with Coomassie Blue or were transferred to nitrocellulose for Western blots (Towbin et al., 1979) using 125I-protein A and autoradiography for detection of bound antibody.

The isoelectric point of LBP was determined by isoelectric focusing with 75% saturated ammonium sulfate. The precipitate, resuspended in column buffer and washed with the same buffer, was loaded onto a hydroxyapatite column (Sigma). A gradient from 0 to 500 mM KCl in 2 mM K₂HPO₄ containing β-mercaptoethanol was used to elute luciferin, measured by its absorption at 390 nm. This luciferin was used as a stock solution and stored in 1-ml aliquots at -70 °C under argon, at a typical concentration of 4 μM as determined both by absorption at 390 nm (ε₉₀ = 2.8 x 10⁵ cm⁻¹ M⁻¹) and by the quanta emitted in the luciferase reaction (Dunlap and Hastings 1981a, 1981b)

**Equilibrium Dialysis**—Equilibrium dialysis was carried out using lucite microcombs (Eisen, 1964) which contained two small glass chambers separated by a polycarbonate membrane (Spectrum Co.). The total volume was 300-400 μl. Because of the great sensitivity of free luciferin to inactivation by oxygen, all solutions were repeatedly degassed under vacuum and saturated with argon (<1 ppm oxygen).

**Results**

**Luciferase Kinetics**—As shown in Fig. 1, light emitted by the luminescent reaction rapidly reaches a maximum and then decays. The decay in light emission approximates first order kinetics until the reaction is at least 50% complete. As shown in Fig. 2, at a fixed initial luciferin concentration, both I₀ and k (i.e. In 2/t₀) are proportional to the luciferase concentration over at least a 10-fold range. On the other hand, at constant luciferase, I₀ is proportional to added substrate.

![Fig. 1. Luminescent decay curve. Light intensity produced from the oxidation of luciferin released from 20 μg of purified LBP in assay buffer catalyzed by 0.25 μg of luciferase. The amount of luciferin can be approximated by I₀ × t₀/(ln 2 × 0.22 N) (where I₀ = initial light intensity; t₀ = 5 s; N is Avogadro's number) as 0.055 nm.](image)

![Fig. 2. Kinetic parameters as a function of luciferase concentration. Both the rate constant k (or in 2/t₀, in s⁻¹) (●) and the initial light intensity (I₀, in quanta/s) (○) increase with increasing luciferase concentration. The amount of substrate (calculated from I₀/(0.22 N × k) to be 0.27 μM) is thus constant for all assays.](image)
while \( k \) remains constant over a wide range of luciferin concentration (Fig. 3). The first order exponential approximation holds for luciferin concentrations up to 1 or 2 \( \mu M \) but deviates as the enzyme begins to become saturated.

**Purification of LBP**—The purification of LBP, summarized in Table I, resulted in a protein purified almost 100-fold with a recovery of about 30%. From SDS-PAGE, the protein at the final stages of purification was estimated to be greater than 95% pure (Fig. 4, lane 4), and to have a molecular mass of 72 kDa. A 65-kDa degradation product with normal luciferin binding capacity was sometimes observed in purified preparations. However, it did not occur with columns which had been washed with 0.1 N NaOH before reusing them.

The apparent molecular mass of the un-denatured LBP was determined by gel filtration chromatography on a calibrated S-300 column to be close to 150 kDa (Fig. 5), suggesting that the native protein is a dimer of the 72-kDa protein observed on SDS-PAGE. Isoelectric focusing of purified LBP gave a single band (pI = 6.7) under both denaturing and non-denaturing conditions, indicating that the two subunits are identical.

**Luciferin Binding** —The binding affinity was measured by equilibrium dialysis under argon of purified LBP

![Graph](image)

**Fig. 3.** Kinetic parameters as a function of luciferin concentration. The initial light intensity \((I_0, \text{quanta/s})\) \((\bigcirc)\) increases with increasing luciferin concentration while \( k \) (or \( \ln 2/t_{1/2}, \text{s}^{-1} \)) \((\bullet)\) remains constant. The amount of luciferin added was determined from the absorbance at 390 nm (see methods). \( I_0 \) deviates from linearity above a concentration of 1 \( \mu M \). The concentration of luciferase was 0.2 \( \mu g/ml \), or 1.5 \( nM \).

