APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1989, p. 2607–2612
0099-2240/89/102607-06$02.00/0

Vol. 55, No. 10

Bioluminescence of the Insect Pathogen *Xenorhabdus luminescens*

THOMAS M. SCHMIDT,[1]† KARL KOPECKY,[2] AND KENNETH H. NEALSON[1]*

Center for Great Lakes Studies, University of Wisconsin–Milwaukee, 600 East Greenfield Avenue, Milwaukee, Wisconsin 53204,[1] and Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2[2]

Received 5 May 1989/Accepted 19 July 1989

Luminescence of batch cultures of *Xenorhabdus luminescens* was maximal when cultures approached stationary phase; the onset of in vivo luminescence coincided with a burst of synthesis of bacterial luciferase, the enzyme responsible for luminescence. Expression of luciferase was aldehyde limited at all stages of growth, although more so during the preinduction phase. Luciferase was purified from cultures of *X. luminescens* Hm to a specific activity of 4.6×10^{13} guanta/s per mg of protein and found to be similar to other bacterial luciferases. The *Xenorhabdus* luciferase consisted of two subunits with approximate molecular masses of 39 and 42 kilodaltons. A third protein with a molecular mass of 24 kilodaltons copurified with luciferase, and in its presence, either NADH or NADPH was effective in stimulating luminescence, indicating that this protein is an NAD(P)H oxidoreductase. Luciferases from two other luminous bacteria, *Vibrio harveyii* (B392) and *Vibrio cholerae* (L85), were partially purified, and their subunits were separated in 5 M urea and tested for complementation with the subunits prepared from *X. luminescens* Hb. Positive complementation was seen with luciferase subunits among all three species. The slow decay kinetics of the *Xenorhabdus* luciferase were attributed to the alpha subunit.

Xenorhabdus luminescens is one of the few nonmarine luminous bacterial species; strains are found both as symbionts with terrestrial nematodes of the family *Heterorhabditidae* and as isolates from human wounds (6). In the former state, the nematode and its bacterial symbiont form a virulent insect-pathogenic pair that is active against a variety of insects (1, 8, 15, 20). When an insect is infected by the bacteria, the carcass becomes visibly luminescent because of the bioluminescence of *X. luminescens* (21). Studies with cell extracts have shown that the light-generating activity is similar to that of bacterial luciferase from marine luminous bacteria in that light emission requires both reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic aldehyde (21).

Bacterial luciferase is a heterodimeric enzyme with a molecular mass of approximately 80 kilodaltons (kDa) which displays variable decay kinetics depending on the aldehyde used in the assay (10–12). The subunits (alpha and beta) can be separated by 5 M urea column chromatography and recombined to yield active hybrids (7, 9). Subunit complementation has been used to demonstrate relatedness between luciferases from several different species of marine luminous bacteria (17, 23). By the use of the subunit complementation approach, mutants with altered decay kinetics or defective flavin binding were localized in the alpha subunit (4), suggesting that this subunit contains the active site.

In addition to luciferase, the light-emitting reaction in vivo requires an NAD(P)H:flavin oxidoreductase to supply $FMNH_2$ (12, 29). This enzyme has been purified and studied by several laboratories (14, 18, 25–27), and various reductases exhibit considerable differences in subunit size, substrate (pyridine nucleotide) usage, and mechanism of action. No direct evidence for complex formation between a luciferase and its oxidoreductase has been published; for one study, indirect evidence of interaction was presented (26).

Among many species of luminous bacteria, several factors are know that control the synthesis and activity of the luminous system (12). These include a mechanism known as autoinduction, regulation by oxygen tension, regulation by iron concentration, regulation by osmolarity, and catabolite repression. These control systems vary in different species of luminous bacteria, and these variations may well be a reflection of the ecological niche of the luminous bacteria (12). Another level of control of luminescence by *X. luminescens* is the formation of spontaneous dim variants referred to as secondary forms, which have been isolated from several strains of *Xenorhabdus* (1, 3); they differ from the parent strains (primary forms) with regard to pigment production (3), antibiotic production (1, 3, 22), protease production (3, 24), and luminescence (3).

In this paper, we describe the pattern of luminescence and luciferase production of the primary form of *X. luminescens* (Hm) and report on the characteristics of luciferase from *X. luminescens* and the ability of the luciferase subunits to form active hybrids with the subunits from the luminous bacteria *Vibrio harveyi* and *V. cholerae*.

MATERIALS AND METHODS

Strains and growth conditions. The bacteria used in this study were a marine strain of *V. harveyi* B392, formerly referred to as MAV (8); a low-salt (presumably estuarine) strain of *V. cholerae* subsp. *albensis*, L85; and two strains of the insect pathogenic strain of *Xenorhabdus luminescens*, Hb, (ATCC 29999) and Hm, obtained from J. Ensign (University of Wisconsin–Madison).

V. harveyi was grown and maintained on seawater complete medium which contains, per liter of 75% seawater, 3 g of Bacto-Peptone (Difco Laboratories), 0.5 g of yeast extract (Difco), and 3 ml of glycerol. *X. luminescens* and *V. cholerae* were grown in a low-salt seawater complete medium containing 5 g of Bacto-Peptone and 2.5 g of yeast extract in 1 liter of 25% seawater, or in LB (19), which contains, per liter

* Corresponding author.
† Present address: Department of Biology, Jordan Hall 138, Indiana University, Bloomington, IN 47405.

of distilled water, 10 g of Bacto-Tryptone, 5 g of yeast extract, and 10 g of NaCl. The cultures were incubated at 25 or 30°C with shaking for growth experiments or in a 20-liter carboy with vigorous aeration for luciferase purification.

All growth experiments were performed with 100 ml of medium in a 500-ml flask at 30°C on a rotary shaker at 150 rpm. At timed intervals, samples were removed for measurement of optical density (optical density at 560 nm [OD_{560}] measured on a Bausch & Lomb Spectronic 20 spectrophotometer). When the OD_{560} reading was greater than 0.5, dilutions were made into LB to a value less than 0.5.

Luminescence measurements. Luminescence was measured by using the photomultiplier-photometer apparatus previously described (9, 19), and calibration was done by using a light standard provided by J. W. Hastings (13).

Luciferase activity was determined by using the flavin-induced assay previously described (10). A sample (5 to 50 μl) of the luciferase preparation was added to 0.5 ml of assay buffer (50 mM phosphate [pH 7.2], 0.01% bovine serum albumin, 10 mM EDTA, 10 mM dithiothreitol [DTT]). Twenty-five microliters of an aldehyde emulsion (0.01% decanal or dodecanal in double-distilled H_2O, sonicated for 3 min) was then added, and the solution was gently mixed. Light emission was initiated by the addition of 1.0 ml of catalytically reduced (hydrogen gas and platinized asbestos) flavin mononucleotide ($FMNH_2$). Luciferase activity was recorded as the peak luminescence from the resulting flash as recorded on a Perkin-Elmer model 56 strip chart recorder.

Decay rates of luciferase were also determined by using the flavin-induced assay. The strip chart data were accumulated for at least a 50-fold decrease in luminescence and plotted on semi-log paper, and the decay constants were calculated.

For detection of NAD(P)H:oxidoreductase activity, either NADH or NADPH was added to a reaction mixture containing oxidized flavin mononucleotide, dodecanal, and the luciferase preparation (14, 17, 26). With this (coupled) assay, light emission is continuous until the NAD(P)H is depleted, which may take many minutes.

Isolation of *X. luminescens* (Hm) luciferase. Cells were grown as described above, harvested by centrifugation, and stored at −20°C until a total wet weight of 150 g was obtained. The frozen pellet was thawed and diluted to 500 ml with lysis buffer which contained 1 mM EDTA and 0.1 mM DTT. Cells were lysed by passage through a Stansted cell disruptor at 15,000 lb/in^2.

Ammonium sulfate was added to the crude extract to a concentration of 40% (wt/vol) and stirred overnight. The precipitate was removed by centrifugation at 8,000 × *g* for 30 min. Ammonium sulfate was then added to the supernatant to a final concentration of 75% (wt/vol), and the solution was again stirred overnight. The precipitate was recovered by centrifugation at 8,000 × *g* for 30 min and suspended in 10 mM phosphate buffer. The resuspended protein was dialyzed against three 5-liter volumes of 10 mM phosphate buffer containing 1 mM EDTA.