**Table I**

| Purification of LBP from night phase cells |
|-----------------|-----------------|-----------------|-----------------|
| Total protein\(^a\) | Total luciferase\(^b\) | Total \( \text{LH}_2 \) bound\(^c\) | Yield % |
| mg | units | nmol | nmol |
| Crude extract | 470 | 1.43 | 16 | 40 | 100 | 0.08 |
| Centrifuged | 260 | 1.82 | 20 | 45 | 110 | 0.17 |
| P-10 | 56 | 1.15 | 19 | 44 | 110 | 0.78 |
| DEAE | 6.8 | 0.090 | 9.8 | 28 | 70 | 4.1 |
| S-300 | 1.8 | 0.017 | 4.5 | 14 | 35 | 7.7 |
| Hydroxylapatite | 1.5 | 0.008 | 4 | 12 | 30 | 8 |

\(^a\) Milligrams protein relative to a bovine serum albumin standard in the Lowry assay.

\(^b\) Standard units using a substrate concentration of 0.2 \( \mu M \).

\(^c\) Measured both before \((-\text{LH}_2)\) and after \((+\text{LH}_2)\) saturation of the LBP with luciferin.

\(^d\) One mg of LBP (144 kDa) is equivalent to 7.2 nmol.

![Graph](image)

**Fig. 4.** SDS-PAGE of LBP at different stages of purification. The fractions are taken from the steps given in Table I. Lane 1, 100 \( \mu g \) of centrifuged crude extract; lane 2, 50 \( \mu g \) of P-10; lane 3, 25 \( \mu g \) of DEAE; and lane 4, 10 \( \mu g \) of hydroxylapatite. The molecular mass of LBP is 72 kDa as judged by comparison to molecular mass standards.

![Graph](image)

**Fig. 5.** Gel filtration chromatography of luciferin-binding protein. Purified LBP was chromatographed through an S-300 column. The amount of luciferin carried through the column is shown and apparent molecular mass values of standard proteins are given above.

against different concentrations of purified luciferin. A Scatchard plot of the data (Fig. 6) shows that one molecule of luciferin is bound per LBP dimer at pH 8 in agreement with the stoichiometry of luciferin bound to the purified protein (8 nmol of luciferin bound per mg protein, Table I). The luciferin is bound with a \( K_r \approx 5 \times 10^7 \text{M}^{-1} \). Fig. 6 also shows that when the equilibrium dialysis is carried out at pH 6.7 (the isoelectric point of LBP) only one-half as much luciferin is bound per mole although the binding constant remains approximately the same. This suggests that when the pH drops below neutrality, LBP undergoes a conformational change to a form that no longer binds luciferin. This change is not simply a dimer to monomer conversion, as the apparent molecular mass (144 kDa) by gel filtration is the same at pH 6 and 8. The circular dichroism spectrum of LBP is also the same at both pH values, confirming the fact that the conformational
It is of interest that a similar curve can be drawn through the data of Krieger (1972) on the effect of pH on luciferase activity (Fig. 7).

Circadian Changes in Amounts of Extractable LBP—The amount of luciferin which can be bound by *G. polyedra* cell extracts has been previously reported to depend upon the time of day the extractions are made, with a maximum during the night (Sulzman et al., 1978). As shown in Table II, both by luciferin binding assay and by Western blots probed with affinity purified anti-LBP, purified LBP was run as a standard.

The present studies, when taken together with the earlier observations of Krieger and Hastings (1968), may be explained by assuming a series of independent (or separate) reactions. The first is the equilibrium between LBP and luciferin at alkaline pH.

\[
\text{LBP + LH}_2 \rightleftharpoons \text{LBP-LH}_2
\]  

As we have shown, this reaction has an association constant of about \(5 \times 10^7 \text{ M}^{-1}\), and the equilibrium is relatively independent of small changes in pH. The second reaction involves a major conformational change of the binding protein to a form that is no longer capable of binding luciferin, even though the two subunits remain bound to one another.

The effect of pH on binding of luciferin is shown in Fig. 7. The data are best fitted to the theoretical curve for a 4-electron transfer:

\[
\text{pH} = 0.25 \log \frac{\text{LBP-LH}_2}{\text{LBP}}
\]

It is of interest that a similar curve can be drawn through the change does not involve dissociation into monomers. Similar ND measurements using purified luciferin were also identical at the two pH values.

The effect of pH on binding of luciferin is shown in Fig. 7. The data are best fitted to the theoretical curve for a 4-electron transfer:

\[
\text{pH} = 0.25 \log \frac{\text{LBP-LH}_2}{\text{LBP}}
\]

Finally, the released luciferin is oxidized by luciferase in the light producing reaction:

\[
\text{LBP-LH}_2 + 4\text{H}^+ \rightarrow \text{H}_4\text{LBP} \text{ (inactive form)} + \text{LH}_2
\]
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\[
\text{LH} + \text{O}_2 + \text{Luciferase} \rightarrow \text{LH} = \text{O} + \text{H}_2\text{O} + \text{hv}. \tag{5}
\]

Within the cell during night phase, the amount of LBP may reach a maximum of 1% of the total cellular protein. Based on the light produced during exhaustive mechanical stimulation of *Gonyaulax* (10⁶ quanta/cell; Seliger et al., 1969), the amount of luciferin has been estimated to be of the same order of magnitude. This suggests that virtually all of the cellular luciferin is bound to LBP. Luciferin is thus protected from inactivation by autoxidation, and can be liberated in small amounts to react with luciferase and O₂ to produce a flash when stimulated by a small pH change. The localization of luciferin-specific fluorescence in the scintillons (Johnson et al., 1985) is in good agreement with immunochemical studies suggesting that LBP is located only in the scintillons.

From Table I it may be calculated that the crude extract from 10⁶ night phase *Gonyaulax* cells contains about 5 mg of LBP. Assuming a scintillon diameter of 0.5 μm and about 400 scintillons/cell, it may be calculated that the total scintillon volume in 10⁶ cells is about 3 μl. Considering the approximations involved in arriving at these figures, it is remarkable that we can conclude within an order of magnitude that the scintillon volume is sufficient to accommodate the entire bioluminescent system.

Because *Gonyaulax* scintillons contain a 20-fold or greater excess both of binding protein and luciferin over their luciferase content, only a slight decrease in pH will result in release of sufficient luciferin to cause a flash of light. Such small changes in pH could be caused by an action potential triggered by mechanical stimulation, as has been shown to be the case in *Noctiluca miliaris* (Eckert and Sibaoka, 1968; Nawata and Sibaoka, 1979). As in *Noctiluca*, we would expect that such a stimulated response could be repeated many times within a given scintillon. Indeed isolated scintillons of *Gonyaulax*, brought to pH 6 in vitro, are also stimulated to flash. They will flash again if brought back to neutrality, provided with fresh luciferin and subjected to a second pH drop.

The maximum rate of synthesis of LBP (and presumably luciferase and luciferin as well) occurs during the early hours of night phase, soon after evolution of O₂ by photosynthesis has stopped. After reaching a maximum, the amount of LBP remains constant for about 6 h and then rapidly decreases, until during day phase only about 10% remains (Morse et al., 1969b). When photosynthesis stops at the onset of night phase, the pH will rapidly fall to a low value, and it seems probable that the cytoplasmic pH will slowly decrease due to accumulation of the products of glycolysis. Toward the end of night phase, *Gonyaulax* cells emit light as a steady glow which terminates soon after daylight (Johnson and Hastings, 1986; Hastings and Dunlap, 1986). This suggests that a drop in cytoplasmic pH might be related to the onset of the glow. However, this possibility is not easily reconciled with early experiments, in which cells were shown to emit successive glow peaks, about 24 h apart, after being placed in constant darkness (Sweeney and Hastings, 1958; Hastings, 1960). The onset of the glow peak coincides also with the disappearance of the scintillons and their contents from the cells, which might well be its most immediate cause.

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1 Non-specific binding to bovine serum albumin at concentrations of 10⁻⁹ M or greater also protects luciferin against autoxidation (Bode and Hastings, 1963).

2 L. Fritz, D. Morse, M. T. Nicolas, and J. W. Hastings, unpublished data.

3 For unknown reasons, we are not able to reproduce these results with our present strains of *Gonyaulax*. 