The dialyzed protein was passed twice through a DEAE-cellulose (Sigma Chemical Co.) column equilibrated with 50 mM phosphate buffer. The sample that passed through the DEAE-cellulose was then loaded onto a DEAE-Sepharose (Sigma DFF-100) column equilibrated with 50 mM phosphate. The column was washed with 50 mM KCl in 50 mM phosphate, and the protein was eluted with 150 mM KCl in 50 mM phosphate. The sample was concentrated and dialyzed in Amicon microconcentrators (molecular mass cutoff, 30,000 Da) and loaded onto a hydroxyapatite column equilibrated with 10 mM phosphate at pH 7.2. Luciferase activity eluted at 150 mM phosphate.

Polyacrylamide electrophoresis of the protein samples was done by the method of Laemmli (16) with 15% acrylamide gels, and the gels were silver stained by the method of Wray et al. (28). Affinity chromatography, utilizing blue Sepharose CL-6B, was performed as described by Tsai (25).

Cross-reaction of luciferase subunits. Preparation of the luciferases for subunit complementation studies was with slight modifications of previously published methods (10). Frozen cell pellets of B392 and L85 were thawed in an equal volume of 3% NaCl, and the resulting mixture poured into 8 to 9 volumes of buffer A (10 mM EDTA, 10 mM DTT) while frozen cells of Hb were mixed directly with 10 volumes of lysis buffer. These mixtures were stirred gently at 0°C, and the release of luciferase from lysed cells was monitored. Cells of L85 were sonicated for several minutes to enhance luciferase release. Cellular debris was removed from the preparation by centrifugation at 10,000 rpm. DEAE-cellulose (Whatman DE-32) which had been hydrated and equilibrated with 10 mM phosphate buffer (pH 7.0) was added in portions to the viscous supernatant until 90% of the luciferase activity was removed from solution. After filtration, the DEAE-cellulose was washed five times with an equal volume of distilled H_2O at 4°C. Luciferase was eluted from the DEAE-cellulose by batchwise treatment with increasing concentrations of phosphate buffers (pH 7.0) at 0°C. Ammonium sulfate precipitation (40 to 75% cut) was used to concentrate the luciferase. The precipitate was dissolved in phosphate buffer (pH 7.0) (10 mM DTT, 10 mM EDTA) and either used directly or stored as a precipitate at −20°C. Before chromatography, the solution was dialyzed (100× volume) against the same phosphate buffer for 6 to 8 h with one change of buffer. The sample was then applied to a column (0.9 by 50 cm) of DEAE-Sephadex (A-50) and eluted with increasing concentrations of phosphate buffer. Column fractions were analyzed for luciferase activity and OD_{280}. The fractions rich in luciferase activity were pooled and reprecipitated with ammonium sulfate (40 to 75% cut) redissolved in buffer A containing phosphate buffer (0.15 M [pH 7.0]) and were stored at −20°C.

Separation and recombination of luciferase subunits. The luciferase preparations were thawed and dialyzed against a 100× volume of buffer A (containing 40 mM phosphate at pH 7.0) for 10 h with two buffer changes. The dialyzed solution was treated with 1.2 volumes of 8 M urea which had been stored over Amberlite MB-3 resin at 4°C and then applied to a column (0.9 by 50 cm) of DEAE-Sephadex which had been equilibrated with buffer A and 5 M urea. The subunits were eluted at 4°C with a linear phosphate buffer gradient from 40 to 120 mM. Fractions were analyzed for OD_{280}.

For subunit recombination, 10-μl samples (either 10 μl from a given sample or 5 μl each from two different samples) were added to 0.5 ml of recovery buffer (100 mM sodium phosphate [pH 7.0], 0.2% bovine serum albumin, 1 mM DTT, 1 mM EDTA) at 4°C. These recovery mixtures were assayed for luciferase activity with time, and recovery was monitored until no significant increase in activity could be seen (usually 24 to 36 h). Alpha and beta subunits of *V. harveyi* were identified as originally specified by Friedland and Hastings (7), and these subunits were used to identify the alpha and beta subunits of the other two species, with which they showed positive subunit complementation.

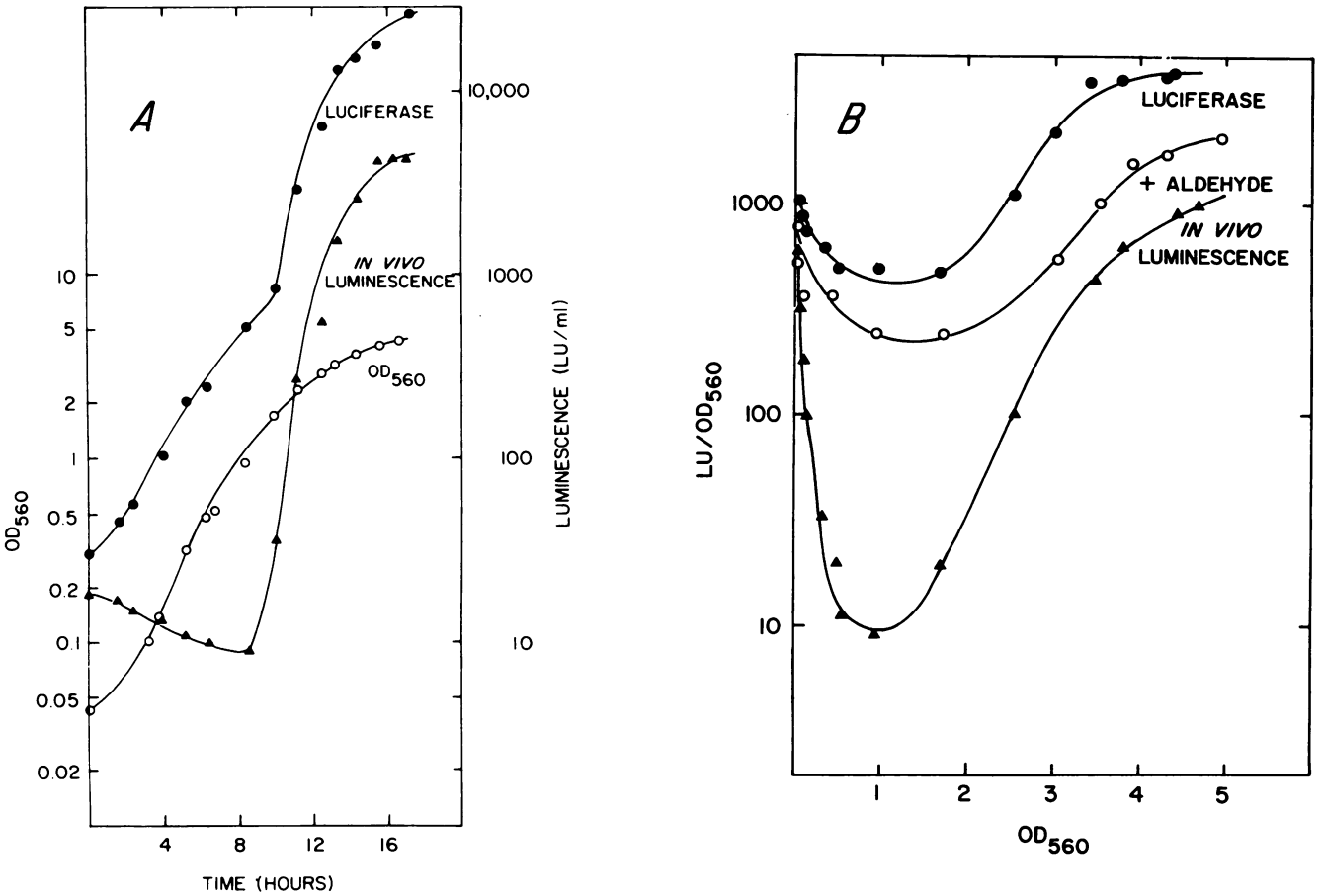


FIG. 1. (A) Growth and luminescence of the primary form of *X. luminescens* Hm. Growth (○) was measured as OD_{560}. In vivo luminescence (▲) and the activity of extractable luciferase (●) were measured at each time point. One light unit (LU) equals approximately 1.06×10^6 quanta/s. (B) Luminescence as a function of growth of the primary form of *X. luminescens* Hm. In vivo luminescence per OD_{560} (▲), in vivo luminescence in the presence of added decanal (○), and the concentration of luciferase (●) were measured at each time point and plotted versus OD_{560}.

RESULTS

Cultures of *X. luminescens* Hm are capable of logarithmic growth in complex media, with a doubling time of approximately 1.5 h at 30°C (Fig. 1). Luciferase activity accumulated in proportion to cell growth during logarithmic growth, but as the culture neared stationary phase there was rapid synthesis of luciferase coincident with an increase in luminescence (Fig. 1A). The luminescence of *X. luminescens* cells was aldehyde limited at all times during the growth cycle (Fig. 1B).

Luciferase was purified to a specific activity of 4.6×10^{13}

TABLE 1. Purification of luciferase from *X. luminescens* Hb

Fraction	Total protein		Total activity		Sp act (quanta/s per mg of protein)
	mg	g	Quanta/s	%	
Crude extract	7,300	100	3.6×10^{14}	100	4.9×10^{10}
40 to 75% AS[a]	2,516	35	3.1×10^{14}	85	1.2×10^{11}
DEAE-cellulose	610	8.3	2.9×10^{14}	80	4.8×10^{11}
DEAE-Sepharose	1.8	0.026	8.3×10^{13}	23	4.6×10^{13}

[a] AS, Ammonium sulfate.

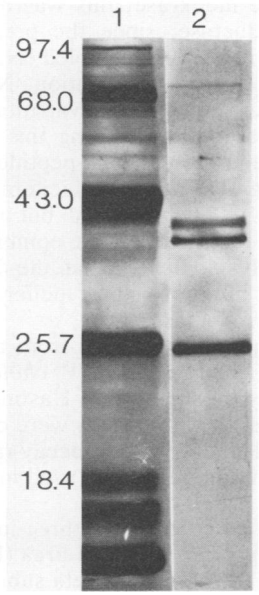
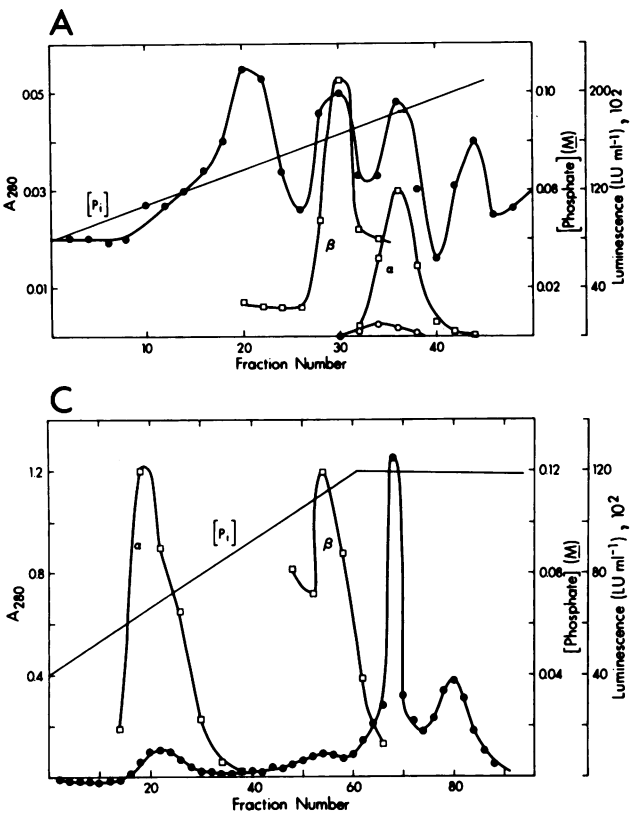


FIG. 2. Silver-stained sodium dodecyl sulfate-polyacrylamide electrophoretic gels of molecular mass markers (in kilodaltons) (lane 1) and the luciferase preparation following hydroxyapatite chromatography (lane 2).



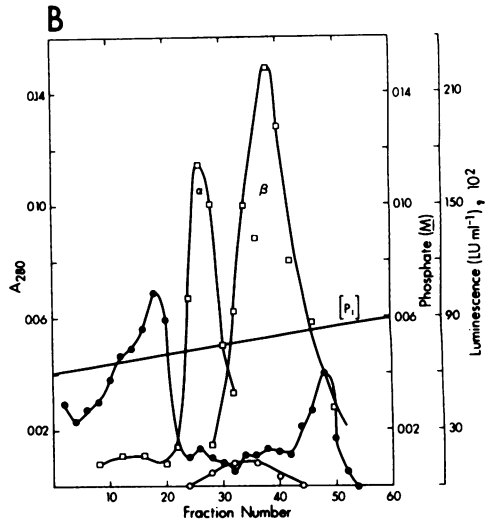


FIG. 3. Separation of luciferase subunits from crude preparations of luciferase from *V. harveyi* B392 (A), *V. cholerae* L-85 (B), and *X. luminescens* Hb (C). Samples were chromatographed through DEAE-Sephadex with phosphate buffer (40 to 120 mM) containing 5 M urea, 1 mM DTT, and 1 mM EDTA (pH 7.0). Symbols: ●, OD_{280}; □, luminescence activity after recombination with complementary subunit; ○, luminescence activity after renaturation with no complementary subunit added. One light unit (LU) equals 1.9×10^{11} quanta/s.

quanta/s per mg of protein (Table 1). As with other luciferases, *X. luminescens* luciferase is an alpha-beta heterodimer; for this species, the respective alpha and beta subunit molecular masses were approximately 39 and 42 kDa (Fig. 2). A third protein with a molecular mass of 24 kDa copurified with the luciferase; this was assumed to be an NAD(P)H:oxidoreductase, since the preparation retained the ability to emit light in the coupled [NAD(P)H-initiated] assay to the final stage in purification. None of the techniques attempted, including hydroxyapatite chromatography (Fig. 2), were capable of separating this protein from the luciferase. However, when the polypeptides were denatured by urea chromatography and the luciferase subunits were separated (Fig. 3C), flavin-induced but not NAD(P)H-induced activity was restored with the combination of the two larger subunits. This indicates that the 24,000-molecular-weight subunit was not required for luciferase activity and is probably the oxidoreductase.

Luciferase was partially purified from three different luminous bacteria (*V. harveyi* B-392, *V. cholerae* L-85, and *X. luminescens* Hb) by the methods of Hastings et al. (10). The decay kinetics of all three enzymes were classified as slow, with Hb showing a notably slower decay rate than the other two enzymes when dodecanal was used in the assay (Table 2).

Chromatography of each of the three luciferase preparations on DEAE-Sephadex with 5 M urea (Fig. 3) resulted in good separation of the alpha and beta subunits. All of these renatured with opposite subunits from the same species and with opposite subunits from each of the other species to yield enzyme activity. Since Friedland and Hastings (7) have previously reported that the beta subunit of *V. harveyi* luciferase is eluted first from the DEAE-Sephadex column, it was possible to identify the alpha and beta subunits of L-85 and Hm by assaying for complementation of the *V. harveyi* subunits. When hybrid enzymes were prepared, the decay rate of the hybrid was found to be identical with that of the parent enzyme from which the alpha subunit was obtained (Table 2).

Under similar chromatography conditions, the alpha subunits of strains L-85 and Hb eluted prior to the beta subunit (Fig. 3), as was previously found for *V. fischeri* luciferase (9). The phosphate concentrations at which the subunits

TABLE 2. Decay constants of luciferases produced by complementation of subunits from different luciferases[a]

Subunit[b]		Total activity[c]	Decay constant (min^{-1})[d]
Alpha	Beta		
Hb	Hb	0.73	0.029
Hb	B392	0.80	0.029
Hb	L85	0.08	0.030
B392	B392	2.17	0.044
B392	Hb	6.90	0.044
B392	L85	1.98	0.048
L85	L85	0.31	0.048
L85	B392	0.34	0.044
L85	Hb	0.13	0.044

[a] Luciferases were partially purified from *X. luminescens* (Hb), *V. harveyi* (B392), and *V. cholerae* (L85), the subunits were separated by chromatography in 5 M urea, and then the subunits were recombined in various combinations.

[b] The following volumes of each subunit were used, on the basis of estimates of protein concentration (OD_{280}):B392, 50 μl; Hb, 15 μl; L85, 6 μl.

[c] Maximal luminescence (the peak height observed in the flavin-initiated reaction) is expressed in 10^9 quanta/ml per s. Activities were measured 25 h after mixing the subunits in recovery buffer.

[d] Decay constants were determined at 25°C as described in Materials and Methods.

eluted also varied for the luciferase subunits, with the beta subunit of Hb luciferase not eluting until nearly 0.12 M phosphate (Fig. 3).

DISCUSSION

The primary form of *X. luminescens* is the form that is usually isolated from nematodes and infected insects. The primary cultures of Hm are not visibly luminescent during the early stage of growth because of limitation for aldehyde and low levels of luciferase. During late logarithmic growth they are readily visible to the eye; this rapid rise in luminescence coincides with a burst of luciferase synthesis (Fig. 1), the timing of which is similar to that of other secondary metabolites (3, 22, 24).

By the criteria applied in our studies (i.e., molecular weight, subunit sizes, substrate specificity, and subunit complementation), the luciferase of *X. luminescens* is similar to those of other known luminous bacteria. By the use of similar methods of purification (10), the luciferase from strain Hm was purified to a specific activity of 4.6×10^{13} quanta/s per mg of protein. Luciferases of *V. harveyi*, *V. fischeri*, and *Photobacterium phosphoreum* have been purified to specific activities of 2×10^{13}, 4×10^{14}, and 4×10^{13} quanta/s per mg, respectively (10). Separation of the subunits was achieved by using standard techniques, although the beta subunit of *Xenorhabdus* luciferase eluted at a phosphate concentration somewhat higher than any other so far reported.

A notable difference between the *Xenorhabdus* luciferase and other luciferase preparations lies in the apparently close association of the luciferase and the oxidoreductase activities. The final protein to be removed from luciferase during purification is frequently an oxidoreductase (14, 17, 25–27), but there has been considerable argument in the literature as to whether oxidoreductase actually forms a complex with luciferase. In strain Hm, an oxidoreductase activity also copurified with luciferase, but it was not possible by using the nondenaturing methods we tried to separate this activity from luciferase. Blue Sepharose CL-6B, which has been used to remove oxidoreductase from *V. harveyi* preparations (25), did not separate these proteins. These results suggest that the luciferase and the oxidoreductase in this system may indeed form a reasonably stable complex in *Xenorhabdus* species.

On the basis first of amino acid sequence analysis (2, 29) and more recently of nucleotide sequence data of cloned *lux* genes (5), it has been hypothesized that the alpha and beta subunits of luciferase evolved via an original gene duplication and that the luciferases from several species are very closely related. The latter hypothesis is supported by results of subunit complementation studies of several laboratories (17, 23) and the data presented here. Studies by Gunsalus-Miguel et al. (9) originally reported no subunit complementation between subunits from *V. harveyi* and *V. fischeri*. However, subsequent studies (17) reported positive complementation between *V. harveyi* and *P. phosphoreum*. Ruby and Hastings (23) reported positive complementation between the beta subunits of *P. phosphoreum* and *P. leiognathi* and alpha subunits of *V. fischeri*, as well as between the beta subunit of *V. fischeri* and the alpha subunit of *P. leiognathi*. These data, along with the subunit complementation results presented here, suggest that all bacterial luciferases (even those from the taxonomically and physiologically distinct *Xenorhabdus* group) share a common ancestry. An unexpected, and as yet unexplained, result of these studies was that the combination of *V. harveyi* (B392) alpha and *X. luminescens* (Hb) beta subunits yielded active luciferase in higher amounts (by a factor of three) than any of the other combinations. Since the functions of the beta subunit are as yet unknown, it is difficult to speculate as to whether this difference is due to efficiency of renaturation of the pair or to some factor that affects enzyme activity. As seen in Table 2, the decay rate of the complex was identical with that of the source of alpha subunit.

Even for *X. luminescens*, which is phylogenetically and ecologically separated from its marine luminous counterparts, the structural genes for luciferase would appear, on the basis of these data and other reports, to be conserved. In contrast, it is known that the regulation of luminescence in marine species differs substantially (12). While it would seem reasonable that the regulatory control mechanisms of the luminous system for *Xenorhabdus* species would be distinct from those of the marine forms, the details of regulation and the magnitude of divergence of these mechanisms remain to be investigated.

ACKNOWLEDGMENT

This work was supported by grant 85CRCR-1-1663 to K.H.N. from the U.S. Department of Agriculture.

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